

Induction of gene expression by 5-Aza-2'-deoxycytidine in acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) but not epithelial cells by DNA-methylation-dependent and -independent mechanisms

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The methylation inhibitor 5-Aza-2'-deoxycytidine (5-Aza-CdR, decitabine) has therapeutic efficacy in acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS). Using microarray analysis, we investigated global changes in gene expression after 5-Aza-CdR treatment in AML. In the AML cell line OCI-AML2, Aza-CdR induced the expression of 81 out of 22000 genes; 96 genes were downregulated (≥ 2 -fold change in expression). RT-PCR analysis of 10 randomly selected genes confirmed the changes of expression in AML cells. Similar results were obtained with primary AML and MDS cells after treatment with 5-Aza-CdR *ex vivo* and *in vivo*, respectively. In contrast, significantly fewer changes in gene expression and cytotoxicity were detected in normal peripheral blood mononuclear and bone marrow cells or transformed epithelial cells treated with 5-Aza-CdR. Interestingly, only 50.6% of the induced genes contain putative CpG islands in the 5' region. To further investigate the significance of promoter methylation in the induced genes, we analyzed the actual methylation status of randomly selected 5-Aza-CdR-inducible genes. We detected hypermethylation exclusively in the 5' region of the myeloperoxidase (MPO) gene. DNA methylation inversely correlated with MPO expression in newly diagnosed untreated AML patients ($P \leq 0.004$). In contrast, all other analyzed 5-Aza-CdR-inducible genes revealed no CpG methylation in the promoter region, suggesting a methylation-independent effect of 5-Aza-CdR.

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Introduction

Epigenetic gene silencing through DNA methylation plays a crucial role in leukemogenesis.¹ Aberrant hypermethylation in cancer cells predominantly occurs in the CpG-rich 5' region of tumor suppressor genes and is mediated by DNA-methyltransferases (DNMTs) subsequently to cell division.² Elevated expression levels of one of the four DNMTs have been found in leukemic blast cells compared to normal blood cells in acute myeloid leukemia (AML) patients.³ Promoter hypermethylation probably follows a tumor-type-specific pattern.⁴ In leukemias, DNA methylation has been reported for several genes, including the estrogen receptor,⁵ E-cadherin,⁶ HIC1 and calcitonin.⁷ Hypermethylation of the p15INK4b tumor suppressor gene is associated with its inactivation in at least 50% of patients with acute leukemia.^{8,9} Furthermore, hypermethylation of p15INK4b

is concomitant with disease progression in myelodysplastic syndrome (MDS).¹⁰

Cytosine analogues such as 5-Aza-2'-deoxycytidine (5-Aza-CdR) have been shown to reactivate the expression of a variety of genes including tumor suppressors in malignant cells *in vitro* and *in vivo* by inhibiting DNMTs.¹¹ In recent reports, the usage of low-dose 5-Aza-CdR as a therapeutic agent (decitabine) was very encouraging in older patients with MDS and AML.^{12,13} Recent data demonstrate that DNA-incorporated 5-Aza-CdR not only binds and inactivates DNMTs but in addition acts as an inhibitor of the methylation of histones, both modifications leading to gene reactivation.¹⁴ This may account for several reports showing that 5-Aza-CdR can induce expression of genes with unmethylated CpGs, like CDKN2D (p19INK4d) or Apaf-1.^{15,16} In addition, 5-Aza-CdR's ability to bind DNMTs has been attributed to its cytotoxic activity as a methylation-independent function.^{17–19} In summary, the mechanism of action of 5-Aza-CdR accounting for its clinical benefit in myeloid leukemias is not completely understood. Recent studies have analyzed changes in global gene expression by 5-Aza-CdR focusing on epithelial cell lines.^{20–22} Using an oligonucleotide microarray and RT-PCR, we investigated global changes in gene expression induced by both *ex vivo* and *in vivo* treatment of AML or MDS cells with 5-Aza-CdR. We show that 5-Aza-CdR preferentially alters gene expression in malignant myeloid but not in nonmalignant blood cells or transformed epithelial cells. Only 50% of the induced genes contain CpG islands in their 5' region. Moreover, hypermethylation was only detectable in a minority of the 5-Aza-CdR-inducible genes. Our study indicates an additional, methylation-independent effect of 5-Aza-CdR in myeloid cells.

Materials and methods

Cell culture, 5-Aza-CdR treatment and RNA preparation

A total of 1×10^7 cells of the human AML cell line OCI-AML2 were treated for 3 days with a single pulse of $1 \mu\text{M}$ 5-Aza-CdR (Sigma, Taufkirchen, Germany) or were left untreated in MEM α medium (Invitrogen, Karlsruhe, Germany). For control experiments, AML cell line KG-1, HeLa, 293 cells (DSMZ, Braunschweig, Germany) as well as PBMCs and normal bone marrow cells isolated using Ficoll gradient centrifugation of whole blood cells or bone marrow from healthy donors were treated with a single pulse of 0, 1 or $5 \mu\text{M}$ 5-Aza-CdR for 3 days. In addition, primary MDS bone marrow mononuclear cells after *in vivo* treatment with 5-Aza-CdR as described elsewhere as well as primary AML blasts (heparinized peripheral blood) were isolated using Ficoll gradient centrifugation.¹¹ The local ethics committees had approved this study and written consent was obtained

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from the patients. Total RNA was extracted with RNeasy RNA preparation Kit (Qiagen, Hildesheim, Germany) according to the manufacturer's instructions. For microarray analysis, two independent experiments were performed and the corresponding total RNAs were pooled.

