Research article

ANTILEUKEMIC ACTIVITY OF COMBINED EPIGENETIC AGENTS, DNMT INHIBITORS ZEBULARINE AND RG108 WITH HDAC INHIBITORS, AGAINST PROMYELOCYTIC LEUKEMIA HL-60 CELLS

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Abstract: DNMT inhibitors are promising new drugs for cancer therapies. In this study, we have observed the antileukemic action of two diverse DNMT inhibitors, the nucleoside agent zebularine and the non-nucleoside agent RG108, in human promyelocytic leukemia (PML) HL-60 cells. Zebularine but not RG108 caused dose- and time-dependent cell growth inhibition and induction of apoptosis. However, co-treatment with either drug at a non-toxic dose and all-trans retinoic acid (RA) reinforced differentiation to granulocytes, while 24 or 48 h-pretreatment with zebularine or RG108 followed by RA alone or in the presence of HDAC inhibitors (sodium phenyl butyrate or BML-210) significantly accelerated and enhanced cell maturation to granulocytes. This occurs in parallel with the expression of a surface biomarker, CD11b, and early changes in histone H4 acetylation and histone H3K4me3 methylation. The application of both drugs to HL-60 cells in continuous or sequential fashion decreased DNMT1 expression, and induced E-cadherin promoter demethylation.

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Abbreviations used: AcH4 – acetylated histone H4; APL – acute promyelocytic leukemia; DMSO – dimethyl sulfoxide; DNMT – DNA methyltransferase; GAPDH – glyceraldehyde-3-phosphate dehydrogenase; H3K4me3 – trimethyl histone H4 methylated at lysine 4 (K4); HDAC – histone deacetylase; HPR – horseradish peroxidase; NBT – nitro blue tetrazolium; PB – sodium phenyl butyrate; PBS – phosphate-buffered saline; PE – phycoerythrin; PI – propidium iodide; zebularine – 1-(β-D-ribofuranosyl)-2(1-H)-pyrimidone; PMA – phorbol myristate acetate; RA – all-trans retinoic acid; RG108 – 2-(1,3-dioxo-1,3-dihydro-2H-isouindol-2-yl)-3-(1H-indol-3-yl) propanoic acid
and reactivation at both the mRNA and the protein levels in association with the induction of granulocytic differentiation. The results confirmed the utility of zebularine and RG108 in combinations with RA and HDAC inhibitors to reinforce differentiation effects in promyelocytic leukemia.

**Key words:** HL-60, Differentiation, RG108, Zebularine, HDAC inhibitors, E-cadherin, Histones

**INTRODUCTION**

The reversal of epigenetic alterations, such as DNA methylation and histone modifications, serves as an attractive strategy in cancer therapy. DNMT and HDAC inhibitors represent promising drugs in epigenetic-based therapy, and the marked synergy between these agents may be employed in combination with other drugs for clinical efficacy. Aberrant DNA methylation is the most frequent alteration found in acute leukemia and can lead to silencing of tumor suppressor genes [1-5]. DNMTs are highly expressed in leukemia cells in a type- and stage-specific manner [6]. Inhibition of DNMT activity is achieved by DNMT inhibitors as attractive drugs for novel approaches in cancer therapy. Some of them, such as 5-azacytidine (Vidaza) and 5-aza-2'-deoxycytidine (Decitabine), have been used clinically in the treatment of hematological malignancies, including the myelodysplastic syndrome and acute leukemia [7-11]. These nucleoside agents are incorporated into DNA after its modification through metabolic pathways, causing DNMT trapping and degradation. However, both agents are characterized by significant cytotoxicity and low stability. Zebularine, a novel nucleoside inhibitor with increased stability and low toxicity, may be used as a demethylating agent [12, 13] that preferentially targets tumor cells [14-16]. Growth-inhibitory and demethylation effects of zebularine in different bladder cancer, AML cell lines and primary AML samples have been shown as well [17-19]. Recently, diverse non-nucleoside DNMT inhibitors have been proposed. RG108, a novel small-molecule DNMT inhibitor, which blocks the active site of human DNMTs, is an attractive candidate for new drug development. RG108 showed low toxicity and significant demethylation of epigenetically silenced genes with increased specificity for hypermethylated tumor suppressor genes [20]. The suppressing activity of epigenetic defects may be alleviated additionally by inhibition of histone deacetylation. In cancer cells, HDAC inhibitors can modulate chromatin configurations and mediate silencing of cancer genes as components of repressive complexes with DNMT inhibitors [21]. Treatment regimens combining DNMT and HDAC inhibitors may increase the efficacy of chemotherapy in a synergistic manner [22-24].

Hypermethylation of the CpG islands in the promoter regions results in the inactivation of cell-cycle regulating and tumor suppressor genes [25-28]. In AML, aberrant DNA methylation can be observed in multiple functionally relevant genes such as p15, p73, ID4, and RARβ2 [26]. CpG hypermethylation of *E-cadherin* was found in bladder cancer cells [29] and in 32-82% of AML
cases [30-32]. Significant re-activation of silenced E-cadherin was determined in cell lines following treatment with demethylating agents [30, 33]. Therefore, hypermethylation of E-cadherin was proposed as a diagnostic and prognostic tool in myelogenous leukemia [32].

In this study, we compared the effects of two functionally diverse DNMT inhibitors, zebularine and RG108, on human promyelocytic leukemia HL-60 cell growth, survival and differentiation. Zebularine but not RG108 showed significant antiproliferative and proapoptotic activity against HL-60 cells. Both agents were effective in the enhancement of RA-induced differentiation and reactivation of methylation-silenced E-cadherin. Combined treatments with HDAC inhibitors, PB and BML-210, had additional differentiation effects in association with early changes in modification status of histones, AcH4 and H3K4me3. In summary, this study supports the potential of zebularine and RG108 as candidates for targeted therapeutic approaches in promyelocytic leukemia.

