The novel histone deacetylase inhibitor BML-210 exerts growth inhibitory, proapoptotic and differentiation stimulating effects on the human leukemia cell lines

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Abstract

Histone deacetylase inhibitors have a potent role in the strategy for the treatment of leukemias. BML-210 (N-(2-Aminophenyl)-N′-phenyloctanol diamine) is the novel histone deacetylase inhibitor, and its mechanism of action has not been characterized. In this study, we examined the in vitro effects of BML-210 on the human leukemia cell lines (NB4, HL-60, THP-1, and K562). We found that BML-210 inhibits the growth of all cell lines and promotes apoptosis in a dose- and time-dependent manner. BML-210 alone induces HL-60 and K562 cell differentiation (up to 30%) to granulocytes and erythrocytes, respectively, and in combination with differentiation agents — all-trans retinoic acid and hemin, markedly potentates it. Those treatments cause G1 arrest and histone H4 acetylation, affects transcription factor NF-κB and Sp1 binding activity to their consensus sequences, the p21 or the FasL promoters, and influences expression of Sp1, NF-κB, p21 and FasL. These findings suggest that BML-210 could be a promising antileukemic agent to induce apoptosis and to modulate differentiation through the modulation of histone acetylation and gene expression.

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1. Introduction

Histone deacetylase (HDAC) inhibitors comprise a diverse group of compounds that block histone deacetylation resulting in hyperacetylation of core histones, transcription factors and proteins involved in transcription (Strahl and Allis, 2000). Transient acetylation of core histones is a prerequisite for chromatin remodeling and gene transcription (Strahl and Allis, 2000). This modification leads to loosening of histone-DNA contacts and facilitates the accessibility of a variety of factors to DNA (Wolffe et al., 1997). A number of transcriptional repressors and corepressors have been shown to recruit the HDAC complex to the promoter regions. In acute promyelocytic leukemia (APL) cells, the promyelocytic leukemia–retinoic acid receptor alpha (PML–RARα) fusion protein, generated by chromosomal translocation, represses the gene expression required for myeloid leukemia by recruiting many HDAC molecules and promoting the development of leukemia (Grignani et al., 1999). Pharmacological doses of all-trans retinoic acid (ATRA) can bind to the fusion protein with a loss of HDAC activity and thereby induce reinitiating of differentiation (Lin et al., 1999). APL cells containing promyelocytic leukemia zinc finger–retinoic acid receptor alpha (PLZF–RARα) are resistant to ATRA, but transcriptional repression mediated by HDAC can be released with HDAC inhibitors (Redner et al., 1999). The clinical benefits of HDAC inhibitors and their implications for differentiation therapy currently are under active investigation. The addition of HDAC inhibitors has been shown to inhibit proliferation and to stimulate differentiation and apoptosis in transformed cells both in vitro and in vivo (Yoshida et al., 2003).

Growth inhibitory effects have been documented in virtually all transformed cell types, including cell lines that arise from hematological (leukemia’s, lymphoma’s and myeloid’s) and
epithelial (breast, bladder, ovarian, prostate, lung) tumors (Marks et al., 2001a). This effect leads to the activation of transcription of a finite number of genes (about 2–8%) via histone acetylation (Chambers et al., 2003). HDAC inhibitors invariably inhibit proliferation of transformed cells in culture (Marks et al., 2001b). It is, however, not yet understood which genes are targeted by histone acetylation and how their expression is modulated by the inhibitors. The mechanism of gene expression selectivity currently is also under intensive study.