Oligonucleotide microarray analysis

Total RNA (10 μ g) isolated from treated and untreated OCI-AML2 cells were used for cDNA synthesis with Superscript II (Invitrogen, Karlsruhe, Germany) and an oligo-dT primer with a T7 RNA polymerase binding site (Tibmolbiol, Berlin, Germany) for 1 h at 42°C. After second-strand cDNA synthesis, *in vitro* labeling was performed according to the manufacturer's instructions using the High Yield RNA labeling kit (Enzo, London, UK). Fragmented cRNAs (15 μ g) in 6 \times SSPE-T hybridization buffer (1 M NaCl, 10 mM Tris (pH 7.6), 0.005% Triton) were loaded on the Affymetrix probe array cartridges HG U133A (Affymetrix, London, UK) and hybridized for 16 h, followed by washing, staining and scanning procedure as described in the user manual.

Methylation-specific PCR (MSP)

Bisulfite-modified genomic DNA as template and the primers MPO-meth. sense 5'-CGGCGGGAAAGGTGGGGTAC-3' and MPO-meth. antisense 5'-GCTACGAAACCGCTACTTAATAC-3' were used for amplification of CpG-methylated sequences of MPO. The PCR conditions (94°C for 5 min, 94°C for 30 s, annealing for 30 s at 64.6°C, elongation at 72°C for 1 min and a final cycle at 72°C for 7 min) were optimized with OCI-AML2 genomic, bisulfite-converted DNA as a positive and HL60 DNA as a negative control. For the 'unmethylated' PCR, the primers MPO-unmeth. sense 5'-TGGTGGGAAAGGTGGGGTAT-3' and MPO-unmeth. antisense 5'-CAACCCTCACCACCATCCTA-3' under conditions: 94°C for 5 min, 94°C for 30 s, annealing for 30 s at 64.0°C, elongation at 72°C for 1 min and a final cycle at 72°C for 7 min were used. Here, HL60 DNA functioned as positive and OCI-AML2 DNA as negative control.

Results

Global changes in gene expression induced by 5-Aza-CdR in AML cells

To assess global changes in gene expression induced by 5-Aza-CdR in myeloid leukemia cells, we performed a microarray analysis. The AML cell line OCI-AML2 was exposed to a single pulse of low-dose 5-Aza-CdR (1 μ M for 3 days) or was left untreated. Drug concentration and incubation time were chosen to achieve a distinct effect on gene expression without inducing excessive cell death (<15%; see Figure 2b). We used expression level and methylation status of p73, which is known to be regulated by promoter hypermethylation in AML cells as a positive control (data not shown²³).

After treatment of the AML cell line OCI-AML2, microarray analysis using oligonucleotide arrays HG U133A (Affymetrix, London, UK) containing 22 000 genes was performed. Comparing mRNA expression level of treated vs untreated AML cells, we identified 81 genes with an increase in expression after 5-Aza-CdR exposure (Supplementary Table 1), while 96 genes were downregulated (data not shown). The differentially

expressed genes could be categorized in regulators of cell cycle control, differentiation, transcription factors, cell adhesion, apoptosis, cytokine signaling, stress response and metabolism. As a proof of principle of the system, we found HLA-DR, myeloperoxidase (MPO) and p21/WAF1/Cip1 to be upregulated (Supplementary Table 1), CpG-rich genes known to be regulated by methylation in leukemias.^{24–26} The p15 gene was already expressed in uninduced cells and not significantly regulated in these cells (data not shown).

Fifty percent of genes induced by 5-Aza-CdR do not contain CpG islands

In all, 70–80% of methylated cytosines are found in (so-called) CpG islands.¹ To find putative methylated target sites in the 5' region of 5-Aza-CdR-induced genes, we searched for CpG islands in the promoter region using the cpGplot software (<http://www.ebi.ac.uk/emboss/cpgplot/>). Interestingly, only 50.6% of the analyzed upregulated genes displayed CpG islands in the 5' region of their gene sequence comprising a minimum of 500 bp upstream of the transcription start site, exon 1 and the 5' end of intron 1 (Supplementary Table 1). Therefore, most genes activated by 5-Aza-CdR may not result from the direct inhibition of promoter methylation in AML cells.

5-Aza-CdR selectively induces genes in myeloid leukemia cells

To ensure that microarray data represent real changes in expression in OCI-AML2 cells, we randomly selected 10 genes with a ≥ 2 -fold increase in expression after treatment with 5-Aza-CdR for further analysis. Semiquantitative RT-PCR analysis confirmed the induction of gene expression after treatment with 5-Aza-CdR in OCI-AML2 (Figure 1a). The expression of all 10 genes, that is, for the surface antigen CD14, Rab13, a gene coding for a member of the Ras oncogene family, the cell cycle regulator genes CCNA1 (cyclin A1) and CDKN1A (p21/CIP/Waf), the MPO, glyco, encoding for the glycoprotein, PTPN22, encoding for a tyrosine phosphatase, calgranulin (cal), ERK1 (ERK) and RPGR encoding for the retinitis pigmentosa GTPase regulator, showed upregulated expression after treatment. The changes of expression were quantified by scanning and analyzing with the NIH image software (Figure 1a). Comparable results were observed for both methods. Additionally, we could confirm these results for most of the genes in the AML cell lines KG1 and EOL1 (Figure 1b) suggesting a panel of 5-Aza-CdR-induced genes that might reflect AML-specific targets of the drug.