MATERIAL AND METHODS

Materials

RG108 and RA were purchased from Sigma Chemical Co. (St. Louis, MO), PB from Calbiochem (Darmstadt, Germany), BML-210 from Biomol Research Laboratories (USA). The stock solutions of zebularine (100 mM in DMSO), RG108 (100 mM in ethanol), RA (2 mM in ethanol), PB (1 mM in water) and 10 mM BML-210 (in DMSO) were stored at -40°C. Rabbit polyclonal antibodies against pan-cadherin (ab16505) and mouse monoclonal antibodies against GAPDH (ab8245) were obtained from Abcam, Cambridge, UK. Antibodies against histones, AcH4 (penta) and H3K4me3, were obtained from Upstate, Lake Placid, USA. Mouse monoclonal anti-human CD11b, C3bi receptor conjugated with PE and goat anti-rabbit (or anti-mouse) HPR-linked secondary antibodies were from Dako Cytomation A/S, Lustrum, Denmark.

Cell culture

The human promyelocytic leukemia HL-60 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco, Grand Island, NY) at 37°C in a humidified 5% CO2 atmosphere. In each experiment, logarithmically growing cells were seeded in 5 ml of medium at a density 5 x 10³ cells/ml. In pretreatment experiments, cells were exposed to DNMT inhibitor for 24 or 48 h, then the drug was washed out and cells were resuspended in fresh media at the initial density and incubated with the differentiation inducer 1 µM RA alone or in the presence of HDAC inhibitors, 1 mM PB or 5 µM BML-210. Treated cells were cultured and harvested at time points indicated.
Cell proliferation, differentiation and viability assays

Cell proliferation was evaluated by the trypan blue exclusion test. Viable and dead (blue colored) cell numbers were determined by counting in a hemocytometer. Growth inhibition was calculated from: \[
\frac{[(C_x - C_0) - (T_x - T_0)]}{(C_x - C_0)} \times 100,
\]
where \(C_0, C_x, T_0, T_x\) represent the total number of cells/ml in untreated (C) and treated (T) cultures at days 0 and \(x\) (3 or 4), respectively. The degree of differentiation was assayed by the ability of cells to reduce NBT to insoluble blue-black formazan after stimulation with PMA [34]. Cell suspension (100 \(\mu\)l) was mixed with an equal volume of 0.2% NBT in PBS containing PMA (40 ng/ml), and incubated at 37\(^\circ\)C for 30 min. NBT-positive cells were counted in a hemocytometer. At least 400 cells were scored for each determination. For morphological evaluation cells were fixed in ice-cold 3:1 methanol-glacial acetic acid a few times for 2 hours, spread on ice-cold microscopic slides, dried and stained with Wright-Giemsa (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer’s instructions. Morphology of cells was then examined by light microscopy.

For statistical analysis, Student’s \(t\) test was used to determine the significance of differences between groups, and significance was set at \(P \leq 0.05\) (*), \(P \leq 0.001\) (**) and \(p \leq 0.0001\) (***)

Determination of CD11b surface biomarker

Control and treated HL-60 cells (5 \(\times\) 10^5 cells/sample) were washed twice in PBS (pH 7.4), then exposed to mouse monoclonal anti-human CD11b conjugated with PE for 30 min in the dark at 4\(^\circ\)C. Cells were washed with PBS, fixed in PBS containing 2% paraformaldehyde for 30 min on ice and the pellet was resuspended in PBS. Eight thousand events were analyzed for each sample by immunofluorescence in FACSaria (BD Biosciences with BD FACS Diva software). Proliferating cells were used as a control. A negative control was performed using an isotype-specific mouse PE-conjugated IgG1 (Santa Cruz Biotechnology, Inc., Heidelberg, Germany).

Flow cytometric analysis of cell cycle distribution and apoptosis

Cell cycle progression and apoptosis were monitored by quantification of cellular DNA content after staining with PI. Control and treated cells were collected by centrifugation, suspended in PBS and fixed in ice-cold 70% ethanol (ratio 1:10) for 24 h at -20\(^\circ\)C. After centrifugation at 500 x g for 5 min, cells were suspended in PBS containing PI (50 \(\mu\)g/ml) and RNase (0.2 mg/ml) and incubated at room temperature for 30 min. The tubes were then taken at 4\(^\circ\)C in the dark until flow cytometric analysis in FACSCalibur (Becton-Dickinson, USA). The percentage of cells in G0/G1, S and G2/M was evaluated with CellQuest software. Apoptotic cells were quantified on the PI histogram as a hypodiploid peak (SubG1) and the data were registered on a logarithmic scale. Detection of early and late apoptotic cells was performed using dual staining with Annexin V-FITC and PI (Kit-AX from Xebio, Prague, Czech Republic).
Control or treated cells (3 x 10^5 cells/sample) were washed twice in cold PBS (pH 7.4), resuspended in 100 µl of 1 x binding buffer with 5 µl of Annexin V-FITC and 5 µl PI and incubated for 15 min in the dark at room temperature. After centrifugation, the cell pellet was resuspended in 1 x binding buffer and analyzed by flow cytometry (BD FACSCanto II, USA with BD FACSDiva software).

**Preparation of proteins**

Cells (5 x 10^6 to 10^7) were harvested by centrifugation at 500 x g for 6 min, washed twice in ice cold PBS and lysed twice in Nuclei EZ lysis buffer (Sigma, St. Louis, MO) for 5 min on ice. Cell homogenates were centrifuged at 500 x g for 5 min at 4°C. Supernatant, corresponding to the cytosolic fraction, was clarified by centrifugation at 15,000 x g for 15 min and then frozen at -76°C or immediately used for electrophoresis. Nuclei were washed in the same cold Nuclei EZ buffer, completely suspended in Nuclei EZ storage buffer (Sigma, St. Louis, MO) for storage at -76°C or immediately used. Protein concentrations were measured using a commercial RCDC protein assay (Bio-Rad, Munich, Germany).

For preparation of histones, nuclei from control and treated cells were suspended in 5 vol. of 0.4 N H₂SO₄ by stirring and incubated overnight at 0°C. The supernatant was collected by centrifugation at 15,000 x g for 10 min at 4°C and the sediment was extracted once more. After centrifugation, both extracts were combined and histones were precipitated by adding 5 vol. of ethanol at -20°C overnight. The precipitated histones were collected by centrifugation, washed several times with ethanol and stored at -20°C until analysis.

