

**Characterization of proteins involved in
differentiation and apoptosis of human leukemia
and epithelial cancer cells**

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Cover: confocal microscopy image of alpha-Dystrobrevin (red) and HSP90 (green) co-localization in HeLa cells (presented in paper IV).

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ABSTRACT

Today, cancer is understood as an epigenetic as well as a genetic disease. The main epigenetic hallmarks of the cancer cell are DNA methylation and histone modifications. The latter changes may be an optimal target for novel anticancer agents. The main goal of using histone deacetylase inhibitors (HDACIs) would be restoration of gene expression of those tumor-suppressor genes that have been transcriptionally silenced by promoter-associated histone deacetylation. However, HDACIs have pleiotropic effects that we are only just starting to understand. These may also be responsible for the induction of differentiation, cell-cycle arrest and pro-apoptotic effects.

There are now so many HDACIs available, with such different chemical structures and biological and biochemical properties, that it is hopeful that at least some of them will succeed, probably in combination with other agents or therapies.

In our studies we focussed ourselves on studies some new HDACIs, that can be useful for treating cancers, including leukemia and epithelial cancer. To do that, we used novel HDACIs, like BML-210, and their combination with the differentiation inducer all-trans retinoic acid (ATRA). Cell differentiation and proliferation in general, and specific gene expression require *de novo* protein synthesis and/or post-translational protein modifications. So, we tried to identify proteins in general and specifically the proteins that could be important for the cell differentiation process, and when and where in the cell the proteins appear.

We delineated that HDACIs inhibited leukemia (NB4 and HL-60) cell growth in a time- and dose-dependent way. Moreover, BML-210 blocked HeLa cell growth and promoted apoptosis in a time-dependent way. Combining of BML-210 with ATRA induced a differentiation process in leukemia cell lines that lead to apoptosis. This correlated with cell cycle arrest in G0/G1 stage and changes in expression of cell cycle proteins (p21, p53), transcription factors (NF- κ B, Sp1) and their binding activity to consensus or specific promoter sequences. We also assessed histone modifications, i.e. H3 phosphorylation and H4 hyperacetylation due to HDACI, leading to chromatin remodeling and changes in gene transcriptions.

We have also studied changes in protein maps caused by HDACIs and differentiation agents, identifying differences for a few proteins due to growth inhibition

and induction of differentiation in NB4 cells using BML-210 alone or in combination with ATRA. These proteins are involved in cell proliferation and signal transduction, like Rab, actin and calpain. One of them was alpha-dystrobrevin (α -DB). To further study possible roles of the latter, we determined changes of α -DB protein isoform expression that correlated with induction of differentiation. We thus identified a novel ensemble of α -DB interacting proteins in promyelocytic leukemia cells, including tropomyosin 3, actin, tubulin, RIBA, STAT and others, being important in cytoskeleton reorganization and signal transduction. Using confocal microscopy, we determined that α -DB co-localizes with HSP90 and F-actin in NB4 and HeLa cells. We also revealed that it changes sub-cellular compartment after treatment with ATRA and/or BML-210. α -DB silencing affected F-actin expression in HeLa cells, further supporting the idea that α -DB is involved in cytoskeleton reorganization in cells. Altogether, our results suggest that α -DB may work as a structural protein during proliferation and differentiation processes of human cancer cells.

Based on our findings, we suggest that HDACIs, like BML-210, can be promising anticancer agents, especially in leukemia treatment, by inducing apoptosis and regulating proliferation and differentiation through the modulation of histone acetylations and gene expression.

POPULÄR SVENSK SAMMANFATTNING

Leukemi är cancer i kroppens blodbildande vävnad, som innefattar benmärgen och det lymfatiska systemet. Ordet "leukemi" betyder "vitt blod" på grekiska. Sjukdomen startas oftast bland de vita blodkropparna. Under normala betingelser är de vita blodkropparna kraftfulla kämpar mot olika typer av infektioner. De växer och delar sig oftast normalt på ett kontrollerat sätt, på det sätt vår kropp behöver dem. Men leukemi bryter denna process. Vid leukemi producerar benmärgen ett stort antal onormala celler. De ser annorlunda ut och fungerar ej. De kan tom blockera cellernas normala funktioner exempelvis i infektionsförsvaret. De stör också bildning och funktion hos röda blodkroppar, bla syretransport och koagulation hos blodplättar.

Behandlingen är komplex och beror på åldern hos individen, vilken typ det är och om den är spridd. Kemoterapi är den främsta behandlingsformen och syftar vanligen till att döda de felaktiga cellerna. Behandlingen innefattar vanligen flera olika substanser.

Så det är viktigt att finna nya ämnen och metoder för att behandla leukemicellerna och förstå deras funktionssätt. Målet med vår forskning är att testa nya molekyler som inte är skadliga för kroppen utan är specifikt riktade mot tumörcellerna. Vi är särskilt intresserade av ämnen som blockerar histondeacetylaser (HDACI) hos leukemiceller och solida tumörer. Vi har funnit belägg för att vissa, tex BML-210, i sig själva och tillsammans med vitamin A syra (ATRA) kan inhibera de onormala cellerna, eller få dem att gå i "programmerad celledöd" (apoptos). Vi har också funnit intressanta egenskaper hos ett protein α -dystrobrevin i dessa processer och specifika interaktioner med andra proteiner i cellernas cytoskelett och i olika cellsignaler.