Next, we treated the epithelial cell lines HeLa and 293 as well as PBMC (peripheral blood mononuclear cells) of healthy donors with a single pulse of 0, 1 or 5 μ M 5-Aza-CdR for 3 days. Even at the higher concentration of 5 μ M, semiquantitative RT-PCR analysis revealed strikingly less changes in expression in transformed epithelial cells (293 and HeLa) and normal PBMC compared to OCI-AML2 cells (Figure 1c). This is unlikely due to abrogated drug uptake in these cells since limited gene induction was detectable for cyclin A1 and glyco (Figure 1c). In summary, the drug was more effective in AML cells than in epithelial and nonmalignant blood cells.

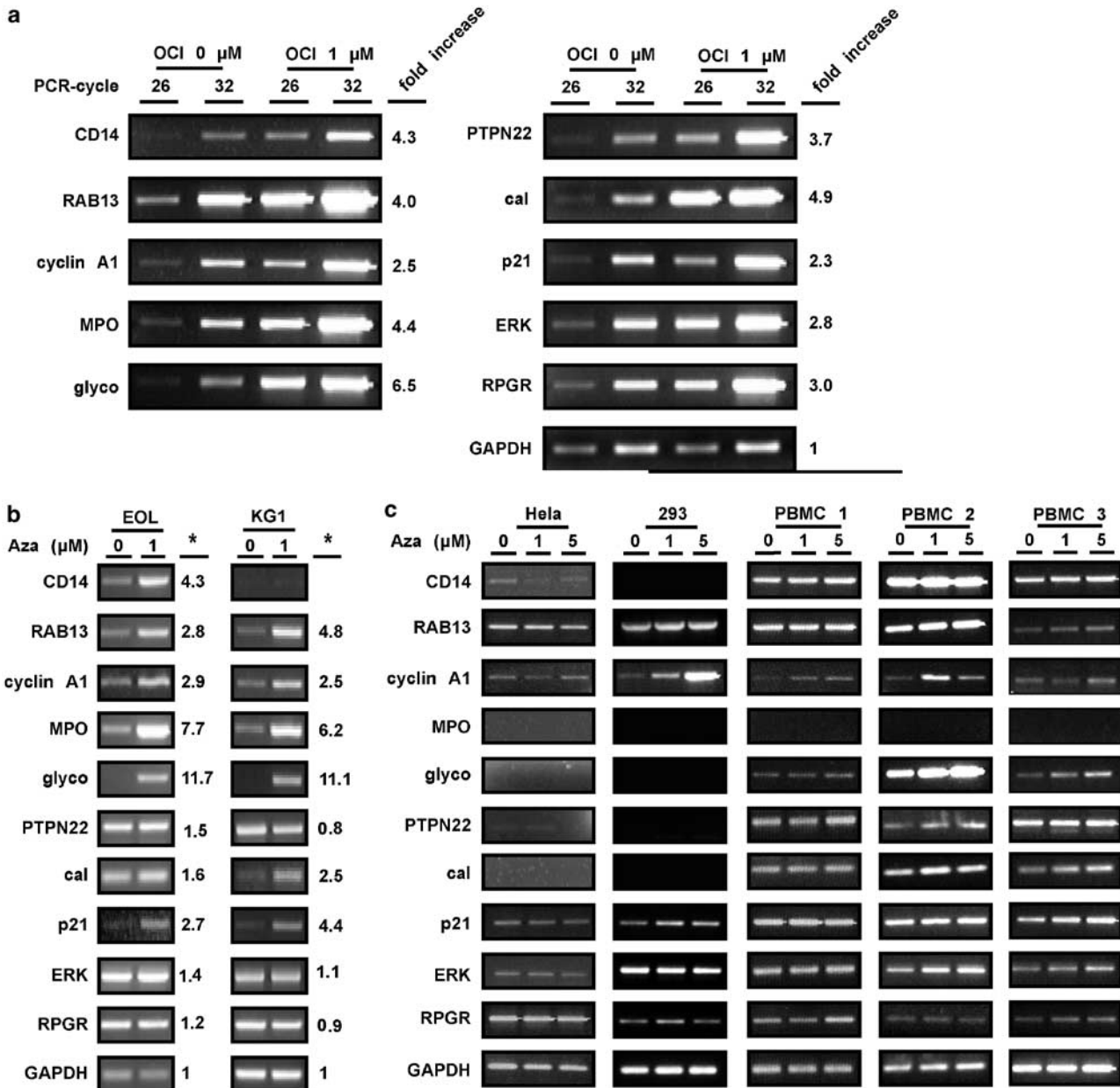


Figure 1 5-Aza-CdR selectively alters gene expression in AML but not in epithelial cells or normal PBMCs. (a) Semiquantitative cycle-dependent RT-PCR analysis of gene expression from one out of two independent experiments with OCI-AML2 cells after 3 days of treatment with a single pulse of 5-Aza-CdR is shown. GAPDH expression was used as loading control. Fold increase in expression after quantification with the NIH image software is indicated (mean of two PCR cycles and two independent determinations). (b) Similar results were observed in KG-1 and EOL cells. (c) Gene expression data from 293 cells, HeLa cells and three different PBMC preparations after 3 days of treatment with a single pulse of 0, 1 or 5 μ M 5-Aza-CdR. GAPDH expression level was used as a control.