**Gel electrophoresis and Western blot analysis**

Cytosolic proteins were run on a 7–15 % polyacrylamide gradient SDS-PAGE gel using Tris-glycine buffer. After SDS/PAGE, proteins were transferred to a PVDF membrane (Immobilon P, Millipore, Bedford, MA) and then the filters were blocked with 5% BSA dissolved in PBS containing 0.18% Tween-20 by incubation overnight at 4°C. After washing in PBS-Tween-20, the filters were probed with primer antibodies against pan-cadherin (1:500 dilution), AcH4 (penta) (1:8,000 dilution), H3K4me3 (1:7,000 dilution) or GAPDH (at 1:10,000 dilution) for 1 h at room temperature. Primary antibodies were prepared in solution containing 0.18% Tween-20, 0.35 M NaCl and 1% BSA. The filters were subsequently washed four times with PBS-Tween-20 and then incubated with HPR-linked goat anti-mouse or goat anti-rabbit secondary antibody for 1 h at room temperature. Immunoreactive bands were detected by enhanced chemiluminescence using ECL™ Western blotting detection reagents (Amersham Pharmacia, Sweden) according to the instructions of the manufacturer.

Histones (5 µg) were dissolved in a buffer containing 0.9 M acetic acid, 10% glycerol, 6.25 M urea and 5% β-mercaptoethanol, and separated on 15% polyacrylamide gel containing 6 M urea and 0.9 M acetic acid by using 0.9 M acetic acid as a buffer [35]. After electrophoresis, gel was stained with Brilliant Blue G-Colloidal (Sigma, St. Louis, MO) or transferred to Immobilon™ PVDF
transfer membrane, and probed with antibodies against AcH4 (penta) or H3K4me3 and secondary antibodies as otherwise indicated. Immunoreactive bands were detected by enhanced chemiluminescence according to the manufacturer’s instructions. The non-acetylated histone H1 band served as a control of histone extraction and protein loading.

**Methylation-specific (MS) PCR analysis**

The methylation status of DNA promoters was determined by using EZ DNA methylation-Direct™ kit (Zymo Research, USA). Briefly, cells (1 x 10⁵) were digested in the reaction mixture with proteinase K at 50°C for 20 min. Bisulfite conversion of DNA was performed according to the manufacturer’s instructions. After conversion of all unmethylated uracils to cytosines, the modified DNA was purified using a Zymo-Spin™ IC column and used for PCR amplification. The primers, forward (F) or reverse (R) for methylated (M) and unmethylated (U) promoters of the target genes were: E-cadherin (MF) 5’- CAA TTA GCG GTA CGG GGG GC-3’, E-cadherin (MR) 5’-CGA AAA CAA ACG CCG AAT ACG-3’; E-cadherin (UF) 5’-TTA GTT AAT TAG TGG TAT GGG GGG TGG-3’; E-cadherin (UR) 5’-ACC AAA CAA AAA ACA CCA AAT ACA-3’. Cycling conditions (40 cycles) for E-cadherin: 95°C 5 min, 95°C 30 s, 59°C 30 s, 72°C 30 s, 72°C 10 min, 4°C. The product size for E-cadherin was 170 bp. The products were electrophoresed on 3% agarose gel, stained with ethidium bromide, and photographed. DNA methylation levels in representative examples were quantitated by scanning densitometry and expressed as the percentages of methylated and unmethylated DNA in the E-cadherin promoter region.

**Reverse transcription polymerase chain reaction (RT-PCR)**

Total RNA was obtained using the Trizol (Invitrogen, Carlsbad, USA) method and cDNA was prepared using the RevertAid™ Premium First strand cDNA synthesis kit (Fermentas, Vilnius, Lithuania) following the manufacturer’s protocols. The primers (5’-3’ orientation) used in PCR reactions were: for E-cadherin (F, +37 (exon 1), melting temperature (MT) 59.3°C) 5’-GGA AGT CAG TTC AGA CTC CAG CC-3’; for E-cadherin (R, +365 (exon 3), MT 57.4°C) 5’-AGG CCT TTT GAC TGT AAT CAC ACC. As an internal control, the primers for GAPDH were: (F, +593, MT 59.3°C) 5’-GGA AGT CAG TTC AGA CTC CAG CC-3’; (R, +1044, MT 57.4°C), 5’ AGG CCT TTT GAC TGT AAT CAC ACC. The size of PCR products were: 352 bp for E-cadherin, 471 bp for GAPDH. The products were electrophoresed on 3% agarose gel, stained with ethidium bromide, and photographed.

**RESULTS**

**Comparative effects of zebularine and RG108 on HL-60 cell proliferation, survival and differentiation**

The antiproliferative effect of two structurally diverse DNMT inhibitors, zebularine and RG108, was evaluated during 4 days of treatment with different
doses of zebularine (20-60 μM) or RG108 (25-100 μM) after staining with trypan blue. Zebularine inhibited cell proliferation in a dose- and time-dependent manner with a moderate retardation at concentrations of 20 or 40 μM (Fig. 1A). The drug at doses of 50-100 μM caused significant growth inhibition (47-60%) at day 3 with the maximal increase to 95% at a dose of 200 μM on day 4 (Fig. 2A). By contrast, RG108 at most doses tested (25-200 μM) had only minor antiproliferative and growth inhibitory (9-21%) effects at day 4, whereas only a very high dose of 300 μM reached the maximal effect (78%) at day 4 (Fig. 1A, 2A).