POPULIARI DISERTACIJOS SANTRAUKA

Pastaruoju metu pasaulyje pastebimas padidėjęs sergančiųjų vėžinėmis ligomis skaičius. Tai susirgimų grupė, kuriai yra būdingas nekontroliuojamas genetiškai pakitusių ląstelių dauginimasis ir šių ląstelių gebėjimas naikinti aplinkinius audinius bei išplisti į kitas kūno vietas. Iki šiol nėra tiksliai nustatyta kodėl žmogus suserga šia liga. Manoma, kad jos atsiradimą gali įtakoti ir aplinkos veiksniai, genetinės modifikacijos ar net paveldimumas. Svarbu išaiškinti, kas pakinta organizme susergant vėžiu ir šias žinias panaudoti gydymui.

Histonų Deacetilazių Inhibitoriai (HDACI) - tai nauja cheminių medžiagų grupė, kuri gali sulėtinti vėžinių ląstelių augimą, sąlygoti ląstelės ciklo sustabdymą, indukuoti vėžinių ląstelių diferenciaciją ar užprogramuotą ląstelių mirtį. Kai kurie HDACI jau yra naudojami klinikoje ir yra gan efektyvūs chemoterapiniai agentai. Tačiau šių medžiagų veikimo molekulinis mechanizmas iki šiol nėra galutinai ištirtas. Tikimasi, kad HDACI poveikio sąlygotų vėžinių ląstelių apoptozės bei diferenciacijos molekulinį mechanizmą išaiškinimas gali atverti naujų vėžio gydymo būdų perspektyvas.

Šio darbo tikslas - nustatyti naujų HDAC inhibitorių, kaip BML-210, poveikį kraujo bei epitelio vėžinių ląstelių augimui, diferenciacijai ir programuotai mirčiai. Darbo metu identifikuoti nauji baltymai, kurie gali būti svarbūs šiuose procesuose, bei sudarytas jų sąveikų tinklas, parodantis šių baltymų funkcijas ląsteliniuose procesuose. Eksperimentiniais tyrimais parodėme, jog vienas iš nustatytų baltymų, α -dystrobrevinas, yra svarbus ląstelių signalo perdavimo procesuose. Apibendrinant darbo rezultatus, galime teigti, kad HDACI BML-210 bei jo kombinacija su diferenciacijos induktoriumi retinoine rūgštimi gali būti potencialūs cheminiai agentai, sąlygojantys vėžinių ląstelių augimo stabdymą, diferenciacijos ir programuotos mirties indukciją.

LIST OF PAPERS

This thesis is based on the following articles, which will be referred to in the text by their Roman numerals:

- I. **J. Savickiene, V.V. Borutinskaite, G. Treigyte, K.-E. Magnusson and R. Navakauskiene. 2006.** The novel histone deacetylase inhibitor BML-210 exerts growth inhibitory, proapoptotic and differentiation stimulating effects in the human leukemia cell lines. *Eur. J. Pharmacol.* 549: 9-18;
- II. **V.V. Borutinskaite, J. Savickiene, R. Navakauskiene and K.-E. Magnusson.** Apoptotic effects of the novel histone deacetylase inhibitor BML-210 on HeLa cells. *Submitted.*
- III. **V.V. Borutinskaite, K.-E. Magnusson and R. Navakauskiene. 2005.** Effects of retinoic acid and histone deacetylase inhibitor Bml-210 on protein expression in NB4 cells. *Biology* 4: 88-93;
- IV. **V.V. Borutinskaite, R. Navakauskiene and K.-E. Magnusson.** Multiple roles of alpha-dystrobrevin in human cancer cells during proliferation and differentiation processes. *Submitted.*

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ABBREVIATIONS

α -DB- alpha-dystrobrevin

AML- acute myeloid leukemia

APL- acute promyelocytic leukemia

ATRA- All-trans-retinoic acid

CBF β - Core Binding Factor beta

CD- cluster of differentiation

C/EBPs – CCAAT/enhancer binding proteins

CREB- Cyclic-AMP response element binding protein

DAPC- dystrophin-associated protein complex

Flt3- FMS-like tyrosine kinase 3

GM-CSF- granulocyte-macrophage colony stimulating factor

G-CSF- granulocyte colony stimulating factor

HDACI- histone deacetylase inhibitor

HSC- hematopoietic stem cell

HSP- heat shock protein

IL-interleukin

M-CSF- macrophage colony stimulating factor

NF- κ B- nuclear factor κ B

PML- promyelocytic leukemia

RA- retinoic acid

RAR α - retinoic acid receptor alpha

Sp- specificity protein

INTRODUCTION

1. Blood cell development (Hematopoiesis)

1.1. Introduction to hematopoiesis

Hematopoiesis (from ancient Greek: *haima* blood; *poiesis* to make) is the formation of blood cells in living body, especially in the bone marrow.

It starts with a pluripotent stem cell that is capable of self-renewal and can give rise to the separate cell lineages. Thus when steady-state stem cells divide, only 50 % of daughter cells on average differentiate; the remaining 50 % do not differentiate, but maintain stem cell number. Hematopoietic stem cells (HSCs) differentiate into hematopoietic progenitor cells that are capable of exponential proliferation as well as continuing the process of differentiation. These cells are broadly divided into "lymphoid" and "myeloid" cells (Fig. 1). Lymphoid cells differentiate into T and B cells, natural killer cells, and dendritic cells. Myeloid cells include red blood cells, platelets, monocytes/macrophages and granulocytes. The life span of differentiated cells can range from years, as in the case of T and B cells involved in immunological memory, to 3 months for red blood cells, and to days for granulocytes.