5-Aza-CdR inhibits proliferation and induces apoptosis in AML but not in epithelial cells

To correlate change of expression with cell biology, proliferation assays were performed using myeloid and epithelial cell lines after treatment with 5-Aza-CdR. There was a dose- and time-dependent inhibition of proliferation in OCI-AML2 and KG-1 cell lines (Figure 2a). In contrast and fitting with expectations based on gene expression data, no changes in proliferation rate were seen in 293 and HeLa cells after 5-Aza-CdR treatment (Figure 2a). Similar results were obtained in apoptosis assays using FACS analysis to quantify DNA fragmen-

tation (Figure 2b). These results indicated significantly higher sensitivity of AML than epithelial cells to the proapoptotic effects of 5-Aza-CdR.

Gene induction after in vivo and ex vivo 5-Aza-CdR treatment of MDS patients and AML cells

To verify that the genes extracted by microarray analysis and RT-PCR are relevant for the *in vivo* situation, we treated primary AML cells (cultivated under proliferating conditions) with a single pulse of 0, 1 or 5 μ M 5-Aza-CdR for 5 days *ex vivo*.

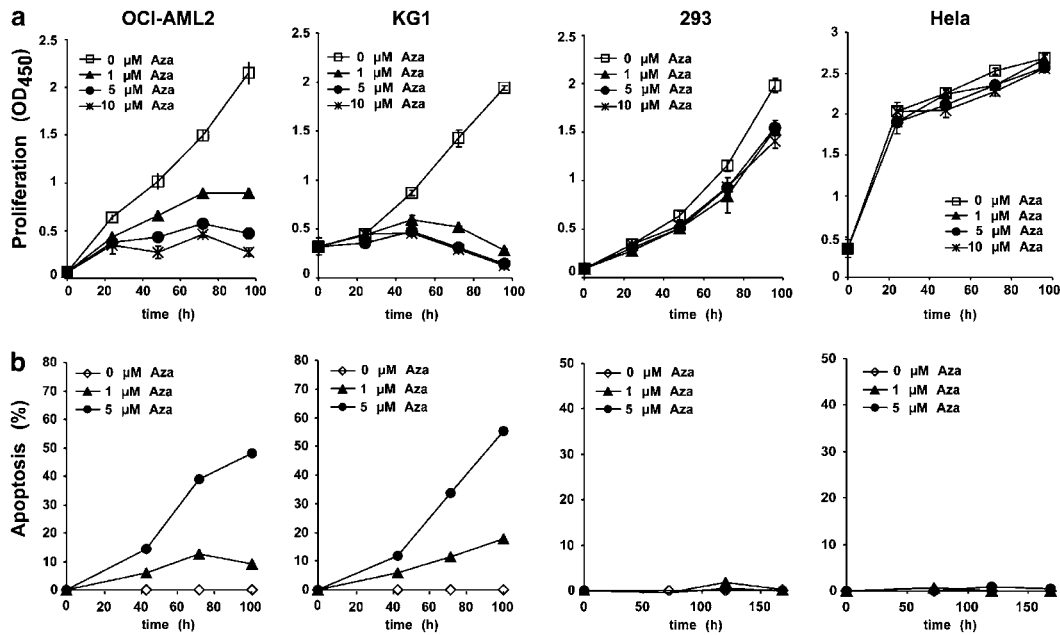


Figure 2 5-Aza-CdR has an antiproliferative and cytotoxic effect on AML but not on epithelial cells. (a) The AML cell lines OCI-AML2 and KG-1 as well as HeLa and 293 cells were treated with 0–10 μM 5-Aza-CdR for the indicated time points. Proliferation was measured using XTT assay by adding 50 μl XTT solution (1 mg/ml XTT, 50 μM PMS, Sigma, Taufkirchen, Germany) to 100 μl cell suspension in RPMI medium without Phenolred at the indicated time points. After incubation with XTT for 3 h at 37°C, proliferation was detected as OD₄₅₀ (mean \pm s.d. for four determinations). (b) Apoptosis induced by 5-Aza-CdR was determined by measurement of DNA fragmentation after fixation of the cells in 2% formaldehyde, RNase (50 $\mu\text{g}/\text{ml}$) digest and staining with 50 $\mu\text{g}/\text{ml}$ propidium iodide. Data are given as the percentage of hypodiploid cells using a FACScan cytometer equipped with the CELLQuest software (Becton Dickinson; Heidelberg, Germany), that is, cells in sub-G1, reflecting the number of apoptotic cells.

Semiquantitative RT-PCR was performed and quantified by NIH image analysis (Figure 3b). After *ex vivo* 5-Aza-CdR-treatment of AML cells from two of three patients with newly diagnosed, untreated AML, we could confirm gene induction for most of the 10 randomly selected genes (Figure 3b).

Importantly, similar results were obtained with primary MDS samples after *in vivo* treatment of MDS patients with low-dose decitabine (achieving estimated plasma levels of 100–500 nM) in a phase II clinical study.¹² Bone marrow mononuclear cells were collected before, during and after treatment with decitabine. Again, semiquantitative RT-PCR confirmed the induction of most of the genes after treatment *in vivo* as shown in Figure 3a, while normal bone marrow cells incubated under the same conditions displayed no expression changes (Figure 3c). Our data suggest that the isolated genes represent *in vivo* targets for 5-Aza-CdR in malignant myeloid cells.