![Fig. 1. Antiproliferative effects of zebularine and RG108 applied alone and in combination with RA in HL-60 cells. A – Cells were treated with zebularine or RG108 at the indicated concentrations for 4 days. B – Cells were pretreated with 50 μM zebularine or 50 μM RG108 for 48 h and then after removal of the inhibitor cultivated with 1 μM RA for the next 4 days. Viable cell number was determined daily by counting in a hemocytometer after staining with 0.2% trypan blue. Results are mean ± SEM (n = 3).](chart)

For all subsequent experiments, we established low toxic culture conditions using DNMT inhibitors at a concentration of 50 μM. Cell cycle analysis of PI stained cells by flow cytometry at 48 h (Fig. 2B) demonstrated a decrease in the proportion of cells in S-phase (by 27% from control) and 2-fold augmentation in G2/M-phase after treatment with 50 μM zebularine, whereas 50 μM RG108 caused a slight decrease in S-phase (by 20% from control) with minor changes in G2/M. The inducer of differentiation (1 μM RA) led to pronounced cell arrest at G0/G1-phase (to 59% over 41% in control) and did not cause relevant changes in G2/M. Thus, the induction of cell cycle arrest at G0/G1 by RA and at G2/M by zebularine may lead to cell differentiation and apoptosis, respectively. As shown in Fig. 2A and C, zebularine elicited a dose-

![chart]
dependent increase in growth inhibition and cell death, which was significant at a dose of 200 μM on day 4 (up to 90%). Cell staining with trypan blue revealed a small number of dead cells in populations treated with RG108 at a broad range of concentrations (Fig. 2C) but remarkable cell killing (up to 60%) and growth inhibition (up to 50%) were observed at a dose of 300 μM (Fig. 2A and C). Zebularine at a relatively low dose of 50 μM induced apoptotic cell death (to 31.2%) as was determined by flow cytometry after cell staining with PI on day 3 (Fig. 3A), whereas 50 μM RG108 did not yield apoptosis (8.3%) as compared to the spontaneous apoptosis in the non-treated control (6.3%). Flow cytometric analysis using two-color staining with Annexin-V-FITC demonstrated a dose-dependent increase in the proportion of early and late apoptotic cells after 72-h treatment with 50-100 μM zebularine and 50-200 μM RG108 (Fig. 3B). Zebularine at 50 μM caused elevated levels of early+late apoptosis (30.6%) as compared to 50 μM RG108 (9.5%).

![Fig. 2. Effects of zebularine and RG108 on HL-60 cell growth inhibition, survival and cell cycle distribution. Cells were treated with zebularine or RG108 at the indicated concentrations for 2-5 days. A – The percentage of cell growth inhibition. B – Flow cytometric analysis of cell cycle distribution between G0/G1, S and G2/M phases (%) after treatment with 50 μM zebularine, 50 μM RG108 and 1 μM RA for 48 h. C – Cell death was evaluated after staining with 0.2% trypan blue and expressed as the percentage of non-viable cells from total cell number. Results are mean ± SEM (n = 3). P ≤ 0.05 (*), P ≤ 0.001 (**) and P ≤ 0.0001 (***) indicate significant differences from untreated cell samples.](image)

For differentiation experiments, HL-60 cells were treated with a pharmacological dose of RA (1 μM) and DNMT inhibitors at a relatively non-toxic concentration of 50 μM added in continuous and temporal fashion. Pretreatment with DNMT
inhibitors for 48 h before treatment with RA had additive antiproliferative (Fig. 1B) and apoptotic (Fig. 3B) effects caused by zebularine (33.9 %) but not by RG108 as compared to the single RA usage (25.7%). Zebularine and RG108 did not induce HL-60 cell differentiation as was determined by NBT assay and the expression of an early differentiation marker CD11b (Fig. 5). However, co-treatment with RA and either DNMT inhibitor for 3 days produced 1.3-fold greater cell differentiation toward granulocytes compared to treatment with RA alone (Fig. 4A). Zebularine or RG108 applied for 24 h before the induction of differentiation caused a greater (1.5-fold) increase in the extent of differentiation at the same time point (Fig. 4A). Of note, the continuous presence of DNMT inhibitors for 48 h before treatment with RA had additive antiproliferative (Fig. 1B) and apoptotic (Fig. 3B) effects caused by zebularine (33.9 %) but not by RG108 as compared to the single RA usage (25.7%). Zebularine and RG108 did not induce HL-60 cell differentiation as was determined by NBT assay and the expression of an early differentiation marker CD11b (Fig. 5). However, co-treatment with RA and either DNMT inhibitor for 3 days produced 1.3-fold greater cell differentiation toward granulocytes compared to treatment with RA alone (Fig. 4A). Zebularine or RG108 applied for 24 h before the induction of differentiation caused a greater (1.5-fold) increase in the extent of differentiation at the same time point (Fig. 4A). Of note, the continuous presence of DNMT inhibitors for 48 h before treatment with RA had additive antiproliferative (Fig. 1B) and apoptotic (Fig. 3B) effects caused by zebularine (33.9 %) but not by RG108 as compared to the single RA usage (25.7%). Zebularine and RG108 did not induce HL-60 cell differentiation as was determined by NBT assay and the expression of an early differentiation marker CD11b (Fig. 5). However, co-treatment with RA and either DNMT inhibitor for 3 days produced 1.3-fold greater cell differentiation toward granulocytes compared to treatment with RA alone (Fig. 4A). Zebularine or RG108 applied for 24 h before the induction of differentiation caused a greater (1.5-fold) increase in the extent of differentiation at the same time point (Fig. 4A). Of note, the continuous presence of DNMT inhibitors for 48 h before treatment with RA had additive antiproliferative (Fig. 1B) and apoptotic (Fig. 3B) effects caused by zebularine (33.9 %) but not by RG108 as compared to the single RA usage (25.7%). Zebularine and RG108 did not induce HL-60 cell differentiation as was determined by NBT assay and the expression of an early differentiation marker CD11b (Fig. 5). However, co-treatment with RA and either DNMT inhibitor for 3 days produced 1.3-fold greater cell differentiation toward granulocytes compared to treatment with RA alone (Fig. 4A). Zebularine or RG108 applied for 24 h before the induction of differentiation caused a greater (1.5-fold) increase in the extent of differentiation at the same time point (Fig. 4A). Of note, the continuous presence of DNMT inhibitors for 48 h before treatment with RA had additive antiproliferative (Fig. 1B) and apoptotic (Fig. 3B) effects caused by zebularine (33.9 %) but not by RG108 as compared to the single RA usage (25.7%). Zebularine and RG108 did not induce HL-60 cell differentiation as was determined by NBT assay and the expression of an early differentiation marker CD11b (Fig. 5). However, co-treatment with RA and either DNMT inhibitor for 3 days produced 1.3-fold greater cell differentiation toward granulocytes compared to treatment with RA alone (Fig. 4A). Zebularine or RG108 applied for 24 h before the induction of differentiation caused a greater (1.5-fold) increase in the extent of differentiation at the same time point (Fig. 4A). Of note, the continuous presence of DNMT
inhibitor with RA in such sequential treatments slightly increased differentiation (1.6-fold) (Fig. 4A). The extension of pretreatment time to 48 h using two moderate concentrations (25 or 50 μM) of zebularine or RG108 demonstrated more pronounced differentiation levels in a dose-dependent manner (Fig. 4B, C). Interestingly, a remarkable acceleration of differentiation among pretreated cells was observed at 24 and 48 h of RA treatment, with the enhancement seen at 72 h (Fig. 4B and C) or later (data not shown).