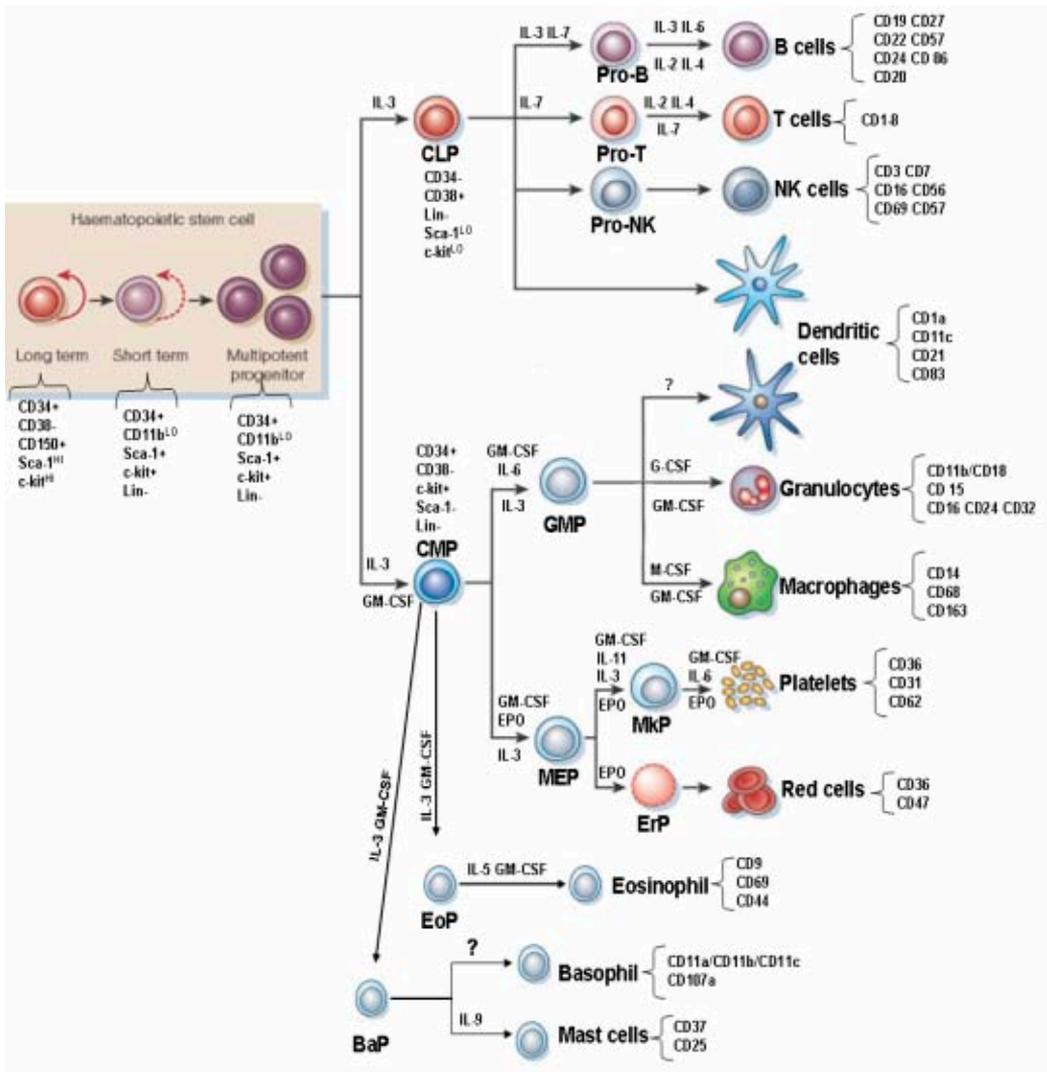


Fig.1. Scheme of hematopoiesis (Reya et al., 2001): bone marrow pluripotent stem cell and the cell lines that arise from it after stimulation with specific cytokines and hematopoietic growth factors. The various progenitor and mature blood cells can be identified by the type of colony they form and the type of expressed molecules on the surface.

BaP- basophil progenitor; CD- cluster of differentiation; c-kit- mast/stem cell growth factor receptor; CLP- common lymphoid progenitor; CMP- common myeloid progenitor; EoP- eosinophil progenitor; EPO- erythropoietin; ErP- erythrocyte progenitor; GMP- granulocyte/monocyte progenitor; Lin- lineage phenotype; MEP- megakaryocyte/ erythrocyte progenitor; MkP- megakaryocyte progenitor; NK- natural killer; Sca-1- stem cell antigen 1.

1.2. Signalling pathways

HSCs must establish a balance between the opposing cell fates of self-renewal and initiation of hematopoietic differentiation. The Wnt (Wingless-type MMTV integration site family), FGF (fibroblast growth factor), Notch, Hedgehog and BMP/TGF β (bone morphogenetic protein/ transforming growth factor β) signalling networks are all implicated in the maintenance of tissue homeostasis by regulating self-renewal of normal stem cells as well as proliferation or differentiation of progenitor (transit-amplifying) cells. Breakage of the stem cell signalling network (Fig. 2) leads to carcinogenesis.

Wnt signalling is highly conserved and Wnt proteins can trigger at least three intracellular signalling pathways: the canonical β -catenin pathway, the non-canonical calcium pathway and the c-Jun N-terminal kinase pathway (Nemeth et al., 2007; Scheller et al., 2006). Three members of Wnt gene family, Wnt5A, Wnt2B and Wnt10B, are expressed to varying levels in hematopoietic cell lines derived from T cells, B cells, myeloid and erythroid cells (Van Den Berg et al., 1998). The number of identified Wnt-associated genes has expanded dramatically. The target genes of canonical Wnt/ β -catenin pathway, such as c-myc (myelocystomatosis oncogene cellular homolog), cyclin D1, c-jun, fra-1 (Fos-related antigen 1) and PPAR δ (peroxisome proliferators-activated receptor) are important in cell cycle regulation that leads to cell proliferation. The non-canonical pathway affects genes involved in cell-cell interactions. Dysfunctional Wnt/ β -catenin signalling, which creates continuous transcription of the many target genes supporting cell proliferation, has now been documented in a wide range of cancers, including colorectal cancer, melanoma, gastric cancer, and tumors derived from hepatic, breast, and prostate tissue (Luu et al., 2004; Reya and Clevers, 2005).

