Histone Deacetylase Inhibitors Induce a Very Broad, Pleiotropic Anticancer Drug Resistance Phenotype in Acute Myeloid Leukemia Cells by Modulation of Multiple ABC Transporter Genes

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Abstract

Purpose: Histone deacetylase inhibitors (HDACi) are being studied in clinical trials with the aim to induce cellular differentiation, growth arrest, and apoptosis of tumor cells. Recent reports suggest that the multidrug resistance-1 (MDR1) gene is regulated by epigenetic mechanisms. To investigate whether additional drug transporters are regulated by HDACi and how this affects cytotoxicity, acute myeloid leukemia (AML) cells were examined.

Experimental Design: AML cells were cultured in the presence of phenylbutyrate, valproate, suberoylanilide hydroxamic acid, or trichostatin A and analyzed for drug transporter expression and function as well as sensitivity to anticancer drugs.

Results: MDR1, breast cancer resistance protein (BCRP), and multidrug resistance-associated proteins (MRP) 7 and 8 were induced in a dose- and time-dependent manner as shown by semiquantitative PCR. The pattern of gene induction was cell line specific. Phenylbutyrate induced P-glycoprotein and BCRP expression and the efflux of drugs as determined with labeled substrates. KG-1a cells treated with phenylbutyrate developed resistance to daunorubicin, mitoxantrone, etoposide, vinblastine, paclitaxel, topotecan, gemcitabine, and 5-fluorouracil; as a result drug-induced apoptosis was impaired. Chromatin immunoprecipitation revealed the hyperacetylation of histone proteins in the promoter regions of MDR1, BCRP, and MRP8 on valproate treatment. Furthermore, an alternative MRP8 promoter was induced by HDACi treatment.

Conclusions: Exposure of AML cells to HDACi induces a drug resistance phenotype broader than the "classic multidrug resistance," which might negatively affect treatment effectiveness.

Acute myeloid leukemia (AML) can be treated with nucleoside analogues such as cytosine arabinoside in combination with anthracyclines (1). A major proportion of patients will, however, die from refractory or relapsing leukemia. Extrusion of chemotherapeutic agents by transporter proteins is a major obstacle for successful treatment of cancer. Overexpression of multidrug resistance-1 (MDR1), also known as ATP-binding cassette ABCB1, is an independent prognostic factor for treatment failure in AML (2). The encoded P-glycoprotein (P-gp) is a membrane-bound transporter that extrudes natural toxins across the plasma membrane (Supplementary Table S1; reviewed in refs. 3, 4). Similar patterns of resistance are conferred by breast cancer resistance protein (BCRP; also known as mitoxantrone resistance gene, MXR, or ABCG2) and multidrug resistance-associated proteins (MRP) 1 (ABCC1), 2 (ABCC2), 3 (ABCC3), and 7 (ABCC10; refs. 4–7). BCRP overexpression is associated with increased risk of relapse and a shorter disease-free survival in AML, especially in combination with MDR1 (8). The transport activity of P-gp, MRP1, MRP7, and BCRP is inhibited by relatively nontoxic compounds such as cyclosporine (9, 10).

Other ABC transporters such as MRP4 (ABCC4), MRP5 (ABCC5), or MRP8 (ABCC11) render cells insensitive to nucleoside analogues (7). MRP8 confers resistance to antiviral drugs including 2',3'-dideoxycytidine and fluoropyrimidines (7, 11, 12). Moreover, BCRP confers resistance to nucleoside analogues in addition to transport of natural toxins (13). In contrast, most ABC transporters are not involved in drug resistance (e.g., ABCG1).
Translational Relevance

Many cancers are initially responsive to chemotherapy but later develop cross-resistance to drugs that have not been administered before. The regulation of drug resistance genes is not completely understood. Histone deacetylase inhibitors (HDACi) are being investigated in clinical trials because these compounds can act synergistically with certain anticancer drugs in experimental models and may be useful in combination treatment. We find, however, that exposure of cancer cells to HDACi can induce several drug resistance-associated ABC transporters, leading to a broad-spectrum anticancer drug resistance, which comprises nucleoside analogues in addition to the “classic multidrug resistance” phenotype. The combined treatment may thus result in treatment failure. Furthermore, we find that the sequence of administration is important for the efficacy of drug treatment. Our data will help to design clinical protocols for a combination of chemotherapy with HDACi in which inactivation of anticancer drugs can be avoided.

In clinical treatment, resistance to chemotherapy is frequently observed after initial response despite continued treatment. The MDR1 promoter is inducible by anticancer drugs (14), environmental stress (15), and epigenetic mechanisms such as DNA demethylation (16).