Fig. 4. Effects of zebularine and RG108 on RA-mediated differentiation upon continuous or sequential treatments. A – Cells were treated continuously with 1 μM RA and 50 μM zebularine or 50 μM RG108 or pretreated with either DNMT inhibitor for 24 h before treatment with 1 μM RA alone or in the presence of 50 μM zebularine or 50 μM RG108 for the next 3 days. Cells were pretreated for 48 h with zebularine (B) or RG108 (C) at concentrations of 25 and 50 μM, and after washout cultivated in the presence of RA alone or together with 1 mM PB or 5 μM BML-210 for the next 3 days. (A) Granulocytic differentiation is expressed relative to RA-treated control or as the percentage of NBT-positive cells of viable cell number (B, C). Results are mean ± SD (n = 3). P ≤ 0.001 (**) and P ≤ 0.0001 (****) indicate significant differences from cell samples treated with RA alone (A, B) or RA following pretreatment with DNMT inhibitor (C).
We next investigated whether other epigenetic drugs, such as HDAC inhibitors, combined with demethylating agents could affect RA-induced differentiation. We used two structurally different HDAC inhibitors, 1 mM PB and 5 μM BML-210. Both induced HL-60 cell differentiation to about 5, 15 and 36% during 3 days of treatment (data not shown) and enhanced it in the presence of RA (Fig. 4B and C). The effect was more pronounced when HDAC inhibitors were added together with RA following 48-h pretreatment with zebularine or RG108 (Fig. 4B and C). In such experiments, significantly more mature cells were found in cell culture pretreated with zebularine at a higher concentration of 50 μM in comparison with 25 μM. As compared to the RA-treated cell culture at day 3, the presence of either HDAC inhibitor together with RA caused a 1.6-fold increase in the level of differentiation and a 2-fold increase when both agents were used after pretreatment with a DNMT inhibitor. Pretreatment with 50 μM RG108 for 48 h before the combined treatment with RA and HDAC inhibitors resulted in a remarkable acceleration of cell maturation during the first two days but finally the level of differentiation was comparable with that of non-pretreated cells (Fig. 4C). In the course of HL-60 cell differentiation, after 7 days of treatment with RA alone or in combinations with DNMT and/or HDAC inhibitors, a decrease in the number of mature cells was associated with an increase in cell death even after drug removal on day 3 of the initial treatment, and by day 10-12 all cells in the cultures with a very high level of differentiation were dead (data not shown).

An analogous accelerating effect of granulocytic differentiation was corroborated by flow cytometric analysis (Fig. 5A), showing an increased proportion of CD11b expressing cells (by about 15%) in RA-treated populations following 48-h pretreatment with zebularine or RG108 together with already mature cells (about 50%) determined by an NBT test at 48 h (Fig. 4B and C). The highest increase in the proportion of CD11b-expressing cells (2-fold increase) in parallel with the high amount of cells undergoing terminal granulocytic differentiation was observed after the addition of 1 mM PB with RA to DNMT inhibitor-pretreated cultures (Fig. 4B and C). Light microscopic examination of morphology was performed to determine granulocytic differentiation of HL-60 cells. After 3 days of treatment with RA alone or in combination with DNMT and HDAC inhibitors, differentiated HL-60 cells showed the same morphological alterations (Fig. 5C, b) with the appearance of nuclei segmentation and chromatin condensation. Cell cultivation with either DNMT inhibitor did not induce changes in morphology as compared with untreated control cells (Fig. 5C, a), while apoptotic cells with fragmented nuclei were seen under treatment with 50 μM zebularine (Fig. 5C, c).

These results clearly demonstrate that both zebularine and RG108 reinforced RA-induced granulocytic differentiation with the additive effect caused by HDAC inhibitors. The efficacy of the effect was sequence-dependent, requiring initial exposure to a DNMT inhibitor followed by HDAC inhibition.
Fig. 5. Flow cytometric analysis of CD11b expression and morphology of untreated and DNMT inhibitor treated HL-60 cells. Cells were treated for 48 h with 50 μM zebularine, 50 μM RG108 or 1 μM RA applied alone or pretreated with DNMT inhibitor for 48 h, and after washout cultivated in the presence of RA alone or together with 1 mM PB for the next 48 h. A – Representative FACS-generated histograms of CD11b expression on control and treated cells. B – Expression of CD11b on treated populations relative to the untreated control. Results are mean ± SD (n = 3), P ≤ 0.05 (*), P ≤ 0.001 (**) indicate significant differences from RA-treated cell samples. C – Morphology of control HL-60 cells (a), differentiated to granulocytes (b) and treated with 50 μM zebularine for 3 days (c). After fixation to slides, cells were subjected to Wright-Giemsa staining for light microscopic examination.