Notch proteins are highly conserved cell-surface receptors and their ligation results in cleavage and release of the intracellular domain of Notch receptors. This can enter the nucleus and bind to transcriptional repressor CSL (CBF1/RBP-J κ /Suppressor of Hairless/LAG-1), converting CSL into a transcriptional activator and subsequent induction of target genes expression (Huang et al., 2007). Activated Notch directly increases PU.1 RNA levels, leading to a high concentration of PU.1 protein, which has been shown to direct myeloid differentiation (Schroeder et al., 2003). Notch1 inhibits the development of erythroid/megakaryocytic cells by suppressing GATA1 activity (Ishiko et al., 2005). It is critical during lymphocyte development, and dysregulation of the pathway can give rise to leukemias, including a subset of T-cell acute lymphoblastic leukemias associated with a recurrent t(7;9) translocation of human Notch1 (Screpanti et al., 2003; Virag et al., 2005).

The BMP/TGF β signalling pathway involved multiple steps in hematopoiesis. The BMPs are members of the TGF- β family of cytokines and regulate development and differentiation through phosphorylation of SMADs and their translocation into the nucleus where they target genes, such as Runx2 (Runt-related transcription factor 2). Loss of either Smad1 or Smad5 causes a failure in the generation of definitive hematopoietic progenitors (McReynolds et al., 2007). Also Smad family members activate PKA (protein kinase A) signalling that play crucial role in many different cellular processes.

In mammals there are three Hedgehog (Hh) genes, Sonic, Indian and Desert Hedgehog. Secreted Hh glycoproteins act via the transmembrane proteins Patched1 (Ptch1) and Smoothed (Smo). In the absence of ligand, Ptch1 inhibits Smo, a downstream protein in the pathway. Downstream of Smo is a multi-protein complex known as the Hedgehog signalling complex, which comprises transcription factors, such as zinc-finger Gli, PKA, protein kinase CK1 (formerly casein kinase 1) and glycogen synthase kinase 3 (GSK3) (Callahan et al., 2004). Regulators of Hedgehog

signalling in vertebrates also include megalin, which is a member of the low-density lipoprotein receptor related family and binds Hedgehog (McCarthy et al., 2002) and SIL (stem cell leukemia-interrupting locus protein) which functions downstream of Ptch (Izraeli et al., 2001).

Hedgehog signalling plays a role in many processes during embryonic development and remains active in the adult where it is involved in the maintenance of stem cell populations. Here, activation of the Hedgehog pathway leads to an increase in angiogenic factors (angiopoietin-1 and angiopoietin-2), cyclins (cyclin D1 and B1)), anti-apoptotic genes and to a decrease in apoptotic genes (Fas). It can also promote certain forms of cancer (Izraeli et al., 2001).

The signal transduction pathways triggered, when angiotensin 1 (Ang1) binds to endothelial-specific receptor tyrosine kinase (Tie2), have been extensively studied. Thus, several cell signalling cascades and downstream targets have been identified, including PI3K (phosphatidyl inositol 3-kinase), SHP2 (also called Ptpn11 for protein tyrosine phosphatase, non-receptor type 11), Grb2 (growth factor receptor-bound protein 2), Grb14, Dok-R (Docking protein-related), ShcA ((Src homology 2 domain containing) transforming protein A), that play important roles in inflammation, apoptosis and other cellular processes (Eklund and Olsen, 2005). Arai and co-workers demonstrated by instance that HSCs expressing the receptor tyrosine kinase Tie2 are quiescent and antiapoptotic and comprise a side population of HSCs, that adhere to osteoblasts in the bone marrow niche. The interaction of Tie2 with its ligand, Ang1, induced cobblestone formation of HSCs *in vitro* and maintained *in vivo* a long-term repopulating activity of HSCs. Furthermore, Ang1 enhanced the ability of HSCs to become quiescent and induced adhesion to bone, resulting in protection of the HSC compartment from myelosuppressive stress. These data suggested that the Tie2/Ang1 signalling pathway plays a critical role in the

maintenance of HSCs in a quiescent state in the bone marrow niche (Arai et al., 2004).