Currently, a number of structurally different HDAC inhibitors are being evaluated as a potential new class of therapeutic agents but little is known about the molecular events that control their effectiveness (Marks et al., 2001b; Minucci et al., 2001; Vigushin and Combers, 2002). Short-chain fatty acid compounds, like butyrate, represent a class that is currently approved for the use in the clinic (Newmark et al., 1994), inducing cell differentiation and/or apoptosis programs in a wide variety of neoplastic cells. The limitations of its clinical use because of rapid metabolism and a very short half-life in the blood may be overcome by butyrate derivates or prodrugs of butyric acid (Gozzini et al., 2003). In leukemia cells, HDAC inhibitors, such as butyrate derivates and suberoylanilide hydroxamic acid (SAHA) induce differentiation or apoptosis (Richon et al., 1998; Rivero and Adunyah, 1998; Vrana et al., 1999). Several HDAC inhibitors are now being evaluated clinically: SAHA is currently in a phase II clinical study against T-cell lymphomas and metastatic squamous cell carcinomas of the head and neck, MS-275 is in trial for advanced solid tumors or lymphomas and for poor-risk hematological malignancy; CI-994 is used for treatment of leukemia (Kelly et al., 2003; Marks et al., 2001a). The depsipeptide FK228 is a particularly potent agent with broad clinical potential in chronic leukemia, small lymphoblastic lymphoma, acute lymphoblastic leukemia, cutaneous T-cell lymphoma or small cell non-small cell lung cancer (Yoshida et al., 2003). Recently, other classes of compounds, such as novel hybrid compounds of trapoxin and trichostatin A, called CHAPs derivates (Yoshida et al., 2001), have been proposed as a promising candidate for antitumor drugs with strong HDAC-inhibiting activity. In some cases, HDAC inhibitors can overcome drug resistance in other chemotherapeutical treatments (Redner et al., 1999). However, the use of HDAC inhibitors is limited by progressive constitutional symptoms observed in many patients.

In our study, we have investigated the biological effects of the novel HDAC inhibitor, BML-210, on leukemia cell proliferation, viability, differentiation and transcriptional regulation of cell cycle– and apoptosis–regulating genes by transcription factors Sp1, NF-κB and p53. Based on our findings, we suggest that BML-210 could be a promising antileukemic agent to induce apoptosis and to regulate differentiation through the modulation of histone acetylation and gene expression.

2. Materials and methods

2.1. Materials

BML-210 was obtained from Biomol Research Laboratories (USA). ATRA and hemin chloride were purchased from Sigma Chemical Co. (St. Louis, MO), phenyl butyrate from Calbiochem (Germany). The stock solutions of BML-210 (5 mM in dimethyl sulphoxide (DMSO)), ATRA (500 μM in ethanol) and hemin chloride (25 mg/ml in 1.4 M ammonium hydroxide) were stored at −20 °C.

2.2. Cell cultures

The human leukemia cell lines (promyelocytic HL-60, acute promyelocytic NB4, chronic myeloid K562 and acute monocytic THP-1) were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco, Grand Island, NY). Cells were grown at 37 °C in a humidified 5% CO2 atmosphere and used for assays during exponential phase of growth. Cell viability was assayed by exclusion of 0.2% Trypan blue. Cell number was determined by counting cells in suspension in a hemocytometer. Granulocytic differentiation of HL-60 and NB4 cells was determined by nitro blue tetrazolium (NBT) reduction (Collins, 1987). Hemoglobin production during erythroid differentiation of K562 cells was established by benzidine staining (Jeannesson et al., 1984).

2.3. Cell cycle analysis

Untreated and HDAC inhibitor-treated cells were collected by centrifugation, suspended in PBS and fixed in ice cold 70% ethanol (ratio 1:10) for 24 h at −20 °C. After centrifugation at 500 × g for 5 min, cells were suspended in phosphate-buffered saline (PBS) containing propidium iodide (PI) (50 μg/ml) and RNase (0.2 mg/ml) and incubated at room temperature for 30 min. The tubes were then taken at 4 °C in the dark until analysis by flow cytometer (Becton-Dickinson FACS Calibur, USA). The percentage of cells in G0/G1, S and G2/M was analyzed by flow cytometer (Becton-Dickinson FACS Calibur, USA). The percentage of cells in G0/G1, S and G2/M was evaluated with CellQuest software. Apoptotic cells were quantified on PI histogram as a hypodiploid peak and the data were registered on a logarithmic scale.