Analysis of the DNA methylation status of 5-Aza-CdR-inducible genes by bisulfite sequencing

To study the mechanism of action of 5-Aza-CdR in more detail, we analyzed DNA hypermethylation of five randomly selected genes, which were induced by 5-Aza-CdR in AML and MDS cells, that is, RPGR, CD14, PTPN22, calgranulin and MPO (Figures 1 and 3). Sequence analysis using the cpGplot software proposed CpG islands in the promoter region of RPGR and CD14 gene but not in the sequence of PTPN22 and calgranulin (Supplementary Table 1 and Figure 4 a–d). To analyze the actual DNA methylation status of these genes, we performed bisulfite sequencing. Genomic DNA of untreated OCI-AML2 cells was bisulfite-modified and subsequently amplified by PCR. The PCR

fragments were cloned and used for bisulfite genomic sequencing comprising the proposed CpG islands and mapping between –356 and +277 and –109 and +497 in the 5' region of RPGR (Figure 4a) and CD14 (Figure 4c), respectively. In the genomic sequence of PTPN22 and calgranulin, a region between –257 and +162 as well as –301 and +92 was analyzed (Figure 4b and d). Remarkably, none of these four genes displayed methylated CpGs in their promoter region (Figure 4a–d). Therefore, we conclude that the mechanism of gene induction of RPGR, CD14, PTPN22 and calgranulin was not related to a direct methylation inhibitory function of 5-Aza-CdR. However, induction of these genes by 5-Aza-CdR could be a result of demethylation of upstream regulators.

Hypermethylation of the 5' region of the MPO gene in AML cells

MPO is an abundant enzyme in primary mature granulocytes catalyzing the synthesis of hypochlorous acid as an antimicrobial substance.²⁵ MPO mRNA is transcribed during the late myeloblast and promyelocyte stage of myeloid development. DNA methylation of the 5' region of MPO regulates the development-dependent expression of the gene in myeloid cells.²⁵

In our study, MPO was the only gene out of five analyzed that showed a DNA methylation in the 5' gene region (Figure 5a). Since the genomic fragment comprising the promoter region (–185 to +499) displayed only two CpGs (with 100% methylation), we expanded the bisulfite genomic sequencing to a 631 bp fragment (MPO fragment 2) spanning 13 CpGs within exons 2 to 3 covering +446 to +1077 (Figure 5a). The

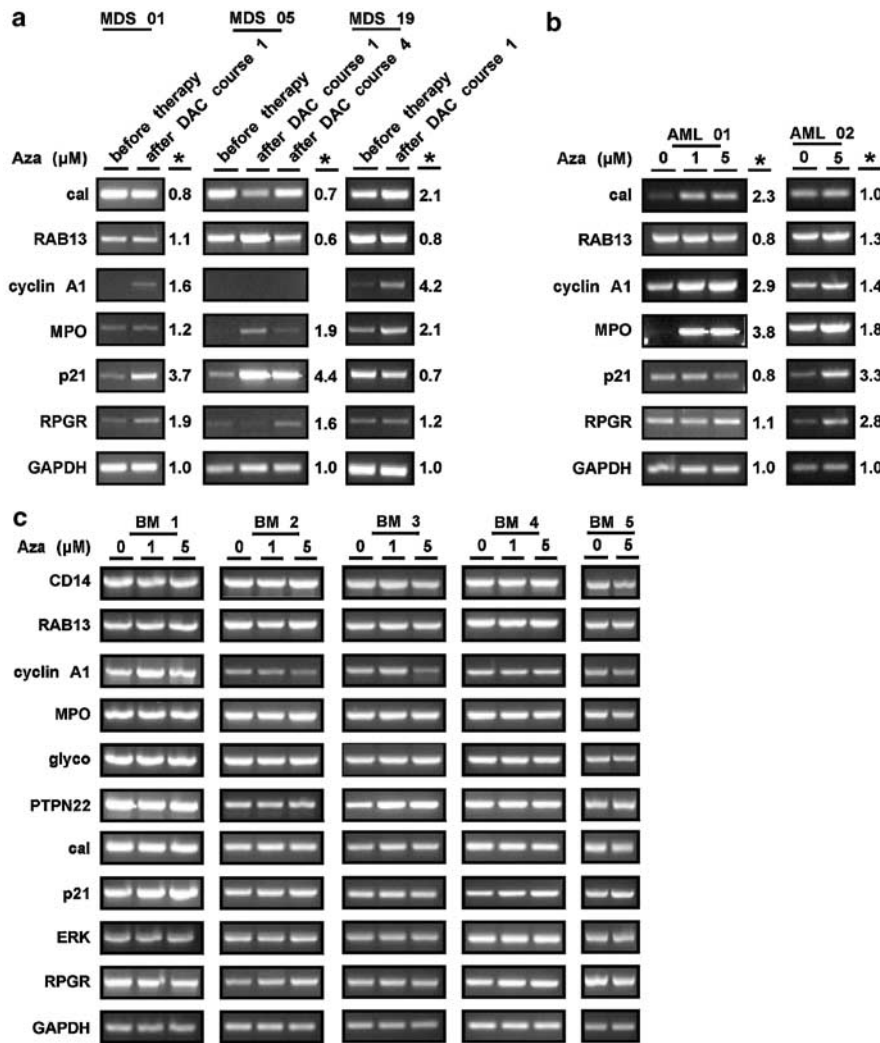


Figure 3 Induction of gene expression in primary AML and MDS but not in normal bone marrow cells by 5-Aza-CdR. (a) RT-PCR analysis of primary bone marrow mononuclear samples of MDS patients before and after *in vivo* treatment with different courses of decitabine (DAC) as described in Daskalakis *et al.*¹¹ (b) AML blasts were treated *ex vivo* (single pulse) with 0 μM (0), 1 μM (1) or 5 μM (5) 5-Aza-CdR for 5 days in a proliferation medium containing 0.2 ng/ml GM-CSF, 0.2 ng/ml IL-3 and 0.2 ng/ml SCF. Fold change of expression is indicated by (*) after quantification with NIH image software. AML1: FAB subtype AML M1 with 84% blast; AML2: AML M2 with 27% blasts. (c) Freshly isolated and Ficoll separated normal bone marrow aspirates were treated *ex vivo* with 0 μM (0), 1 μM (1) or 5 μM (5) 5-Aza-CdR for 5 days without stimulating conditions (BM 1–3) or were cultivated in the same proliferating medium (s. above) (BM 4 and BM 5).