Reactivation of E-cadherin in response to treatments with zebularine and RG108 or in their combinations with RA and HDAC inhibitors

In order to evaluate the demethylating potential of zebularine and RG108 in HL-60 leukemia cells, we tested their ability to affect the chromatin-modifying enzyme DNMT1, which is overexpressed in myeloid leukemia in association with promoter hypermethylation of a number of genes [6, 31, 32]. Western blot analysis (Fig. 6A and B) demonstrated that both DNMT inhibitors decreased DNMT1 expression in a dose-dependent manner, with a more remarkable effect by zebularine. Combined treatment resulted in greater depletion of DNMT1, as was observed in RA-treated cells following 48-h pretreatment with 50 μM zebularine or 50 μM RG108 (to 34 or 31% of control, respectively), compared to treatment with either single drug (to 38 or 58% of control) or RA alone (to 54% of control).

Next, we examined the ability of zebularine and RG108 to reactivate epigenetically silenced E-cadherin [30-32], known as a metastasis suppressor [30-32, 36]. In the initial experiments methylation-specific (MS) PCR analysis was used to determine the effect of either DNMT inhibitor on DNA methylation
Fig. 6. Zebularine and RG108 reactivate E-cadherin in coordination with changes in the expression of DNMT1, E-cadherin mRNA and protein. Cells were treated with the indicated concentrations of zebularine and RG108 or 1 μM RA applied alone, or pretreated for 48 h with 50 μM zebularine or 50 μM RG108 before treatment with RA for the next 3 or 5 days. A – Western blot analysis was performed for DNMT1 on the total cell lysate, and B – blots from three experiments were quantified densitometrically, DNMT1 expression levels were normalized to GAPDH and expressed as the percentage of the untreated control (mean ± SD, n = 3). C – Methylation-specific (MS) PCR was done for the E-cadherin promoter (M-methylated DNA, U-unmethylated DNA). D – The methylation status expressed as the percentages of methylated and unmethylated DNA in the E-cadherin promoter. Results are mean ± SD (n = 3). E – E-cadherin mRNA expression at each time point was determined by RT-PCR analysis using GAPDH as a loading control. F – Cytosolic proteins were isolated at day 3 from control and treated cells as indicated and Western blotting was performed using antibodies against pan-cadherin and GAPDH as a loading control. The data are representative of three independent experiments that gave similar results. P ≤ 0.05 (*), P ≤ 0.001 (**) indicate significant differences from untreated cell samples.

status in E-cadherin promoter. As shown in Fig. 6C and D, the promoter region investigated had hypermethylated status in untreated HL-60 cells. Treatment with RA alone for 3 days showed the appearance of an unmethylated band (28% over 11% in control). The unmethylation-specific band increased after 3 days of treatment with 50 μM zebularine (to 35%) and 50 μM RG108 (to 47%). An equal proportion between methylated and unmethylated bands (about 50%) was found after prolonged treatment for 5 days with either DNMT
inhibitor as well as under combined sequential treatments at day 3 (51-55%). After all treatments, the methylation-specific band of the *E-cadherin* promoter region still existed, suggesting the moderate hypomethylating property of zebularine or RG108 at a relatively low dose of 50 μM. The ability of DNMT inhibitors to reactivate *E-cadherin* was confirmed by RT-PCR and Western blot analysis. Corresponding to changes in the promoter hypomethylation, RT-PCR revealed an increase in E-cadherin mRNA level in DNMT inhibitor-treated samples and elevated levels after sequential treatment with RA following 48-h pretreatment with either DNMT inhibitor at day 3 (Fig. 6E). The changes in mRNA levels paralleled the levels of E-cadherin protein expression as determined by Western blot analysis using rabbit polyclonal antibodies to pan-cadherin, which recognize all known members of the cadherin family and detect a band of approximately 130 kDa specific for E-cadherin (Fig. 6F). A markedly higher expression of E-cadherin was detected in differentiating cultures under combined treatments than in proliferating cells treated with DNMT inhibitors. Thus, combined treatments with a demethylating compound and RA induced greater depletion of DNMT1 and greater de-repression of *E-cadherin* at both the mRNA and the protein levels.

**Changes in modification status of acetylated histone H4 and histone H3K4me3 in response to zebularine and RG108 or their combinations with RA and PB**

To test the mechanisms by which epigenetic agents, DNMT and HDAC inhibitors, act by altering chromatin structure, we investigated two histone modifications, i.e. acetylation of histone H4 and methylation of histone H3K4me3, which represent the active chromatin state and lead to opening of the chromatin structure. HL-60 cells were treated with DNMT inhibitors (50 μM zebularine and 50 μM RG108), HDAC inhibitor (1 mM PB) and 1 μM RA as single agents or in combination. Taking into account that HDAC inhibitors induce rapid and substantial increases in the global levels of histone acetylation with a high rate of turnover, histones were extracted from control cells after treatment for 18 h. Of note, this time point also represents the initiation stage of RA-induced differentiation. Histones were examined on acid-urea gels that led to resolution of the distinct acetyl isoforms of histone H4 after staining with Brilliant Blue G-Colloidal. As shown in Fig. 7A, untreated cells contained non-acetylated, mono- and di-acetylated histone H4, as did cells treated with RA or zebularine. Cells treated with HDAC inhibitor (1 mM PB) as a single agent or together with RA or zebularine contained highly acetylated histone H4 isoforms up to tetra-acetylated. A similar experiment using 50 μM RG108 showed the same pattern of histone H4 isoforms (data not shown). To characterize histone modifications in greater detail, blots of the same gel were probed with antibodies against AcH4 and H3K4me3, which better recognize higher forms of histone modifications. As shown in Fig. 7B, control cells and cells treated with RA or either DNMT inhibitor exhibited the most abundant lower forms of acetylated histone H4 and a low extent of methylated H3K4me3. Zebularine or RG108...
used together with RA caused a change in histone H4 acetylation toward tri-acetylated status. Treatment with PB alone or together with RA induced an accumulation of higher acetylated forms, and the addition of DNMT inhibitor to this combination maintained the extent of H4 hyperacetylation and increased methylation of H3K4me3. In contrast, neither DNMT inhibitor influenced the level of this modification but their combination with PB showed higher H3-K4 methylation.