Histone deacetylase inhibitors (HDACi) such as trichostatin A or valproate induce apoptosis or growth arrest or restore suppressed myeloid differentiation in AML (17). Several HDACi have been introduced into the treatment of leukemias. They were used alone or in combination with DNA demethylating agents or all-trans retinoic acid (18, 19). These studies have shown clinical remissions. HDACi can act synergistically with certain DNA-binding drugs in experimental models (20); thus, combination treatment is being investigated in clinical trials.

Recent reports have, however, suggested that HDACi may modulate MDR1 expression. Leukocytes of patients and colon and renal cancer cells treated with the HDACi depsipeptide or trichostatin A, respectively, display increased MDR1 expression (21, 22). P-gp induction has also been observed in human and murine cells exposed to valproate (23). Treatment with the HDACi apicidine increases rhodamine-123 efflux and induces paclitaxel resistance in HeLa cells (24). Furthermore, depsipeptide combined with all-trans retinoic acid increases MDR1 levels in acute promyelocytic leukemia (25). Thereby, resistance to doxorubicin is induced, which is reversible by the P-gp inhibitor PSC833 (25).

These reports argue for the possibility that combinations of HDACi and anticancer drugs might lead to reduced cytotoxic effects by inducing multidrug resistance-associated drug transport. In the current study, we show that HDACi induce in AML cells the expression of several ABC transporters. We find that exposure to HDACi can induce a pleiotropic anticancer drug resistance, which comprises nucleoside analogues in addition to the “classic multidrug resistance” phenotype.

Materials and Methods

Cells and culture conditions. Cell lines K562, CML, HL-60, KG-1a, and SK-Hep-1, obtained from Deutsche Sammlung von Mikroorganis men und Zellkulturen, were cultured in RPMI 1640 (Invitrogen). The cancer lines KB-3-1 and KB-8-5 were kindly provided by Dr. Michael M. Gottesman (National Cancer Institute, NIH). KB cells were grown in DMEM (Invitrogen). KB-8-5 cells were selected with 10 ng/mL colchicine (Sigma-Aldrich) and transferred to DMEM without colchicine 1 day before experiments were done.

Mononuclear cells were isolated from leukemic AML patients by Fi- col and cultured in Iscove's medium. Medium for cell lines contained 10% fetal bovine serum (PAA), whereas patient cells were grown in 20% fetal bovine serum. All media were supplemented with 2 mmol/L l-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. All cells were cultured at 37°C in a fully humidified atmosphere containing 5% CO2.

Trichostatin A, valproate (Sigma-Aldrich), suberoylanilide hydroxamic acid (SAHA; vorinostat), and phenylbutyrate (all from Alexis Biochemicals) were used alone or in combination with DNA demethylating agents or all-trans retinoic acid (18, 19). These studies have shown clinical remissions. HDACi can act synergistically with certain DNA-binding drugs in experimental models (20); thus, combination treatment is being investigated in clinical trials.

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Analysis of drug efflux by flow cytometry. After culturing in the presence or absence of phenylbutyrate or valproate, cells were harvested and transferred to DMEM/fetal bovine serum and 30 ng/mL BODIPY-paclitaxel (Molecular Probes). After 30 min at 37°C, cells were spun and resuspended in medium with or without 5 μmol/L cyclosporin A (Sigma-Aldrich) in addition to BODIPY-paclitaxel. One hour later, cells were spun, resuspended, and immediately analyzed by flow cytometry. To identify the amount of BODIPY-paclitaxel removed by drug efflux, histograms of cells in which P-gp was inhibited by cyclosporin A were overlaid. The distance between the two peaks thus indicates the amount of drug transport activity. Similarly, for analysis of BCRP transport activity, cells were loaded with 20 μmol/L mitoxantrone; fumitremorgin C (Alexis Biochemicals) at 10 μmol/L was used as an inhibitor.

Accumulation of radiolabeled drugs. [3H]daunorubicin, [3H]pacli- taxel, and [3H]5-fluorouracil (5-FU) were purchased from Hartman Analytic. For drug transport experiments, they were mixed with the respective cold compounds to adjust final drug concentrations as indicated. Cells were washed with PBS and counted. Viable cells (1 × 10⁶) were resuspended in medium containing radiolabeled drugs. After
incubation at 37°C for indicated periods, ice-cold PBS was added to the samples. Samples were collected on ice, spun, and transferred to scintillation tubes. Opti-Fluor solution (Perkin-Elmer) was added, and incorporated activities were assessed with a liquid scintillation counter (Perkin-Elmer). Results are expressed as drug content per 1 × 10^6 cells.