pattern of methylation of the upstream MPO gene region correlated inversely with low MPO mRNA expression in OCI-AML2 cells. Our findings suggest that in contrast to RPGR, PTPN22, CD14 and calgranulin, the gene induction by 5-Aza-CdR of the MPO gene was linked to the inhibition of DNA methylation.

On the basis of the bisulfite genomic sequencing data of the MPO gene, we devised an MSP. OCI-AML2 genomic bisulfite-treated DNA was used as a positive control for the 'methylated' PCR. Here, primers were used that bind to methylated CpG with cytosine remaining unconverted after bisulfite treatment²⁷ (see Figure 5a for primer binding sites). We used the AML cell line HL60 as a positive control for the 'unmethylated' PCR with primers binding exclusively to unmethylated CpGs. The high mRNA expression of HL60 correlated with completely unmethylated CpGs in MPO fragments 2 and 3 as shown by bisulfite genomic sequencing²⁵ (data not shown).

Our analysis of the MPO gene indeed showed that the MPO 5' region is methylated in OCI-AML2 cells. Additionally, MPO expression was inducible by 5-Aza-CdR in AML cell lines as well as in MDS and AML patient samples but not in HeLa, 293 or normal PBMCs (Figures 1 and 3). These findings led us to investigate the methylation status of the MPO gene in AML patient samples. We analyzed 93 bone marrow and peripheral blood cell samples of patients with newly diagnosed AML using MSP for hypermethylation of the MPO gene. Representative results of the MSP with OCI-AML2 and HL60 as controls are shown in Figure 5b. Densitometric analysis of the MSP amplicates was used to quantify the degree of methylation as the ratio of the 'unmethylated' vs 'methylated' PCR product. Samples with neither a positive signal for 'methylated' nor for 'unmethylated' were excluded from the analysis resulting in 78 remaining samples. A total of 25 probes (32.1%) showed only a signal for methylated MPO, of which one sample was completely unmethylated (1.3%), while 52 samples (66.7%)

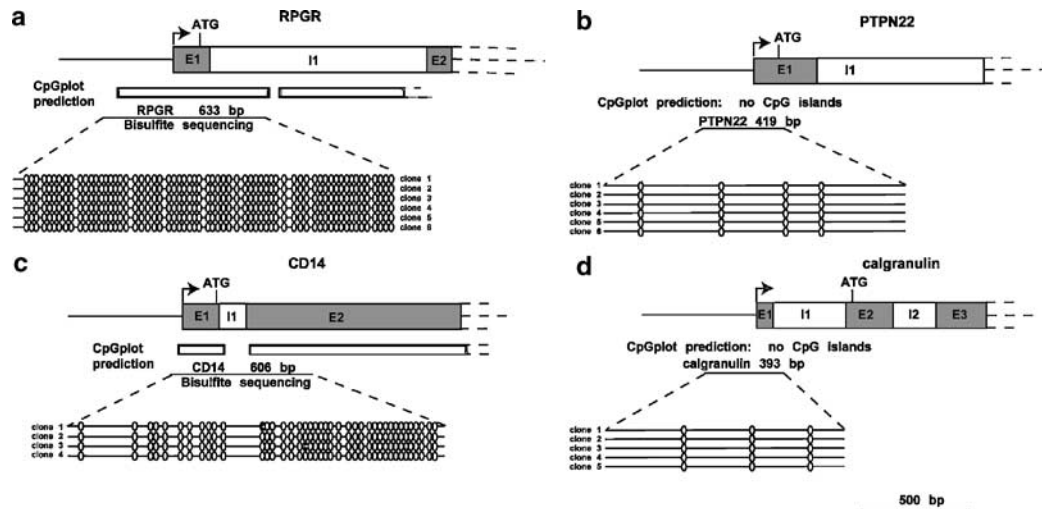


Figure 4 Bisulfite genomic sequencing revealed no methylated CpGs in the 5' gene region of RPGR, CD14, PTPN22 or calgranulin. Genomic DNA of untreated OCI-AML2 cells was bisulfite-modified. Specific genomic fragments for RPGR (a), PTPN22 (b), CD14 (c) or calgranulin (d) upstream regions were amplified as indicated. The proposed positions of CpG islands analyzed using the cpGplot software are shown as black bars. The actual position of CpGs and the result of bisulfite genomic sequencing of four to six independent clones are shown; unmethylated CpG (open circles) and methylated CpG (black filled circles). Position of transcription start site (arrows), exons (E) and introns (I) based on NCBI database.

displayed both methylated as well as unmethylated CpGs. The ratio of unmethylated vs methylated amplicates of the later 52 samples ranged from 0.032 to 3.831. For statistical analysis, the ratio of unmethylated vs methylated amplicates as the result of the MSP was used to group the 78 AML samples in (a) highly methylated (completely methylated and ratio ≤ 0.3), (b) intermediate (ratio 0.3–1.7) and (c) low methylated (ratio > 1.7 or completely unmethylated). We found a significant inverse correlation between intracytoplasmic MPO expression and the degree of MPO DNA methylation (Kruskal–Wallis test, $P \leq 0.004$). No correlation was found between DNA methylation of the MPO gene and clinical parameters like FAB classification, white blood cell count, response to therapy, overall survival and disease-free survival (data not shown). The inverse correlation between MPO gene methylation and MPO expression suggests that methylation is a relevant regulatory mechanism for MPO expression in AML cells.