Next, we performed Western blot analysis of cytosolic proteins isolated at 18 h from cells treated as in the previous experiment. As shown in Fig. 7C, untreated control cells showed minimal E-cadherin expression, whereas co-incubation with RA and zebularine or RG108 at doses of 50 μM resulted in elevated E-cadherin expression even at 18 h of treatment. The HDAC inhibitor PB also induced E-cadherin expression, with the increase observed in cells after co-treatment with RA and either DNMT inhibitor. This was associated with the
appearance of NBT-positive cells under treatment with PB or RA alone (to 10 or 14%, respectively), with an increase in the amount of NBT-positive cells (to about 30%) under combined treatments in the presence of PB (Fig. 4D). Taken together, HDAC inhibitors caused cooperative changes in histone H4/H3 acetylation/methylation in parallel with the acceleration of granulocytic differentiation and re-expression of E-cadherin.

DISCUSSION

The involvement of DNA methylation in leukemogenesis allows the development of new therapeutic strategies using DNMT inhibitors. To date, very few studies have compared the biological effects of different hypomethylating agents [37-39]. In this study, we performed a comparative analysis of two epigenetic drugs, the nucleoside agent zebularine and the non-nucleoside agent RG108, to identify their antileukemic action in human promyelocytic leukemia HL-60 cells. It was found that zebularine acts by the formation of covalent complexes between DNMT and zebularine-incorporated DNA [12, 14], leading to depletion of DNMTs [17]. To date, this compound has been proposed as a strong candidate in several clinical trials because of its stability, minimal toxicity, oral bioavailability and selectivity to target tumor cells [15-18]. However, zebularine requires prolonged exposure and high doses because of its limited DNA incorporation and low metabolism [40]. A novel small-molecule inhibitor, RG108, which functions without being incorporated into the DNA, effectively blocks DNMTs at the active site, and causes demethylation of genomic DNA and reactivation of tumor suppressor genes, with little toxicity in human cancer cell lines [20, 38]. Hypomethylating agents with antitumor potential have already shown utility in treatment of various human cancers [21]. This work represents the first experimental evidence of the efficacy of both zebularine and RG108 to reinforce RA-mediated differentiation to granulocytes in promyelocytic leukemia HL-60 cells. The comparison of the effects of both DNMT inhibitors on HL-60 cell proliferation and viability demonstrated more pronounced time- and dose-dependent growth inhibition and cell death by zebularine than by RG108. Previous reports also mentioned that zebularine at DNA demethylating doses effectively inhibited proliferation of AML cell lines and arrested cells at G2/M [18, 41] or showed no interference with cell-cycle progression at low toxic concentrations [39]. Interestingly, zebularine preferentially targets many cancer cells compared to normal fibroblasts due to different incorporation of the drug into DNA and reduction of DNMT levels and gene expression [42]. Several groups have reported that the antiproliferative effect of zebularine in many human cancer lines is associated with the up-regulation of p21 WAF1, p16 INK4A or p15 INK4B, the inhibitors of cyclin-CDK complexes involved in G1 and S phase progression [17, 18, 43]. As was postulated, p21 WAF1, which is important in G1 cell cycle arrest, can directly affect DNA replication by binding proliferating cell nuclear antigen (PCNA),
which is a target for DNMT1 [44, 45]. Cancer cells that respond to zebularine showed complete depletion of DNMT1 and partial depletion of DNMT3b (both are cell cycle regulated) and DNMT3a (occurs only during the S-phase) [17, 46]. Knockdown or downregulation of DNMTs by DNMT inhibitors, such as zebularine, causes intra-S-phase arrest of DNA replication, represses histone synthesis, decreases its global acetylation during replicative S-phase or triggers cell cycle arrest within G2/M, leading to inhibition of cell proliferation and/or apoptosis [47, 48].

Other mechanisms, such as the induction of apoptosis, may be involved in cancer cell growth regulation by zebularine [49]. For example, zebularine triggers apoptosis in AML cell lines [18, 39] and breast cancer or pancreatic cells by alterations in pro-apoptotic Bax, anti-apoptotic Bcl-2, caspase 3 or PARP cleavage [48, 50]. In our study, zebularine yielded low apoptosis at a low concentration of 50 μM but caused apparent toxicity at higher doses (100-200 μM), while RG108 was non-toxic at a broad range of concentrations. Our previous study indicated that low apoptotic activity of zebularine at low doses in PML cell lines, NB4 and KG1, was accompanied by limited cleavage of procaspase-3 and PARP-1 [51]. The reduced toxicity shown by RG108 may be explained by its inability to affect the methylation of repeated centromeric sequences that may contribute to maintenance of chromosome stability [20].

In our study, we have observed that HL-60 cells treated using RA combinations with DNMT and HDAC inhibitors achieved accelerated differentiation into granulocytes, which subsequently die by apoptosis [52]. If HL-60 cell differentiation is mainly regulated through both retinoic acid receptors (RARs and RXRs), the apoptosis is modulated by activation of RXR, up-regulation of caspase activity [53, 54], and the induction of the mitochondrial rather than the receptor-mediated apoptotic pathway [55]. Both processes are independently regulated in this cell line [56]. In the classical NB4 model of APL, RA-induced differentiation and growth arrest depend on the PML-RARα translocation product [57]. The process of apoptosis is temporarily inhibited until terminal differentiation of NB4 cells is complete. These cells undergo post-maturation apoptosis, which involves the activation of pro-caspase 8 and TRAIL (tumor-necrosis-factor-related apoptosis-inducing ligand) expression, inducing the autocrine and paracrine death pathway [58]. However, rexinoid signaling may trigger apoptosis in immature NB4 cells as a default pathway in the absence of survival factors [59].