Analysis of apoptosis by Annexin V binding. Apoptosis induction was assayed by detection of phosphatidylserine on the plasma membrane as described earlier (26). In brief, cells were stained with FITC-labeled Annexin V (BD Pharmingen) and counterstained with propidium iodide. Numbers of apoptotic, that is, Annexin V-positive cells, were assessed by flow cytometry.

Real-time PCR. For real-time PCR, cDNA was prepared from 2 µg RNA using the Omniscript cDNA synthesis kit (Qiagen). Real-time PCR was done using Platinum SYBR Green qPCR SuperMix (Invitrogen) on a ABI-Prism 7700 (Applied Biosystems). Data analyses were done by comparing Ct values with a control sample set as 1. The unpublished primers are included in Supplementary Table S2.

Analysis of the MRP8 promoter region by rapid amplification of 5'-cDNA ends. CMK cells were incubated with or without 3 mmol/L valproate for 24 h. Total RNA was isolated, and a semiquantitative rt-PCR was done with primers specific for human ABC transporters. β-Actin mRNA expression was used as a control for equal loading. cDNA from SK-Hep-1 hepatoma cells served as a positive control for MRP2 expression. Representative experiment. B, modulation of MDR1, BCRP, MRP7, and MRP8 mRNA expression by SAHA was assayed by rt-PCR. KG-1a and HL-60 cells were cultured for 20 h with or without 2 or 8 µmol/L SAHA. β-Actin expression was used as loading control. C, effects of various HDACi on gene expression was analyzed by rt-PCR. Cells were treated with 165 mmol/L trichostatin A, 3 mmol/L valproate, or 3 mmol/L phenylbutyrate for 24 h or, as a negative control, with medium (M) or the solvent DMSO (D). Representative experiment. Bottom, fold expression of MDR1, BCRP, MRP7, and MRP8 after valproate or phenylbutyrate treatment relative to the expression in untreated cells. Data were calculated after quantification of band intensities with ImageJ software. Fold expression ± SD of three independent experiments. M, medium control. D, reversibility of gene induction. CMK cells were cultured for 24 h in the presence or absence of 3 mmol/L phenylbutyrate or 3 mmol/L valproate. Phenylbutyrate- or valproate-treated and untreated cells were then harvested, transferred to fresh medium, and cultured for another 24 h with or without the respective HDACi. RNA was isolated and rt-PCR was done. M, medium; W, washout.
from total RNA using a gene-specific primer 1 (Supplementary Table S2) and SuperScript II reverse transcriptase. A homopolymeric tail was then added to the 3’-end of the cDNA using terminal deoxynucleotidyl transferase and dCTP. PCR was done using Taq DNA polymerase, a deoxyinosine-containing anchor primer, and a gene-specific primer. The following nested PCR used an Abridged Universal Amplification Primer in combination with further gene-specific primers (Supplementary Table S2). PCR products were separated by agarose gel electrophoresis, isolated with a gel extraction kit (Qiagen), and analyzed by automated sequencing (Supplementary Table S3).

**Chromatin immunoprecipitation.** Chromatin immunoprecipitation was done as described elsewhere (27). Antibodies to acetylated histones H3, H4, and H3K9 were from Upstate. Rabbit IgG (Santa Cruz Biotechnology) served as a negative control. The protocol was slightly modified for background reduction by extension of a pre-clearing step to 3 h and extensive washing. Following reversal of histone-DNA crosslinks, DNA was isolated with the PCR Purification Kit (Qiagen). Fragments from promoter regions of MDR1, BCRP, and MRP8 were amplified by quantitative PCR using the SYBR Green Master Mix for LightCycler 480 (Roche). Specific primers are summarized in Supplementary Table S2.