Discussion

With more knowledge on the importance of epigenetic alterations for malignant growth, there is increasing interest in the DNMT inhibitor 5-Aza-CdR. Many reports show the ability of 5-Aza-CdR to reinduce the expression of a distinct gene by inhibiting its DNA methylation, but few studies analyze more global effects of the drug and none investigated leukemic cells.^{20–22} We therefore performed a genome-wide analysis to detect global changes in gene expression induced by the treatment of an AML cell line with 5-Aza-CdR. We found 81 genes with an increase and 96 genes with a decrease in expression in 5-Aza-CdR-treated vs -untreated OCI-AML2 cells. The relatively low number of altered genes, 81 increased and 96 decreased genes out of 22 000 (0.37 and 0.44%, respectively), indicate a specific effect of 5-Aza-CdR at the molecular level. Parallel analysis of DNA fragmentation showed less than 15% apoptosis under the chosen treatment condition of 1 μM 5-Aza-CdR for 3 days, excluding cytotoxicity as a major inducing signal. The 5-Aza-CdR concentration of 1 μM employed in our

experiment is within a concentration range used for various cell types and is in line with the estimated steady-state serum levels (0.1–0.5 μM) in 5-Aza-CdR-treated patients *in vivo*,²⁸ indicating physiological treatment conditions.

The 5-Aza-CdR is a newly revised therapeutic substance for the treatment of MDS, AML and CML. Its effectiveness is currently investigated in phase I–III clinical studies.^{12,13,29} Therefore, the specificity of 5-Aza-CdR for malignant cells, especially for myeloid malignant cells, is an important issue. In our study, 5-Aza-CdR induced gene expression only in AML and MDS cells but not in epithelial cells. This is remarkable since similar results were obtained in highly proliferating AML cell lines and in primary, *ex vivo*-treated AML cells with a low proliferation rate in cell culture. Finally, gene induction by 5-Aza-CdR could be confirmed for most genes in bone marrow cells of MDS patient samples treated *in vivo*.

Specific gene induction by low-dose 5-Aza-CdR in AML but not in epithelial cell lines or normal PBMC correlated with higher cytotoxicity of the AML cell lines induced by higher drug doses (5 μM). Further studies will be necessary to investigate whether the increased expression of one of the induced genes renders the AML cells more sensitive to cell death or whether cell death is a leukemia-specific but gene induction-independent function even of low-dose 5-Aza-CdR. For example, ERK1, one of the analyzed 5-Aza-CdR-induced genes, is known as part of a signaling cascade, which can result in increased cell death under certain conditions.³⁰ On the other hand, high surface expression of CD14 in myelomonocytic AML is associated with high cellular resistance to AraC.³¹ It is worth noting that CD14, calgranulin and MPO have been characterized as differentiation-related genes. Calgranulin has been proposed to function in myeloid differentiation.³² The differentiation of myelomonocytic cells from pluripotent stem cells to mature macrophages/monocytes and granulocytes is accompanied by the expression of CD14.³³ This is in agreement with earlier *in vitro* studies reporting the differentiation-inducing effect of 5-Aza-CdR that may account for its antileukemic potential.^{34,35}

The major question of this study was whether the mechanism of action of 5-Aza-CdR is restricted to methylation inhibition or