2.4. Assessment of CD11b

NB4 or HL-60 cells (5 × 10^5 cells/sample) were washed twice in PBS, pH 7.4, then treated with monoclonal anti-human CD11b, C3bi receptor/RPE (DakoCytomation, Denmark) in the dark at 4 °C for 30 min. Cells were washed with PBS containing 2% bovine serum albumin (BSA), fixed in 4% paraformaldehyde for 15–30 min on ice and pellet was resuspended in PBS. Eight thousand events were analyzed for each sample by immunofluorescence using flow cytometry. Proliferating cells with and without CD11b antibodies were used as a control.

2.5. Preparation of cytosolic and nuclear proteins

The cells (5 × 10^6 to 10^7) were harvested by centrifugation at 500 × g for 6 min, washed twice in ice cold PBS and suspended in Nuclei EZ lysis buffer (Sigma, St. Louis, MO) for 5 min on ice. The cell homogenates were then centrifuged at 1500 × g for 5 min. Supernatant, corresponding to the cytosolic fraction, was
clarified by centrifuging at 15,000 ×g for 15 min and then it was frozen at −76 °C or immediately used for sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE). Nuclei were washed in the same cold Nuclei EZ buffer, vortexed briefly and set on ice for 5 min. They were pelleted at 500 ×g for 5 min, then completely suspended in Nuclei EZ storage buffer (Sigma, St. Louis, MO) and frozen at −76 °C.

Nuclear protein extracts for electrophoretic mobility shift assay (EMSA) were prepared by lysis of nuclei in buffer (20 mM Tris-HCl, pH 8.0, 200 μM EDTA, 2 mM EGTA, 20% glycerol, 400 mM NaCl) containing 3 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1× protease inhibitor cocktail (Complete, Sigma, St. Louis, MO). After incubation for 1 h on ice, the extracts were centrifuged at 18,000 ×g for 20 min, and used immediately. Protein concentrations were determined by RCDC protein assay as recommended by the manufacturer (Bio Rad Lab, CA).

For SDS-PAGE analysis of total nuclear proteins, nuclei were resuspended (approx. 5 ×10^7/ml) in two volumes (v/v) of 2× lysis solution (100 mM Tris, pH 7.4, 5 mM MgCl₂, 200 mM DTT and 4% SDS), and thereafter 3 volumes of 1× lysis solution and benzonase (Pure Grade, Merck, Germany) was added to give a final concentration of 2.5 units/ml. The lysates were incubated for 1 h at 0 °C and then centrifuged at 15,000 ×g for 30 min. The supernatants were immediately subjected to electrophoresis or frozen at −76 °C.

2.6. Electrophoretic mobility shift assay (EMSA)

The probes used were synthetic oligonucleotides (MWG-Biotech AG, Denmark) representing binding sites: (5′-AGTT GAGGGGACTTTCCACGC-3′) consensus NF-κB; (5′-AAAGCTTGGAACATAGAAATGGGTCCATCTGTACAA AA-3′) NF-κB from the FasL promoter; (5′-ATTCA-1T CGGCGGCGCCGAC-3′) consensus Sp1; (5′-ATTCCATGGTTTCGGGCGGCAGC-3′) mutated consensus Sp1; (5′-GGCCGAGCGCGGGTCCCCTATGGCCCGG-3′) Sp1-3 (element 3) from the p21 promoter; (5′-ATCA GAAATTGTGGGCAACATAGG-3′) mutated consensus Sp1; (5′-ATCCATGGTCCTGGGGCGGCAGC-3′) Sp1 from the FasL promoter; (5′-CTCGCGGGGGGGGGCCGCAGC-3′) Sp1 from the p65 promoter; (5′-CTTCGGCGCCGTTGGGGCCGCAGC-3′) mutated Sp1 from the p65 promoter; (5′-ATCGAGAACAGTCCCAAATCGTTGAGCTTCAGTCG-3′) p53 from the p21 promoter; (5′-ATCGAGAGATCGTCCAAAATCGTTGAGCTTCAGTCG-3′) p53 from the p21 promoter; (5′-GCCTGTTGGGGCCGAGG GAGAGAAATGGGGAACGTTCAGTCG-3′) PU.1 from the human neutrophil elastase promoter.