***Fig. 2.*** HDACi increase the protein level and the transport activity of P-gp and BCRP. A, immunoblot analysis of P-gp and BCRP expression on phenylbutyrate treatment. CMK and KG-1a cells were treated with or without phenylbutyrate at the indicated concentrations for 24 h. β-Actin was used as a loading control. B, drug efflux on HDACi treatment. CMK and KG-1a cells were incubated in complete medium with 30 ng/ml BODIPY-paclitaxel for 30 min at 37°C and incubated for another 60 min at 37°C in 30 ng/ml BODIPY-paclitaxel in the presence or in the absence of 5 μmol/L cyclosporin A. BODIPY-paclitaxel fluorescence was determined by flow cytometry. The drug efflux activity is related to the distance between peaks in the presence (right peak) and absence (left peak) of the P-gp inhibitor cyclosporin A. Mean fluorescence relative to the mean fluorescence of untreated cells. Mean and SD of three independent experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001. C, experiments were done as in B. Mitoxantrone (20 μmol/L) was used with the inhibitor fumitremorgin C (10 μmol/L). Histograms of mitoxantrone in the presence and in the absence of fumitremorgin C are shown in separate panels because of short distance between the peaks. M, medium.
Results

Induction of multidrug resistance-associated genes by HDACi.
To investigate effects of HDACi on drug resistance genes, myeloid leukemia cells were incubated with SAHA. The expression levels of 11 ABC transporter genes were analyzed by semiquantitative rt-PCR. We first studied the time course of gene expression in CMK cells. Exposure to SAHA markedly increased MRP7, MRP8, and BCRP mRNA levels in CMK cells, starting after 8 to 12 h (Fig. 1A). The expression of MDR1 was also up-regulated in these cells but to a lesser extent. In contrast, MRP6 and MRP3 were down-regulated in CMK cells, whereas the effects on other ABC transporters were weak and inconsistent. For further studies, we decided to focus on transporters that were induced in these cells: MDR1, BCRP, MRP7, and MRP8.

SAHA induced MDR1, BCRP, and MRP7 expression in KG-1a and HL-60 cells; MRP8 was increased in KG-1a cells. The induction of drug resistance genes occurred in a dose-dependent manner (Fig. 1A and B). We also investigated valproate, trichostatin A, and phenylbutyrate. All analyzed HDACi induced the expression of ABC transporters; in some cells, >10-fold gene induction was noted (Fig. 1C). The pattern of gene induction is cell line specific.

Next, CMK cells were exposed to phenylbutyrate or valproate for 24 h, and HDACi were removed from the medium. MRP8, BCRP, and MDR1 were induced after 24 h and persisted for at least 48 h only if HDACi were continuously present (Fig. 1D). Removal of the HDACi caused a rapid down-regulation within 24 h. Induction of drug resistance genes was also reversible in KG-1a cells (data not shown).

Modulation of P-gp expression and substrate transport. We next analyzed protein expression. As shown in Fig. 2A, treatment with phenylbutyrate dose-dependently increased the level of P-gp in CMK and KG-1a cells. Induction of BCRP protein was observed in CMK cells. Modulation of the transport activity was analyzed with fluorescent substrates. CMK and KG-1a cells were pretreated with phenylbutyrate or valproate for 24 h. After washing, cells were loaded with BODIPY-paclitaxel. Flow cytometric analysis revealed that phenylbutyrate and valproate caused a significant reduction of the BODIPY-paclitaxel content in comparison with untreated CMK cells (Fig. 2B). Inhibition by cyclosporin A in control samples indicated that this was due to increased drug transport. Valproate-treated KG-1a cells displayed a comparable augmentation of efflux, whereas the modulatory effect of phenylbutyrate was insignificant in these cells (Fig. 2B). Furthermore, a moderate but reproducible decrease of mitoxantrone content was observed in HDACi-treated cells (Fig. 2C). In the presence of fumitremorgin C, a BCRP inhibitor, mitoxantrone accumulation was identical in HDACi-treated and untreated cells (Fig. 2C). We confirmed the modulation of drug transport with a second assay. To this end, accumulation of radiolabeled substrates was analyzed in time course experiments. Multidrug-resistant KB-8-5 and drug-sensitive KB-3-1 cells were used as biological specificity controls (28). Treatment with phenylbutyrate decreased the accumulation of labeled paclitaxel and daunorubicin in CMK cells (Fig. 3). Some reduction of paclitaxel accumulation was also detectable in KG-1a cells. Both CMK and KG-1a cells displayed a strikingly reduced 5-FU content after exposure to phenylbutyrate (Fig. 3). Expectedly, KB-3-1 and KB-8-5 cells accumulated similar amounts of 5-FU. In summary, both assays suggest that HDACi enhance drug efflux.

Analysis of primary hematopoietic cells. Leukemia cell lines do not necessarily reflect the patients’ disease. Therefore, we also analyzed primary hematopoietic cells. Mononuclear leukemic cells from a patient with relapsed AML were cultured with phenylbutyrate, valproate, or SAHA. All HDACi caused a
major increase of BCRP, MDR1, and MRP7 expression, whereas MRP8 was only augmented by phenylbutyrate (Fig. 4A). Functional BODIPY-paclitaxel efflux from the patient cells was increased following a 24 h exposure to phenylbutyrate (Fig. 4B).