The comparative analysis of the response to zebularine and RG108 in different cancer cell lines demonstrated relatively low levels of their demethylating activity as compared with aza-nucleosides [38, 39]. However, dose- and time-dependent demethylation of genomic DNA has been established [20, 38] in parallel with re-expression of methylation-silenced genes, such as p16, p15 or E-cadherin, in response to zebularine [15, 17, 60] or p16, TIMP-3 and SERP-1 in response to RG108 [20]. Moreover, a more recent study indicated different effects of DNMT-inhibiting nucleoside analogues on gene transcription,
involving several genes relevant to leukemogenesis [39]. As indicated previously, in leukemia cell blasts with elevated expression of DNMTs, DNA methylation was associated with the loss of E-cadherin transcript and protein [30, 31]. Similarly, we also determined E-cadherin promoter methylation in HL-60 cells. Our data demonstrated that zebularine and RG108 targeted DNMT1 (Fig. 6) and resulted in partial demethylation of E-cadherin promoter and the gene reactivation as manifested by E-cadherin mRNA expression seen after treatments with zebularine or RG108 even at cytostatic doses of 50 μM (Fig. 6). The differentiation-inducing agent RA applied alone also was able to reactivate E-cadherin, with a higher extent when RA was added after 48-h pretreatment with a demethylating agent. This may be explained by the ability of RA to induce an early and coordinated decrease in the total DNMT enzyme activity and to reduce the interactions of DNMT1, DNMT3a and HDAC1 at chromatin regions of RA target genes [61]. Indeed, RA application to HL-60 cells following a demethylating agent may have an additional effect on E-cadherin reactivation. In spite of very moderate hypomethylating efficiency of zebularine or RG108 in HL-60 cells, E-cadherin re-expression at transcript and protein levels correlated with the level of granulocytic differentiation at the initiation (Fig. 7D) and maturation (Fig. 6F) stages. This also occurs in other PML cell lines in response to zebularine, consistent with our previous observations [51]. This led to E-cadherin being proposed as an indicator useful in monitoring the efficacy of therapy based on DNA demethylation in promyelocytic leukemia.

It is necessary to note that the effects of distinct DNMT inhibitors on gene transcription may be unrelated to direct promoter DNA hypomethylation [39] but may occur through regional enrichment of histone acetylation at the gene promoter. Because DNA methylation and histone acetylation can act synergistically, DNMT inhibitors may be combined with HDAC inhibitors to enhance reactivation of epigenetically silenced genes [28]. This synergy, which is sequence dependent and requires initial exposure to DNMT inhibition followed by HDAC inhibition, has been explored in several clinical trials [62]. For example, PB, as the first clinically available HDAC inhibitor, was used in combination with 5-aza-cytidine in a phase I trial in the treatment of myeloid neoplasm [23]. In this study, we have explored PB and a novel HDAC inhibitor, BML-210, which are able to induce granulocytic differentiation preferentially in HL-60 cells [63]. We reasoned that DNMT and HDAC inhibitors may induce transcriptional activation of E-cadherin by altering chromatin structure.

As is known, HDACs, mostly HDAC1, are overexpressed in nearly all primary AML cells and cell lines [64]. For example, HDAC1, 2 and 6 are the most highly expressed HDACs in HL-60 cells, and during RA-induced differentiation to granulocytes, HDAC1, 2 and 3 are down-regulated with nearly complete disappearance in mature cells [65]. Expression of HDACs is regulated at mRNA and protein levels, but post-translational modification is also involved especially in HDAC2 by the ubiquitin-proteasome pathway [66]. Different HDAC inhibitors, despite their ability to cause histone hyperacetylation, may act as
selective down-modulators of HDAC isoenzymes in the regulation of different target genes to determine cell response to the drug [64]. For example, the inhibition of HDAC1 and 2 activity by sodium butyrate in AML cells is associated with the expression of GM-CSF and CAAT/enhancer binding protein α (C/EBPα), critical factors for the induction of granulocytic differentiation [65, 67]. A significant down-regulation of HDAC2 protein levels by proteosomal degradation and inhibition of the enzyme was demonstrated in K562 human erythroleukemia cells treated with butyrate and valproic acid [66]. Silencing of E-cadherin was also dependent on HDAC1 and 2 activities in a transcriptional repressor complex containing the zinc finger factor Snail in epithelial cell lines and highly metastatic pancreatic cells [68, 69], and treatment with an HDAC inhibitor (TSA) was sufficient to block the repressor effect [69].

Our data demonstrated that continuous and temporal treatment with zebularine and RG108 at non-toxic doses significantly accelerated/enhanced RA-induced HL-60 cell differentiation in the presence of HDAC inhibitors in parallel with re-expression of the mature phenotype-specific protein E-cadherin. PB but not zebularine or RG108 induced early changes in H4 acetylation and H3K4me3 methylation, leading to a relaxed and active chromatin state for transcriptional activation [70-72], with a higher efficacy upon co-treatment with DNMT inhibitor and/or RA (Fig. 7). The data are consistent with other studies, indicating that HDAC inhibitors trigger histone H3 methylation at K4 cooperatively with H3 acetylation in APL cells [73, 74], while DNMT inhibitors, such as zebularine, act by depletion of DNMT1 or up-regulation of genes encoding chromatin-modifying proteins, causing recruitment of histone modifiers to DNA and thereby affecting chromatin structure to unlock repression [14]. Consequently, after demethylation by a DNMT inhibitor, the release of methyl-CpG binding protein (MeCP2) from the silenced gene promoter prepares chromatin to reinforce the histone acetylation [75] and expression of RA-target genes, leading to cell differentiation. This is in agreement with our data, showing the efficacy of zebularine and RG108 to potentiate granulocytic differentiation in sequential treatments with other drugs, such as RA and HDAC inhibitors. The results of our study support the view that the utility of DNMT inhibitor is based not so much on its own DNMT-inhibiting properties but rather on its potential to enhance the activity of other drugs used in combination [39]. Additionally, the combined strategy may contribute to reduction in the doses of each drug and offer the most successful approaches. Here, we propose low toxic culture conditions with short-term exposure (24-48 h) to zebularine and RG108 in sequential combinations with RA and HDAC inhibitor PB that may hold promise for the development of differentiation therapy in acute leukemia.

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