Standard DNA reactions were performed with 10 μg nuclear extracts in a 20 μl of reaction buffer (10 mM HEPES pH 7.9, 3 mM MgCl₂, 0.1 mM EDTA, 40 mM NaCl, 10% glycerol) containing 2 μg BSA, 1 μg poly(dI-dC), 1 pM labeled oligonucleotide for 30 min at room temperature. When desired, unlabeled competitor oligonucleotide was added to protein extracts at 50 or 100 fold molar excess for a 15 min preincubation. DNA-protein complexes were resolved on 5% polyacrylamide gel containing 1× Tris-borate buffer. After electrophoresis, gels were dried and then exposed to X-ray films.

2.7. Isolation and fractionation of histones

Histones were extracted as described previously (Treigyte and Gineitis, 1979). Shortly, histones were extracted from nuclei twice by 0.4 N H₂SO₄ and precipitated by adding 5 vol of ethanol at −20 °C overnight. Histone electrophoresis was carried out essentially as described (Hurley, 1977). Firstly, histones (5 μg) were dissolved in a buffer, containing 0.9 M acetic acid, 10% glycerol, 6.25 M urea and 5% β-mercaptoethanol, then resolved on 15% polyacrylamide gels containing 6 M urea and 0.9 M acetic acid, and transferred to Immobilon™ polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). The membranes were probed with anti-hyperacetylated histone H4 (Penta) antibodies (Upstate, Lake Placid, NY).

2.8. Gel electrophoresis and immunoblot analysis

Cytosolic and nuclear proteins were resolved on 7–15% polyacrylamide gradient gels (Invitrogen, CA), then transferred to Immobilon™ PVDF membranes and probed with antibodies against p21, p53, Sp1, FasL, NF-κB p50 or NF-κB p65 (Santa Cruz, Biotechnology, Inc., CA) at a concentration of 1 μg/ml in PBS containing 0.18% Tween-20, 0.35 M NaCl, and 1% BSA. The membranes were subsequently washed with PBS-Tween and then incubated with horseradish peroxidase-conjugated secondary antibody (DAKO, A/S, Denmark) 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.
3. Results

3.1. Growth inhibition of leukemia cell lines by BML-210

In order to estimate the effects of different doses of BML-210, the leukemia cell lines NB4, HL-60, THP-1 and K562 were treated with HDAC inhibitor alone for 5–6 days. In all cell lines tested, BML-210, as a single agent, inhibited cell growth in a dose- and time-dependent manner (Fig. 1). The optimal concentration of BML-210 for the cells was 5 μM. At a concentration of 10 μM, HL-60 and THP-1 cells were more sensitive for cell growth inhibition and survival. The maximum growth inhibition was achieved with 20–30 μM BML-210. HDAC inhibitor treatment at those doses for 72 h of exposure produced a loss of viability in all cell lines tested. In the apoptosis-resistant cell line K562, the cytotoxic effect was delayed. A combination of BML-210 and ATRA exerted additive growth inhibitory effects in NB4 and HL-60 cells (data not shown). 24 h exposition to lethal doses (20–30 μM) BML-210 caused NB4 or THP-1 cell growth restoration after 6 or 9 days, respectively (data not shown).

3.2. BML-210 enhances leukemia cell differentiation induced by different agents

To examine the differentiating activity of BML-210, leukemia cell lines HL-60, NB4 and K562 were treated with 5–10 μM BML-210. Interestingly, BML-210 alone induced a dose-dependent differentiation of HL-60 cells to granulocytes, and K562 cells to erythrocytes up to 30% on day 4 (Fig. 2B, C). By contrast, BML-210 did not induce NB4 cell differentiation. NBT-positivity (Fig. 2B) and expression of the early differentiation marker CD11b at 24 h (Fig. 3) was noticed in HL-60 cells only. The in vitro differentiation experiments with inducers of differentiation were performed at a lower concentration of BML-210 (5 μM) to avoid cytotoxicity (see Fig. 1). Indeed, BML-210 in combination with 1 μM ATRA accelerated and noticeably enhanced NB4 and HL-60 cell granulocytic differentiation during 4 days, with a stronger effect on HL-60 cells (Fig. 2A). Pretreatment with 5 μM BML-210 for 24 h before subsequent treatment with ATRA alone resulted in greater differentiation effects in comparison with ATRA treatment (Fig. 2A). K562 cell erythroid differentiation by hemin alone was delayed till day 5, while combined treatment with 10 μM hemin and 7 μM BML-210 accelerated the cell maturation to erythrocytes (Fig. 2C).