Six additional AML patient samples were cultured with or without HDACi for 24 h. The expression of MDR1, BCRP, MRP7, and MRP8 was analyzed by real-time PCR. Increased expression of at least one of these drug resistance genes was observed in each sample (Fig. 4C). In two samples, a very strong induction of MDR1, BCRP, or MRP8 was noted. Taken together, HDACi may modulate the expression of MDR1, BCRP, MRP7, and MRP8 in leukemic cells from AML patients, but the pattern and intensity of modulation of these genes differ among patient samples.

Induction of a pleiotropic anticancer drug resistance phenotype by HDACi. Next, we investigated anticancer drug resistance in KG-1a cells. We did not use CMK cells because they showed a modulation of the cell cycle status on phenylbutyrate or valproate treatment as detected by propidium iodide staining and the induction of the cell cycle inhibitor p27 (Supplementary Fig. S1A-C). KG-1a cells were pretreated with phenylbutyrate for 24 h followed by a 72 h anticancer drug exposure. The cytotoxic effect was then determined by assessment of apoptosis using flow cytometry. Exposure to phenylbutyrate decreased the chemosensitivity toward etoposide, daunorubicin, vinblastine, paclitaxel, and topotecan (Fig. 5A). Furthermore, cells were rendered less sensitive to pyrimidine analogues including 2′,3′-dideoxycytidine, cytosine arabinoside, 5-FU, and gemcitabine (Fig. 5A). The phenylbutyrate-mediated protection of drug-induced apoptosis was also shown by inhibition of mitochondrial membrane potential loss and DNA fragmentation (data not shown). Protection from cytotoxic drugs was dose dependent. The percentage of nonapoptotic KG-1a cells in the presence of 4 mmol/L 5-FU, which was 5% without HDACi, was increased to 32% in the presence of 3 mmol/L phenylbutyrate (Fig. 5B). Similar results were obtained when phenylbutyrate and anticancer drugs were added simultaneously. Extension of the phenylbutyrate pretreatment period to 2 days, however, failed to improve the chemoprotection against 5-FU

Fig. 4. Modulation of gene expression and transport activity in primary AML cells by HDACi. A, expression of drug resistance genes in primary AML cells. Mononuclear cells from peripheral blood of a patient with AML were isolated by Ficoll centrifugation. Cells were cultured for 24 h in the presence of phenylbutyrate, valproate, or SAHA at the indicated concentrations or in the absence of HDACi. Total RNA was isolated and semiquantitative rt-PCR was done for assessment of MDR1, BCRP, MRP7, MRP8, and, as a loading control, β-actin. M, medium. B, drug efflux from primary AML cells. Mononuclear cells from the same patient were cultured for 24 h with 6 mmol/L phenylbutyrate or without HDACi. Cells were then analyzed for BODIPY-paclitaxel efflux in the presence (white areas) or absence (shaded areas) of cyclosporin A. The threshold was set at <1% BODIPY-negative cells in medium alone with cyclosporin A. C, real-time PCR for drug resistance genes in primary AML cell samples. Mononuclear cells from six additional AML patients cultured with phenylbutyrate or valproate, respectively, at 3 mmol/L (patients 1-3) or 6 mmol/L (patients 4-6), or SAHA at 4 μmol/L (patients 1-3) or 8 μmol/L (patients 4-6), or were kept in the absence of HDAC-inhibitors. Total RNA was isolated and quantitative real-time PCR was done to determine MDR1, BCRP, MRP7, and MRP8 mRNA expression (normalized to ubiquitin). Each symbol represents one patient: patients 1 (○), patient 2 (●), patient 3 (△), patient 4 (□), patient 5 (↑), and patient 6 (●). Fold expression of MDR1, BCRP, MRP7, and MRP8 following treatment with phenylbutyrate, valproate, or SAHA relative to the amount found in untreated cells.
but increased the number of cells surviving etoposide. Conversely, the chemoprotective effect of phenylbutyrate treatment was lost when cells were first treated with chemotherapeutic drugs, and phenylbutyrate was added later (Fig. 5C).