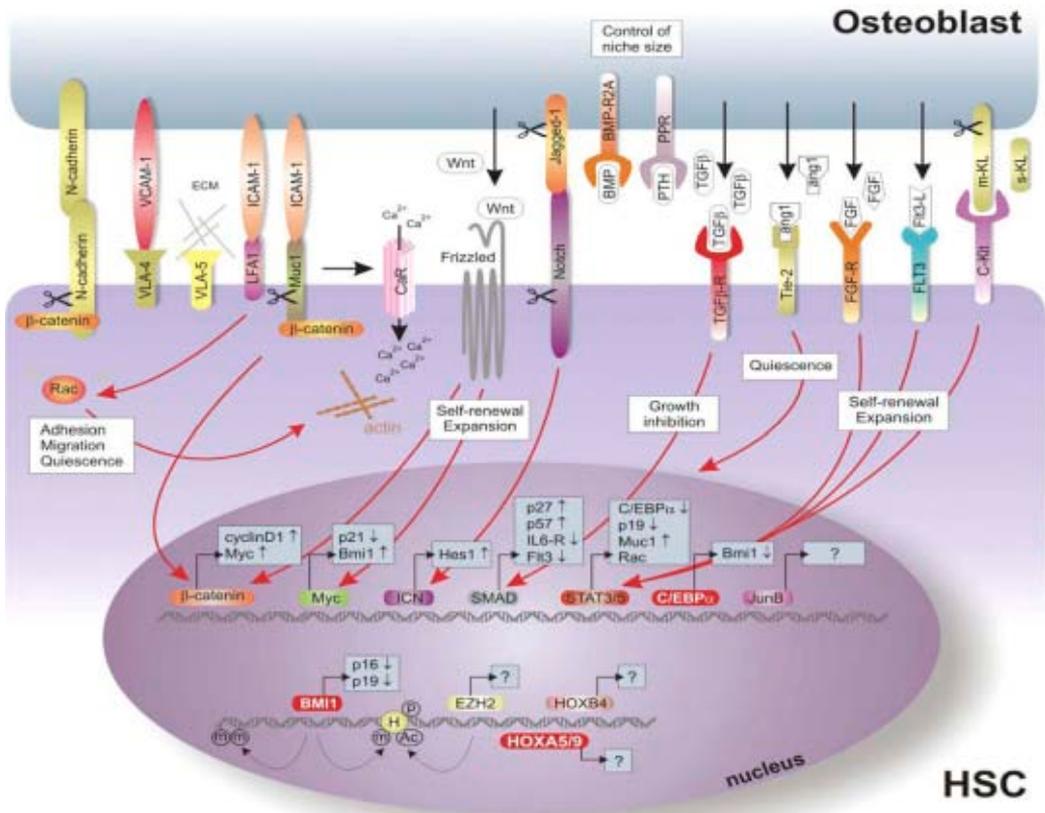


Fig.2. Signal transduction pathways in the HSC niche. A graphical representation of signalling pathways involved in the HSC fate in the niche. In this model, direct physical interaction between the HSC and osteoblast could be mediated by cadherins and integrins which are involved in processes like adhesion and migration of the stem cell. Once appropriately localized within the quiescent niche, processes such as self-renewal, maintenance of quiescence or exit from the niche followed by proliferation and differentiation are highly controlled by growth factors and cytokines locally secreted by osteoblasts and stromal cells. Examples of such molecules are TGF- β , which is a negative regulator of the cell growth, Ang-1 responsible for the stem cell quiescence or Wnts and FGF-1, which promote stem cell expansion. These factors can now dictate HSC fate by triggering specific signalling downstream modulators within the HSC, such as Myc, β -catenin, STATs, SMADs or C/EBP α . The possible intrinsic, that is, epigenetic regulators are represented in the lower part of the figure (Rizo et al., 2006).

The signalling systems discussed here are only some of the major players, and even the description of these is greatly oversimplified because many of these pathways interconnect with one another, i.e. creating "cross talk". Although this "cross talk" seem to create problems not only for our comprehension but also for the cell (!). It is essential for the precise modulation of the genetic response to a variety of ligands reaching the cell at the same time and at varying intensities.

Over the last few years, many reports have extended our knowledge of the novel proteins and signalling cascades that are involved in cell proliferation, differentiation and apoptosis. One such example is the dystrophin-associated protein complex (DAPC). DAPC originally identified in muscle, where it stabilizes the membrane by linking the actin-based cytoskeleton to the basal lamina. It consists of dystrophin, dystroglycan, sarcoglycans, dystrobrevin and syntrophin (Blake et al., 1996). Mutations in some DAPC members result in various forms of muscular dystrophy. The DAPC is also expressed in non-muscle tissues, and it is now considered not only as a mechanical component of the cell but also as a cytoskeletal scaffold on which signalling complexes are assembled (Rando et al., 2001). Our group has earlier shown that α -DB is tyrosine phosphorylated after treatment of acute promyelocytic cells for granulocytic differentiation with ATRA (Kulyte et al., 2002). The role of DBs in intracellular signal transduction, as well as in other cellular functions, is starting to become clearer thanks to the studies of their binding partners. It has been suggested that specific DB isoforms may interact with a specific subset of proteins (Blake et al., 1999; Peters et al., 1998). Several DB-associated proteins have recently been described, e.g. syncoilin, desmuslin, DAMAGE, dysbindin and pancortin (Newey et al., 2001; Mizuno et al., 2001; Albrecht and Froehner, 2004). Since DBs have no enzymatic activity of their own, their involvement in signalling pathways may be dependent on interactions with other

proteins. Indeed, like dystrophin, DBs can bind one or two syntrophin molecules, which in turn bring multiple signalling molecules together by recruiting nNOS, protein kinases, ion channels and membrane protein receptors to the dystrophin complex (Albrecht and Froehner, 2002; Garcia et al., 2000). DAPC components are the target of a variety of protein kinases which regulate the dynamics of their interactions. It has been demonstrated that both α - and β -DB are specific phosphorylation substrates for PKA and that protein phosphatase 2A (PP2A) can associate with DBs. The data suggest a new role for DB as a scaffold protein that may play a role in different cellular processes involving PKA signalling (Ceccarini et al., 2007).

Taken together, the balance between self-renewal and differentiation of hematopoietic cells is of critical importance: too little self-renewal or too much differentiation may jeopardize the ability to sustain hematopoiesis throughout life, whereas excessive self-renewal and/or aberrant differentiation may result in leukemogenesis.

1.3. Regulation of self-renewal and differentiation by extrinsic and intrinsic events

HSCs can differ in self-renewal, clone size (= number of differentiated progeny per HSC), differentiation capacity, migration patterns and primitiveness (Fig. 3). It is unclear how heterogeneous the HSC compartment is and the basis of the heterogeneity has remained speculative (Guenechea et al., 2001; Sieburg et al., 2006). The prevailing view is that HSC heterogeneity is regulated by both extrinsic and intrinsic events.