We compared BML-210 effects on NB4 cell growth and differentiation with those obtained using another well known HDAC inhibitor, phenyl butyrate. This agent at concentrations of 0.5–4 mM inhibited cell growth in a dose-dependent manner, and in combination with ATRA (but not alone) increased the NBT-positivity (data not shown).

3.3. Influence of BML-210 on NB4 and HL-60 cell cycle progression

NB4 and HL-60 cells were incubated with BML-210 (5 μM) alone and in combination with ATRA (1 μM), or pretreated with HDAC inhibitor for 24 h and then transferred to fresh medium...
without or with ATRA, and finally analyzed for cell cycle distribution by flow cytometry. Cell cycle analysis revealed that BML-210 caused a decrease in the proportion of NB4, THP-1 and K562 cells in the S and an increase in the G0/G1 phase (Fig. 4A). In HL-60 cells, 5 µM BML-210 alone caused an increase in the G0/G1 phase up to 62%, and in combination with ATRA up to 75% at 24 h (Fig. 4B). This effect of BML-210 was reversible because a 24 h-pretreated cell population transferred to HDAC inhibitor-free medium for the next 24 h started to proliferate with a marked increase in the S phase (62%). The addition of ATRA to 24 h HDAC inhibitor-pretreated cells decreased the proportion in the S phase to 32% and increased that in the G0/G1 to 55%; there was no additive effect on cell accumulation in the G1 phase. This might be caused by a decrease in a viable cell number, and/or by an increase in apoptotic cells during the first 24 h of HDAC inhibitor treatment (Fig. 4A). Thus, BML-210 and ATRA combination increased growth inhibitory effect, which could be attributed to an arrest in the transition through the cell cycle.

3.4. Induction of apoptosis by BML-210 in leukemia cell lines

BML-210 at a concentration up to 10 µM caused cytotoxic effects on different leukemia cells, as previously shown in Fig. 1. As shown in Fig. 5A, C, 10 µM BML-210 caused THP-1, NB4 and HL-60 cell death, the increase in proportion of cells in the subG1 phase at 24 h and double augmentation at 48 h. The extent of induced cell death caused by 10 µM BML-210 alone at 24–48 h varied among cell lines tested (Fig. 5A). BML-210 did not increase K562 cell apoptosis at 48 h time-point, and the appearance of a higher number of dead cells was delayed till day 6 as shown in Fig. 1 and Fig. 5A. BML-210 combination with inducers of differentiation (ATRA or hemin) caused additive cytotoxic effect (Fig. 5A). Flow cytometric analysis of PI stained cells following NB4 cell treatment with BML-210 demonstrated a dose-dependent induction of apoptosis at 48 h, reaching 58% at a dose of 20 µM (Fig. 5C).

Thus, based on comparable results obtained by Trypan blue exclusion, Acridine orange/Ethidium bromide staining (data not shown) and flow cytometry, we conclude that BML-210 induces cytotoxicity through the pathway of apoptosis.