Histone hyperacetylation of MDR1, BCRP, and MRP8 promoter regions by HDACi. Next, we sought to further investigate the mechanism of drug resistance gene induction by HDACi. The effects of valproate on histone acetylation status within promoter

![Figure 5](https://example.com/figure5.png)

**Fig. 5.** Induction of resistance to anticancer drugs by phenylbutyrate. A, inhibition of apoptosis induction by phenylbutyrate. KG-1a cells were incubated with 3 mmol/L phenylbutyrate for 24 h or cultured without HDACi. Chemotherapeutic agents etoposide (50 μg/mL; Eto), daunorubicin (1 μg/mL; Dauno), mitoxantrone (4 μmol/L; Mitox), vinblastine (0.5 μg/mL; Vin), paclitaxel (5 μg/mL; Pac), topotecan (1 μg/mL; Topo), 2′,3′-dideoxycytidine (5 mmol/L), 5-FU (4 mmol/L), gemcitabine (0.5 μmol/L; Gem), or cytosine arabinoside (5 μg/mL) were added, and cells were cultured for another 72 h. As control, cells were cultured in the absence of chemotherapeutic agents. Cells were harvested, washed, and incubated with FITC-labeled anti-Annexin V and propidium iodide for 15 min at 4°C. Numbers of Annexin V-positive cells were analyzed by flow cytometry and indicate nonapoptotic, Annexin V-negative cells. B, dose dependence of chemoprotection by phenylbutyrate. KG-1a cells were incubated with phenylbutyrate at the indicated concentrations for 24 h. Subsequently, 5-FU (4 mmol/L), mitoxantrone (4 μmol/L), or etoposide (40 μg/mL) was added and cultures continued for 72 h. Percentages of nonapoptotic cells were measured by Annexin V-FITC staining. Mean and SD of three independent experiments. C, HDACi-induced drug resistance depends on the sequence of treatment. KG-1a cells (1.2 × 10^6) were exposed to 20 μg/mL etoposide or 2 mmol/L 5-FU for a total of 72 h. Cells were treated with (gray columns) or without (white columns) 3 mmol/L phenylbutyrate for 24 or 48 h before or after the administration of anticancer drugs or were exposed to phenylbutyrate and anticancer drugs simultaneously. Apoptosis induction was assessed by flow cytometry after FITC-labeled Annexin V staining. Mean and SD of three independent experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
regions of MDR1 and BCRP were studied using chromatin immunoprecipitation assays. Valproate caused hyperacetylation of histone H4 but not of histone H3 within the MDR1 promoter. In contrast, hyperacetylation of histone H4 and additionally histones H3 and H3K9 of the BCRP promoter was shown in CMK cells (Fig. 6A).

MRP8 showed a strong gene induction (Fig. 1A–C). Therefore, we further evaluated the mechanism of MRP8 promoter regulation. We investigated the 5′-untranslated region of MRP8 mRNA by rapid amplification of 5′-cDNA ends PCR in CMK cells. The obtained PCR products (Supplementary Table S3) were compared with MRP8 mRNA sequences from the PubMed Nucleotide database (29, 30). In untreated cells, two of the three identified sequences (Fig. 6B, top transcripts) contained a short sequence next to the translation initiation site, directly linked to an exon located ∼12 kb upstream (Fig. 6B, transcripts 1 and 2). Although similar sequences have been described, a published exon 2 (30) and an upstream portion of exon 3 (29) were skipped in all MRP8 mRNAs obtained. A transcript lacking exon 3 and the ATG site was found, in which exon 4 is directly linked to exon 1 (Fig. 6B, transcript 3). Interestingly, with rapid amplification of 5′-cDNA ends experiments, we found alternative MRP8 transcription initiation sites in valproate-treated cells. A total of three additional transcripts were encoded on valproate treatment, and among those, two different 5′-mRNA ends were detected. Both variants contained the above-mentioned downstream portion of exon 3. One transcript included both exons 1 and 2 sequences but no part of the upstream portion of the exon 3 sequence. This transcript (transcript 4) was similar to the sequences found in untreated CMK cells (Fig. 6B, transcripts 1 and 2). Sequences of exon 3 upstream of the splice site were, however, only detected in valproate-treated cells (transcripts 5 and 6). To characterize these transcripts, primers were designed that exclusively amplify a sequence of the second variant (Supplementary Table S2). Figure 6C shows that CMK and KG-1a cells treated with valproate or phenylbutyrate expressed this transcript variant, whereas it was absent in untreated cells. Thus, we suggest that basal MRP8 expression is controlled by a promoter upstream of the first exon, whereas expression induced by HDACi is driven from an alternative promoter present within the 5′ portion of exon 3 or intron sequences upstream of exon 3.

Unexpectedly, valproate reduced the acetylation of histone proteins H3, H3K9, and H4 from the region upstream of exon 1.