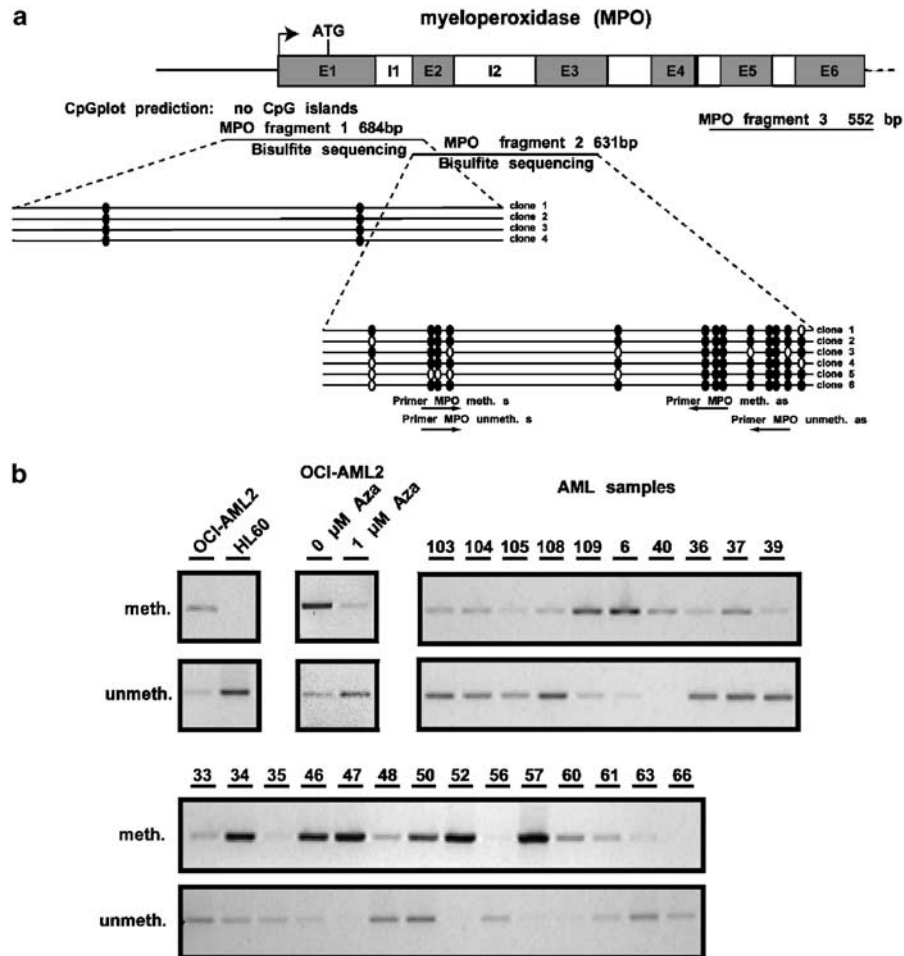


Figure 5 Hypermethylation of the 5' region of MPO. (a) After bisulfite treatment, two genomic fragments (MPO fragment 1, MPO fragment 2) were amplified as indicated and subcloned. The position of CpGs and the result of bisulfite sequencing of four to six independent clones are shown; unmethylated CpG (open circles) and methylated CpG (black filled circles). Position of transcription start site (arrows), exons (E) and introns (I) based on NCBI database. The binding sites of the MSP primers for 'methylated' PCR (MPO-meth. s/as) and 'unmethylated' PCR (MPO-unmeth. s/as) are given. (b) Analysis of the DNA methylation status of MPO gene in primary AML cells by methylation-specific PCR. In all, 93 primary AML blasts were analyzed for their MPO methylation status by MSP using specific primers (MPO-meth. s/as; MPO-unmeth. s/as) to amplify the methylated (meth.) and the unmethylated (unmeth.) bisulfite-modified genomic sequence. Representative results are shown. OCI-AML2 and HL60 genomic DNA was used as control and internal standards to normalize amplification efficiency and signal levels between different PCRs. PCR amplicates were electrophoresed in an agarose gel and ethidium bromide stained for quantification with NIH image software.

whether there are other functions accounting for its therapeutic activity. Therefore, we analyzed the actual methylation pattern of five randomly selected 5-Aza-CdR-induced genes by bisulfite sequencing. According to *in silico* testing for CG density, two of the five genes (CD14 and RPGR) showed a potential CpG island in the promoter region. Interestingly, bisulfite genomic sequencing revealed no methylated CpGs in the 5' region of four induced genes (calgranulin, CD14, PTPN22 and RPGR), implying that 5-Aza-CdR affects their expression independently of direct promoter methylation. On the other hand, 5-Aza-CdR may target upstream regulators that were suppressed by DNA methylation or the minimal promoter comprises other regions than analyzed. However, of the 81 genes showing 5-Aza-CdR-induced upregulation in the microarray analysis, only 50.6% displayed potential CpG islands within their 5' region. This methylation-independent effect of 5-Aza-CdR is in agreement with other studies, indicating that silenced genes with unmethylated CpG islands or without CpG islands can be induced by 5-Aza-CdR.^{15,20,36,37} In addition, effects of 5-Aza-CdR upon

histone methylation and RB phosphorylation have been recently described.^{38,39}

In contrast to this, we found DNA methylation in the 5' region of the MPO gene in AML cells. The hypermethylation status of genomic DNA of the AML cell line OCI-AML2 correlated with gene repression, while a second cell line (HL60) with high MPO mRNA level displayed no methylated CpGs. In addition, 93 samples (bone marrow and peripheral blood cells) of AML patients before therapy were analyzed by MSP for DNA methylation of the 5' region of the MPO gene. The degree of methylation inversely correlated with the expression of MPO in these primary AML cells, implicating that methylation in the 5' region indeed results in decreased protein synthesis. The MPO gene is expressed exclusively in immature myeloid cells; its expression is downregulated during myeloid maturation.²⁵ As myeloid leukemogenesis is characterized by blockage in late myeloid differentiation, MPO is a hallmark of leukemic blasts and a marker for diagnosis of AML. Recently, Matsuo *et al*⁴⁰ found that the percentage of MPO-positive blasts is an

independent prognostic factor in AML. The overall survival of all FAB subtypes (excluded were patients with FAB M3) was best in patients with >50% MPO-positive blasts. In our study, we did not find such a correlation between MPO expression and survival, as there was no significant correlation between degree of methylation in the MPO gene and survival. Further studies will be necessary to associate hypermethylation of the MPO gene with low amount of protein in AML and MDS blasts. Characterization of MPO as a target for 5-Aza-CdR would include the analysis of MPO re-expression as a relevant prerequisite of therapy success.

In summary, using microarray and subsequent RT-PCR analysis, we found genes specifically induced in myeloid blast but not in epithelial cells or normal PBMCs after treatment with 5-Aza-CdR. We detected hypermethylation of the 5' region of the MPO gene in primary AML cells, which correlated inversely with protein expression. In contrast, four out of five analyzed 5-Aza-CdR-inducible genes completely lacked any DNA methylation in their upstream regions challenging the notion that 5-Aza-CdR exclusively acts by demethylating CpG islands in malignant cells. Our data thus support a distinct, alternative mechanism of action of 5-Aza-CdR in myeloid cells.

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Supplementary Information

Supplementary Information is available on the Leukemia website (<http://www.nature.com/leu>).

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