HSCs reside in the bone marrow in adult mammals in a specialized microenvironment or niche. In this niche, most HSCs remain in a quiescent state (G0 state), thereby preserving their capacity to self-renew. The size and character of HSC pool is regulated by the balance between self-renewal and differentiation, symmetric (HSC expansion) and asymmetric (HSC maintenance) cell divisions, survival and apoptosis of HSCs. Extrinsic (environmental) signals are derived predominantly from stromal cells and their products. Homing of HSCs to different types of stromal cell niches should also contribute HSC heterogeneity. In addition to extrinsic signals, intrinsic mechanisms, including transcription factors, signal transducers, epigenetic regulators, and apoptotic proteins, control HSC decisions (Akala and Clarke, 2006; Muller-Sieburg et al., 2002; Oguro and Iwama, 2007).

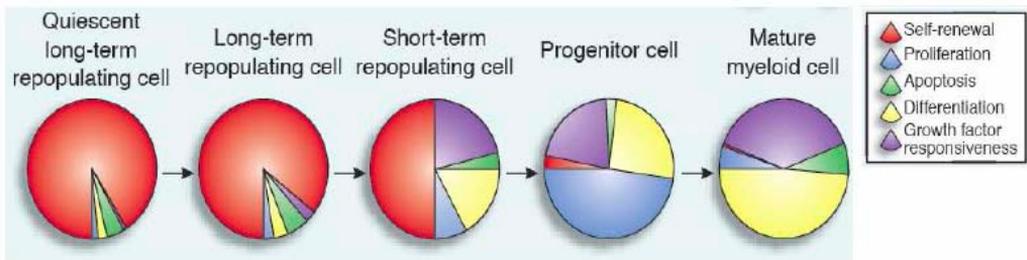


Fig.3. Heterogeneity of HSCs.

1.3.1. Extrinsic factors

Extrinsic control would mean that self-renewal and differentiation can be controlled by external factors, such as cell-cell interactions in the hematopoietic microenvironment or cytokines, and thereby be responsive to demands for increased hematopoietic cell production (Fig. 1). The microenvironment can be provided by stromal matrix cells, that comprise adipocytes, fibroblasts, endothelial cells and

macrophages, and which secrete extracellular molecules such as collagen, glycoproteins, glycolipids, glycosaminoglycans to form the extracellular matrix.

A cell expresses a selection of cell surface glycoproteins and glycolipids, which mediate its interaction with antigen, components of the immune system, and with other cells and tissues. These molecules, i.e. cluster of differentiation (CD) molecules, are used to identify the cell type, stage of differentiation and activity of a cell (Fig. 1). Thus, CD34 has been considered to be the most critical marker for HSCs. CD34 expression is down-regulated on primitive cells as they differentiate into mature cells. CD11b is used as a marker for granulocyte/monocyte differentiation, and it has been established that PU.1 and Sp1 regulate CD11b transcription (Chen et al., 1993; Hickstein et al., 1992).

CD molecules have numerous functions, often acting as receptors or ligands important to the cell. Thereby, a signal cascade is usually initiated, altering the behavior of the cell. Some CD proteins do not play a role in cell signalling, but have other functions as in cell adhesion (CD2, CD11a, CD18, CD33).

Table 1. Hematopoietic growth factors and their main effects on target cells.

Factor	Target cells	Main effects and traits	Cell sources
IL-1	Hematopoietic cells, immune system cells	Inflammatory agent: activates lymphocytes, neutrophils, fibroblasts, NK	Macrophage, somatic cells
TNF	Stromal cells	Inducer of apoptosis, promoter of secondary production of cytokines by stromal cells	Platelets, T cells, somatic cells, macrophages
SCF	Mast cells, early pluripotent cells	Growth factor for stem cells, early lymphoid and myeloid	Stromal cells, fibroblasts

		progenitors, mast cells	
Flt-L	Stem and progenitor cells	Growth factor for stem cell and progenitors	Stromal fibroblasts
IL-3	Early hematopoietic cells	Growth factor for early hematopoietic cells	T lymphocytes
GM-CSF	Hematopoietic progenitor cells in granulocytic, monocytic lineage	Stimulator of early hematopoiesis, mast cells granulopoiesis, monocyte formation	Stromal cells, T lymphocytes, mast cells
IL-6	B cells, megakaryocytes	Promoter of platelet production, immunoglobulin production	Fibroblasts, T cells, macrophages
G-CSF	Granulocyte progenitors, granulocytes	Role in early hematopoiesis, granulocyte production	Stromal cells, macrophages
Trombopoetin	Stem cells, megakaryocytes	Growth factor for platelets	Liver, kidney
M-CSF	Monocyte progenitors, monocytes	Promoter of monocyte production	Stromal cells, macrophages
IL-5	Eosinophils	Role in eosinophil formation	T cells, mast cells
Epo	Erythroid progenitors	Growth factor for red cells	Liver, kidney

IL-interleukin; TNF-tumor necrosis factor; SCF-serum cell factor; Flt-L- ligand for stem cell tyrosine kinase 1; GM-CSF- granulocyte-macrophage colony stimulating factor; G-CSF- granulocyte colony stimulating factor; M-CSF- macrophage colony stimulating factor.

Stromal cells are the major source of growth factors (Table 1). Stem cell factor (SCF, also known as c-kit ligand) is produced by stromal cells. It binds to its receptor c-kit, expressed by hematopoietic stem cells and is essential for normal blood cell production (Keller et al., 1995; Li and Johnson, 1994). The ligand for stem cell tyrosine kinase 1 (Flt3L) is a transmembrane protein and binds to Flt3 on hematopoietic cells. It is important for cell survival and cytokine responsiveness. They can also synergize with other growth factors, such as IL-3 and IL-6, to induce proliferation of these primitive cells and are therefore widely used in various *in vitro* culture systems (Ramsfjell et al., 1996). Erythropoietin (Epo) is synthesized by the kidney and is the primary regulator of erythropoiesis. Epo stimulates the proliferation and differentiation of immature erythrocytes (Inoue et al., 1995). Colony Stimulating Factors (CSFs) are cytokines that stimulate the proliferation of specific pluripotent stem cells of the bone marrow in adults. Granulocyte-CSF (G-CSF) is specific for proliferative effects on cells of the granulocyte lineage. Macrophage-CSF (M-CSF) is specific for cells of the macrophage lineage. Granulocyte-macrophage-CSF (GM-CSF) has proliferative effects on both classes of lymphoid cells (Fleetwood et al., 2007). IL-3, which is secreted by T cells, is also known as multi-CSF, since it stimulates stem cells to produce all forms of hematopoietic cells. Thrombopoietin makes myeloid progenitor cells differentiate to megakaryocytes, i.e. thrombocyte-forming cells (Underhill and Basser, 1999).