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**Figure 6.** Analysis of histone acetylation in the promoter regions of MDR1, BCRP, and MRP8. A, chromatin immunoprecipitation assay. CMK cells were incubated with (gray columns) or without (white columns) 3 mmol/L valproate for 24 h. Antibodies to the indicated acetylated histones were used and the precipitated DNA was amplified by real-time PCR with specific primers for the MDR1 or BCRP promoter or MRP8 promoter regions upstream of exon 1 or within exon 3. Mean and SD of three independent experiments. B, physical mapping of the MRP8 promoter region. CMK cells were incubated with or without 3 mmol/L valproate for 24 h. Rapid amplification of 5′-cDNA ends PCR was done and the amplified PCR products were sequenced. The 5′-mRNA ends of the obtained MRP8 transcripts are displayed schematically and aligned with published MRP8 transcripts variants (11, 29, 30). Exons are designated as described previously (30). C, rt-PCR for detection of MRP8 transcript obtained from the HDACi-induced promoter within exon 3. CMK and KG-1a cells were treated with or without 3 mmol/L phenylbutyrate and 3 mmol/L valproate for 24 h. RNA was isolated and semiquantitative rt-PCR was done with primers that amplify a transcript that starts 20 bp upstream of a putative splice site of exon 3, thus identifying a transcript variant that is found after treatment with valproate. β-Actin was used as loading control. Representative experiment. M, medium.
In contrast, histone proteins in the 5‘-portion of exon 3 were hyperacetylated in valproate-treated cells (Fig. 6A). This finding is consistent with the results from rapid amplification of 5’-cDNA ends, where transcript variants regulated by the region inside of exon 3 are only detected in valproate-treated CMK cells (Fig. 6B, transcripts 5 and 6). In conclusion, our results suggest that the MR8 gene is regulated by different promoters, one of which is inducible by HDACi.

Discussion

In the present study, we show that HDACi induce multidrug resistance-associated genes in leukemia cells. Activation of ABC transporters including MDR1, BCRP, MRP7, and MR8 reduces the sensitivity to natural toxins and to cyclic nucleotides, which are clinically used as antiviral or anticancer agents (12). To our knowledge, induction of resistance to nucleoside analogues by HDACi has not been reported. We conclude that resistance to fluoropyrimidines results from the induction of MR8 and BCRP, respectively, as observed in KG-1a cells. BCRP confers resistance to gemcitabine but not to 5-FU, whereas MR8 expression renders cells resistant to 5-FU (12, 13). Induction of transporters for natural toxins as well as for nucleoside analogues was also detected in AML cells isolated from patients. The broad-spectrum cross-resistance we characterized in KG-1a cells thus exceeds the “classic multidrug resistance” phenotype related to P-gp as well as so-called “atypical multidrug resistance” associated with BCRP or MRP1 expression. It resembles clinical experiences that treatment failure is not limited to the known substrates of one ABC transporter. If cancers relapse after chemotherapy, they may become refractory to any available chemotherapy.

Our finding extends reports showing modulation of the MDR1 or the BCRP gene by HDACi. Depsipeptide increases BCRP expression in cancer cell lines and this modulation was accompanied by histone modifications such as an increase in histone acetylation in the BCRP promoter (22, 31, 32). Induction of MDR1 was shown in various malignant cells (21, 25, 33–35). Stepwise selection of cells with depsipeptide leads to induction of MDR1 expression (34). MDR1 induction by HDACi may, however, be cell type specific because down-regulation was reported in multidrug-resistant cancer cells (36).

In some models, however, trichostatin A fails to modulate MDR1 gene expression despite hyperacetylation of the promoter (37). Trichostatin A increases MRPI expression (38), whereas an epigenetic regulation of MRPI has not been reported. It is conceivable that other drug resistance genes might also be controlled by the chromatin status. In fact, we have noted decreased cytosine arabinoside sensitivity in phenylbutyrate-treated KG-1a cells. Cytosine arabinoside is a known substrate of the investigated ABC transporters; we hypothesize that additional genes associated with drug resistance may be included in a coordinate regulation.