3.5. Histone H4 acetylation in response to BML-210

The two HDAC inhibitors, phenyl butyrate and BML-210, were further examined for their influence on histone H4
hyperacetylation in NB4 and HL-60 cells. In untreated NB4 cells, histone H4 generally is present in Ac 0–Ac 2 forms (Fig. 6A). After 4 h of treatment with both 1.5 mM phenyl butyrate and BML-210, histone H4 appears in hyperacetylated form. This process was dose- and time-dependent, and more pronounced in HL-60 cells. ATRA alone did not induce histone hyperacetylation (Fig. 6B). After the combined treatment with BML-10 and ATRA, histone H4 underwent intense hyperacetylation earlier after around 2 h (Fig. 6E) in comparison with treatment by a single HDAC inhibitor, phenyl butyrate or BML-210 (Fig. 6C, D), or ATRA (Fig. 6B). Here histone H4 deacetylation also started earlier, i.e. after 8 h of treatment. These results demonstrate that HDAC inhibitors and ATRA act synergistically on histone H4 hyperacetylation.

Fig. 6. Time- and dose-dependent hyperacetylation of histone H4 in the response to HDAC inhibitors. (A) NB4 and HL-60 cells were treated with different concentrations of BML-210 for 4–8 h. NB4 cells were treated with 1 μM ATRA alone (B), 1.5 mM phenyl butyrate alone (C), 10 μM BML-210 alone (D) and 10 μM BML-210 in combination with 1 μM ATRA (E) for indicated time. Histones were isolated from untreated or HDAC inhibitor-treated cells resolved on 15% polyacrylamide-acetic acid-urea gels and examined by Western blot analysis using antibodies specific to hyperacetylated histone H4. The five acetylation states of histone H4 are indicated representing non-acetylated (Ac 0), mono-(Ac 1), di-(Ac 2), tri-(Ac 3) and tetra-(Ac 4) acetylated forms.
3.6. Modulation of transcription factors binding activity by BML-210

To study the binding activity of transcription factors Sp1, NF-κB, p53 and PU.1, we performed EMSA with radiolabeled oligonucleotides and nuclear extracts from untreated NB4 cells, and cells treated with BML-210 alone or in combination with ATRA. Previously, we showed a specific Sp1 binding to the consensus sequence and the binding site 3 (Sp1-3) of the p21 promoter, p53 binding affinity to the p21 promoter or NF-κB to the consensus sequence and to the FasL promoter, or PU.1 to the human neutrophil elastase (HNE) promoter (Fig. 7). The binding of those transcription factors to the promoters was efficiently competed by addition of a 50–100 fold molar excess of unlabeled competitor (Cold) or using a probe containing mutated binding site (Mut) from the target promoter. Arrows indicate DNA complexes with Sp1, NF-κB, PU.1 and p53 proteins.

After that we investigated whether treatment with different HDAC inhibitors, BML-210 and phenyl butyrate, affected the binding activity of transcription factors in NB4 and HL-60 cells. By EMSA, both HDAC inhibitors induced NF-κB or Sp1 binding to their consensus sequences and Sp1 to the p65 promoter, or p53 to the p21 promoter during the first 8 h (Fig. 8A). Fig. 8B demonstrates different NF-κB binding activity to consensus sequence in HL-60 and NB4 cells during 4 days of treatment with 10 μM BML-210. This was associated with differentiation induction in HL-60 cells only leading to enhanced complex formation the first two days. NF-κB binding to the FasL promoter increased in both cell lines. In NB4 cells, binding activity of Sp1 to the FasL or Sp1-3 to the p21 promoter was pronounced on days 3–4. In HL-60 cells, we did not observe Sp1 binding, because the Sp1 protein exists in a truncated form, which is not able to bind to the GC-rich elements in DNA (Rao et al., 1999; Savickiene et al., 2005).

Combined treatment with BML-210 and ATRA caused elevated binding capacity of many transcription factors (NF-κB, PU.1, Sp1–3, p53) to the promoters tested during first two days and subsequent decrease on day 3–4 (Fig. 8C). In contrast, Sp1 binding to the FasL promoter markedly increased on day 3–4, which is likely associated with the induction of apoptosis at this time-point.