An important feature of growth factor action is that two or more growth factors may synergize in stimulating a particular cell to proliferate or differentiate (Fig. 1). Moreover, the action of one growth factor on a cell may stimulate production of another growth factor or growth factor receptor.

1.3.2. Intrinsic factors

Differentiation of pluripotent hematopoietic stem cells into mature circulating blood cells is coordinated by complex series of transcription factors. Tissue specific and developmentally correct expression of a given gene is thus not achieved by a single transcription factor. Rather a unique combination of cell-type specific and wide-expressed nuclear factors account for specificity and diversity in gene expression profiles (Fig. 4) and the composition and balance of transcription factors within a cell are critical determinant of cell lineage/differentiation.

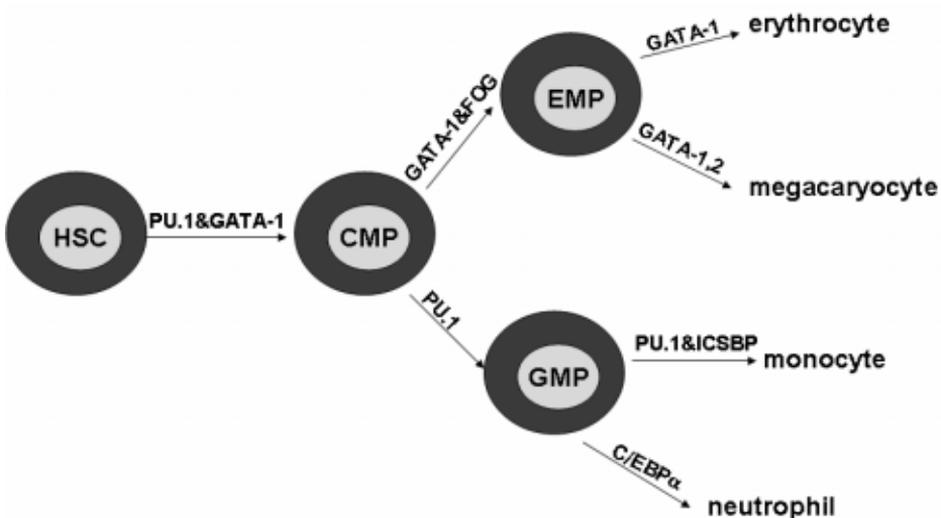


Fig.4. A model for the transcriptional control of hematopoiesis and myeloid lineage commitment.

PU.1/ Spi1 (spleen focus forming virus (SFFV) proviral integration oncogene). The transcription factor PU.1 is a hematopoietic-specific ETS (E26 transformation-specific) family member involved in the development of all hematopoietic lineages (Huang et al., 2008). This nuclear protein binds to a purine-rich sequence known as the PU-box found near the promoters of target genes, and

regulates their expression in coordination with other transcription factors and cofactors (Kastner and Chan, 2008). It has further been demonstrated that Junb (jun B proto-oncogene) and Jun are direct target genes for PU.1 (Fig. 5, A) (Steidl et al., 2006). Junb may play a central role in differentiation and growth arrest during hematopoiesis. Junb target genes are cyclin-dependent kinase inhibitor 2A (p16), Core Binding Factor (CBF)- β and many others. c-Jun is highly responsive to extracellular signals that control proliferative and apoptotic programs (Milde-Langosch et al., 2000; Shaulian and Karin, 2001).

Yoshida et al. (2007) showed that PU.1 directly activates the transcription of the C/EBP ϵ gene and binds PML (Promyelocytic leukemia), which is essential for granulocytic differentiation.

PU.1 also directly controls the expression of critical genes involved in macrophage differentiation and function, e.g. the CD11 integrin, the M-CSF, G-CSF and GM-CSF receptors. PU.1-mediated macrophage differentiation has recently been shown to depend on the induction of the transcription regulators Erg-1/2 (Early Growth Response-1/2) and Nab-2 (NGFI-A Binding Protein 2). These factors act to reinforce the macrophage gene expression program and repress the alternate neutrophil program through repression of Gfi1 (Growth Factor Independent 1) (Dahl et al., 2007).

Deregulation of PU.1 leads to loss of lineage development and possibly leukemia. PU.1 (-/-) mice have complete loss of macrophages and B cells, delayed development of T cells and granulocytes. The block occurs at the CMP stage. It was shown, that the function of PU.1 is down-regulated by AML1-ETO in t(8;21) myeloid leukemia that blocking the differentiation process (Vangala et al., 2003).

ICSBP (Interferon consensus sequence binding protein). ICSBP is a transcription factor that specifically presents in hematopoietic cells and can regulate transcription through multiple DNA elements. However, its own binding ability is

very weak, and to recognize the target sequence tightly, it requires other transcription factors, such as PU.1 (Nakano et al., 2005). Both ICSBP and PU.1 are involved in the regulation of many immune-related genes, and the development of macrophages and dendritic cells (Iwama et al., 2002). ICSBP (-/-) mice, which are immunodeficient and susceptible to various pathogens, also have defects in the macrophage function and develop a chronic myelogenous leukemia (CML)-like syndrome (Tamura et al., 2000).