To link the regulation of ABC transporter mRNA expression with development of anticancer drug resistance, we additionally studied changes of the drug transport function in two leukemia cell models. CMK and KG-1a cells displayed reduced accumulation of radiolabeled and fluorescent substrates following exposure to phenylbutyrate or valproate. The degree of reduction was compared with the efflux activity of colchicine-selected, multidrug-resistant KB-8-5 cells, which express MDR1 mRNA at levels comparable with those observed in clinical samples of refractory cancers (28). The induced drug efflux activities in AML cells are lower than the activity of KB-8-5 cells, suggesting that HDACi confer moderate levels of functional multidrug resistance. Nonetheless, phenylbutyrate protects a fraction of leukemia cells against highly cytotoxic concentrations of anticancer drugs. Our in vitro result may reflect the clinical finding that small populations of surviving cells are then responsible for relapses after chemotherapy. In accordance, the detection of minimal residual disease is predictive for relapse in acute leukemia and is associated with a MDR1 phenotype (39). In our studies, a coinduction of drug resistance genes was observed in cell lines and in primary cells. The patterns of induction vary from one leukemia cell type to another and thereby may reflect the heterogeneity of AML. The investigated AML cell lines display different phenotypes and contain diverse chromosomal abnormalities (40). It is therefore conceivable that HDACi do not cause one single drug resistance phenotype but rather inactivate different drugs in different cancer cells, reflecting the respective patterns of induced ABC transporters. In addition, the cytotoxicity of HDACi alone varies widely between different AML cell lines. Growth arrest resulting from induction of cell cycle inhibitors such as p21 and p27 may contribute to the antiproliferative effects of HDACi (41). On the other hand, cell cycle arrest allows for DNA repair and may reduce sensitivity to chemotherapy. The chemoprotective effects resulting from induction of drug resistance genes may be balanced by the cytotoxic activities of HDACi. HDACi have been shown to increase the sensitivity of cancer cells to anticancer drugs targeting DNA (20). Results presented here show that the sequence of administration may be critical for the effectiveness of such approaches. In summary, combinations of HDACi with anticancer agents achieve increased rates of apoptosis in certain cellular models (42–44) but might also lead to unwanted cellular or clinical outcomes. From our findings, HDACi should be administered after chemotherapy to avoid inactivation of cytotoxic drugs. Our results should, however, be translated into the clinical practice with caution. HDACi concentrations in our investigation are above clinically relevant plasma levels. Although plasma levels of 8 to 11 μmol/L SAHA have been achieved in a phase I study (45), the levels after oral administration are ~10-fold lower. Similarly, although maximum concentrations of phenylbutyrate at 3.5 mmol/L have been achieved (46), the recommended plasma concentration is 0.5 μmol/L (47). The maximum valproate plasma concentration is 0.27 to 0.76 mmol/L (48). Trichostatin A is not used in the clinic because of its toxicity.

To better understand how HDACi control the expression of drug transporter genes, we analyzed the acetylation status of the MDR1, BCRP, and MR8 promoter regions. We find that histones in the promoter regions of MDR1, BCRP, and MR8 are acetylated in the presence of valproate. These findings are in accordance with previous studies, although the histone proteins that are subject to acetylation within the MDR1 promoter region may vary in different AML cells (25, 34). Hyperacetylation of the MDR1 promoter by HDACi is not necessarily accompanied by increased P-gp expression. For instance, trichostatin A fails to induce MDR1 expression in CEM-CCRF cells due to hypermethylation of the promoter (37). Moreover, in K562 cells,

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4 S. Dreyer and T. Licht, unpublished data.
MDR1 mRNA expression is not paralleled by P-gp due to a translational blockade (49). Few investigations have analyzed the MRP8 promoter region (11, 29, 30). Here, we have identified two mRNA variants that differ at their 5’-ends, one of which is observed only after exposure to HDACi. Similarly, the expression of the MDR1 gene is also controlled by two different promoter regions, one of which is active in drug-selected cancer cells but not in normal tissues (50). In conclusion, such as MDR1 and BCRP, the MRP8 gene contains a promoter region that is inducible by changes of the chromatin structure due to treatment with HDACi.

In conclusion, we have identified a coordinate regulation of several multidrug resistance genes on HDACi in malignant hematopoietic cells that causes a pleiotropic resistance to anticancer drugs. This finding may help to explain the clinical experience that cancers can become cross-resistant to drugs that have not been administered before and are not mediated by one single drug resistance protein. Our data suggest that combined treatment with HDACi and chemotherapeutic agents may result in treatment failure. Induction of drug resistance genes is, however, reversible and depends on the sequence of treatment. Hence, it appears feasible to design clinical protocols for a combination of chemotherapy with HDACi in which inactivation of anticancer drugs can be avoided.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


Histone Deacetylase Inhibitors Induce a Very Broad, Pleiotropic Anticancer Drug Resistance Phenotype in Acute Myeloid Leukemia Cells by Modulation of Multiple ABC Transporter Genes

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