3.7. Distribution of some regulatory proteins in the cytoplasm and the nucleus of NB4 cells during treatment with BML-210 and ATRA alone or in combination

The total cytoplasmic and nuclear levels of p21, p53, Sp1, FasL and NF-κB components, i.e. p50 and p65, were estimated by Western blot (Fig. 9). ATRA (1 μM) treatment for 96 h had no influence on either the cytoplasmic or the nuclear levels of p21 and p53 expression, but increased the cytoplasmic level of p50, p65 and FasL. At the same time Sp1 protein expression slightly decreased in the cytoplasm. 5 μM BML-210 decreased
both the cytoplasmic and the nuclear levels of p21, Sp1, p50 and p65 and only p53 cytoplasmic level after 24 h of treatment. Expression of FasL in the nucleus only slightly increased during 96 h of HDAC inhibitor treatment. Cotreatment with BML-210 and ATRA for 24–96 h had no significant changes in the cytoplasmic level of transcription factors examined, but decreased expression of p21, Sp1 and p65 in the nucleus during cell maturation. These results are in agreement with the data obtained in EMSA and demonstrate that investigated regulatory proteins are involved in the processes caused by BML-210 alone or together with differentiation inducer.

4. Discussion

BML-210 is a novel histone deacetylase inhibitor with putative effects on leukemia cell growth, differentiation and apoptosis. We found here that BML-210 is comparable with a well known HDAC inhibitor, phenyl butyrate, in its ability to cause cytotoxic effects dependent on exposure time and dose. The growth of four different leukemia cell lines (NB4, HL-60, K562, and THP-1) was inhibited and the apoptotic process was promoted by BML-210. These cellular responses were associated with the increased accumulation of the cells in G1 and subG1 phases of the cell cycle (Fig. 4). It is known that inhibitor of cyclin-dependent kinases, p21 (Waf1/Cip1), plays an important role in G1 cell cycle arrest (Harper et al., 1993). It has also been reported that HDAC inhibitors, like sodium butyrate, FK228, trichostatin A (TSA), MS-27-275, induce the expression of p21 (Kosugi et al., 1999; Richon et al., 2000; Sasakawa et al., 2002). Our results demonstrate clearly that BML-210 caused p21 expression during the first 24 h-treatment, probably via acetylation of histone H3 and H4 in the p21 promoter region (Kwon et al., 2002). This coincides with transcription activation through Sp1 sites of the p21 promoter, which contains six Sp1 binding sites and plays a major role in the regulation of p21 transcription (Gartel and Tyner, 1999; Sowa et al., 1997). Cell arrest in the G1 was necessary for the induction of apoptosis by higher doses of BML-210. Many HDAC inhibitors cause cell growth arrest and induction of differentiation or apoptosis, although the mechanisms of the induction of apoptosis are cell type (Bernhard et al., 1999; Bernhard et al., 2001) or HDAC inhibitor-specific (Bernhard et al., 2001; Kwon et al., 2002; Peart et al., 2003). Our data show that BML-210 alone or in combination with the differentiation inducer ATRA markedly increased Sp1 binding to the FasL promoter during 4 days of treatment in both HL-60 and NB4 cells (Fig. 8). These results are consistent with an increased number of apoptotic cells and up-regulation of FasL expression in the cytoplasm (Fig. 9) supporting the data about the involvement of Fas/FasL apoptotic pathway (Bernhard et al., 2001; Glick et al., 1999; Kwon et al., 2002).

We found that BML-210 alone induces differentiation in K562 and HL-60 cell lines. We detected the early myeloid differentiation marker CD11b expression and NBT-reduction in HL-60 but not NB4 cells. This difference was associated with a more pronounced histone acetylation in HL-60 cells (Fig. 6) or with direct/indirect alterations of the transcriptional profile (Fig. 8). However, BML-210 accelerated and markedly enhanced HL-60 and NB4 cell granulocytic or K562 cell erythroid differentiation, when used in combination with inducers of differentiation. The data is supported by our morphological and immunohistochemical observations and increased PU.1 binding to the HNE promoter (Fig. 8); the expression of the latter is normally up-regulated during the early phases of granulocytic/monocytic differentiation (Scott et al., 1994). This biological effect was accompanied by histone H4 hyperacetylation and more rapid appearance of this modification during 24 h of cotreatment with BML-210 and ATRA (Fig. 6). It is known that histone modifications play a crucial role in recruitment and the function of pre-initiation complex components on promoters during the activation of certain genes (Fischle et al., 2003; Kouzarides, 2002, 2003). In fact, we have demonstrated that BML-210 alone and in combination with ATRA affects the binding activity of
transcription factors NF-κB, p53, Sp1 to the p21, FasL and others myeloid promoters with the following changes in the expression levels of several regulatory proteins.

NF-κB represents a family of inducible transcription factors participating in the regulation of various cellular processes, such as cell growth, differentiation, apoptosis, inflammation and immunity (Glosh, 1999). NF-κB-mediated gene induction was shown to be enhanced by HDAC inhibitors, like TSA or sodium butyrate (Adam et al., 2003; Chen and Greene, 2003; Quivy and Van Lint, 2004). The most abundant form of NF-κB is a p50/p65 heterodimer, in which p65 contains the transcriptional activation domain. Both the subunits are acetylated upon activation with involvement of p300/CBP (CREB binding protein) (Chen and Greene, 2003; Gerritsen et al., 1997). p65 binds to CBP and its homolog p300 (Roth et al., 2001), while p50 fails to recruit the transcriptional co-activators. This resulted in transcriptional activation, presumably via associated histone-directed acetylation activity, which includes localized chromatin (Kiernan et al., 2003). Our results demonstrate that BML-210 enhances NF-κB binding to its consensus sequence during the first hours of treatment. This early effect was most pronounced in HL-60 cells and coincided with the induction of cell differentiation. In NB4 cells, NF-κB activity on days 3–4 represents the survival of proliferating cells (Fig. 8B). During apoptosis, the decrease in NF-κB binding activity to consensus site and to the FasL promoter on day 4 ran in parallel during our experiments with BML-210 alone or in combination with ATRA (Fig. 8). Alternatively, it has been demonstrated that an important functional role of NF-κB in p21 induction, G1 arrest and cell maturation by HDAC inhibitor, sodium butyrate, and the disruption of NF-κB pathway caused the cells to engage an alternative, apoptotic program (Dai et al., 2003).

Sp1 sites are often found in NF-κB-regulated genes and this can provide a number of combinatorial regulations. The interaction of Sp1 with DNA may raise the expression level of NF-κB (Saccani et al., 2001). HDAC inhibitors would thus increase NF-κB target genes even in the absence of activated NF-κB (Hirano et al., 1998). On the other hand, we detected elevated Sp1 binding activity to the p65 promoter and NF-κB to the consensus sequence during 2–4 h of BML-210 treatment. This correlated temporally with the increased p65 protein level in NB4 cell nucleus, which could account for the transcriptional activation of NF-κB-activable genes. There are two classes of such genes: those containing constitutively and immediately accessible NF-κB binding sites in their promoter and those that need the hyperacetylation to become accessible to NF-κB (Saccani et al., 2001). HDAC inhibitors would thus increase NF-κB-dependent transcription by blocking the HDAC activity associated with p65, thereby resulting in histone hyperacetylation and subsequently a higher level of gene expression.

p53 protein conformation for DNA binding to the promoters may play a role in modulating the rate of its acetylation and further recruitment of chromatin remodeling factors (Dornan et al., 2003; Vogelstein et al., 2000). Recently, Lagger et al. (2003) have indeed demonstrated that p53 cooperates with Sp1 in the activation of the p21 promoter. It is known that HDAC inhibitors, such as sodium butyrate, TSA, apicidin, SAHA and others, are able to induce p21 (Calonghi et al., 2005; Joseph et al., 2005). Our results show elevated binding activity of both Sp1–3 and p53 to the p21 promoter during 24 h-exposure to BML-210 alone or together with ATRA in NB4 cells. After such treatment, p21 protein level increased in both the cytoplasm and the nucleus immediately.

Taken together, our results demonstrate that the novel HDAC inhibitor BML-210 could be a promising agent for leukemia therapy to induce apoptosis and to modulate differentiation via histone acetylation and gene expression.

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