GATA (GATA binding factors). The GATA family is divided into two subfamilies on the basis of the expression profiles of the individual transcription factors. GATA1, GATA2 and GATA3 belong to the hematopoietic transcription factors family, since they are expressed mainly in the hematopoietic system. GATA1 is expressed in primitive and definitive erythroid cells, megakaryocytes, eosinophils and mast cells (Ferreira et al., 2005), and GATA2 in stem and progenitor cells, at a more immature stage compared with GATA1. GATA3 is found exclusively in T cells of hematopoietic lineage. High levels of GATA1 block PU.1 function and thereby direct cells into the erythroid and megakaryocytic lineages.

The expressions of GATA1 and GATA2 genes may influence the regulation of hematopoiesis in the bone marrow stroma (Fig. 5, B) and it is worthy of further explore their roles in pathogenesis and development of leukemia. Over the past few years, mutations in the gene encoding GATA1 have been linked to several human hematologic disorders, including X-linked dyserythropoietic anemia and thrombocytopenia, X-linked thrombocytopenia and beta-thalassemia, and Down syndrome acute megakaryoblastic leukemia (Cantor, 2005). It was shown that GATA1 knockout mice lack definitive erythroid cells (Crispino, 2005).

C/EBPs (CCAAT/enhancer binding proteins). The C/EBPs are a family of transcription factors that regulate cell growth and differentiation. Two members, C/EBP α and C/EBP ϵ , are of critical importance in granulopoiesis. Disruption of

C/EBP α gene in mice results in the loss of production of neutrophils and eosinophils because of loss of G-CSF receptor, whereas mice that lack C/EBP ϵ generate neutrophils and eosinophils with abnormal function, gene regulation, and morphology (Lee et al., 2006). C/EBP α mutations have been observed in acute myeloid leukemia (AML) patients with approximate frequency of 5-14 %.

The C/EBP α transcription factor regulates the balance between cell proliferation and differentiation in hematopoietic and non-hematopoietic tissues. C/EBP α promotes differentiation by the up-regulation of lineage-specific gene products and by the exit from cell cycle that means proliferation arrest (Fig. 5, C). Several models of C/EBP α -induced growth arrest have been described, when different regions of C/EBP α are involved in different protein binding (Fuchs et al., 2008). The main role of C/EBP α is in the development of granulocytes, and it needs to be suppressed at the granulocyte/monocyte progenitors (GMPs) for both basophil and mast cell development.

C/EBP α can cooperate with additional factors to direct monocytic commitment of primary myeloid progenitors. C/EBP α and PU.1 are expressed in HSCs, where C/EBP α binds and activates PU.1 during granulocyte and macrophage development (Friedman, 2007). However, both these transcription factors are down-regulated in megakaryocyte-erythrocyte progenitors (Kummalu and Friedman, 2003).

C/EBP ϵ is expressed exclusively in granuloid cells and essential for the terminal differentiation of committed granulocyte progenitors. It has also been shown, that C/EBP ϵ is directly regulated by Retinoic Acid Receptor (RAR)- α (Du et al., 2002).

CREB (Cyclic-AMP response element binding protein). CREB is a transcription factor that functions in glucose homeostasis, growth-factor-dependent cell survival, proliferation and memory (Kinjo et al., 2005). Signalling by

hematopoietic growth factors, such as GM-CSF, results in activation of CREB and up-regulation of CREB target genes (Kinjo et al., 2005). In resting cells, CREB exists in an unphosphorylated state that is transcriptionally inactive; upon cell activation, it becomes phosphorylated and can bind to CRE on the promoters of target genes (Fig. 5, D). The activation of CREB turns on the transcription of more than 5000 target genes, including proto-oncogenes such as c-fos (FBJ murine osteosarcoma viral oncogene homolog), cell cycle regulatory genes such as cyclin A1 and cyclin D2, antiapoptotic gene Bcl-2 (human B-cell lymphomas), and other genes related to growth, survival such as *erg1*, mitogen activated (MAP)- kinase (Kinjo et al., 2005; Siu and Jin, 2007). CREB overexpression is sufficient for immortalization, growth factor-independent proliferation, and blast-like phenotype (Shankar et al., 2005; Shankar et al., 2005).

Flt3 (FMS-like tyrosine kinase 3). Flt3 is a receptor tyrosine kinase expressed by immature hematopoietic cells and is important for the normal development of stem cells and the immune system. Ligand binding to Flt3 promotes receptor dimerization and subsequent signalling through phosphorylation of multiple cytoplasmic proteins, including Shc1, SHP-2, SHIP (Src homology 2 domain-containing inositol-5-phosphatase), Cbl (Casitas B-lineage lymphoma), Cbl-b, Gab1 (growth factor receptor bound protein 2-associated protein 1) and Gab2, as well as the activation of several downstream signalling pathways, such as the Ras/Raf/MAPK and PI3K cascades. The ligand for Flt3 is expressed by marrow stromal cells and other cells and synergizes with other growth factors to stimulate proliferation of stem cells, progenitor cells, dendritic cells, and natural killer cells (Rosnet et al., 1993). Mutations of Flt3 have been detected in about 30% of patients with AML (Table 2). Patients with Flt3 mutations tend to have a poor prognosis. The mutations most often involve small tandem duplications of amino acids within the juxtamembrane domain of the receptor and result in constitutive tyrosine kinase

activity. Expression of a mutant Flt3 receptor in murine bone marrow cells results in a lethal myeloproliferative syndrome and preliminary studies suggest that mutant Flt3 cooperates with other leukemia oncogenes to confer a more aggressive phenotype (Birg et al., 1992; Choudhary et al., 2005; Kiyoi et al., 1999).

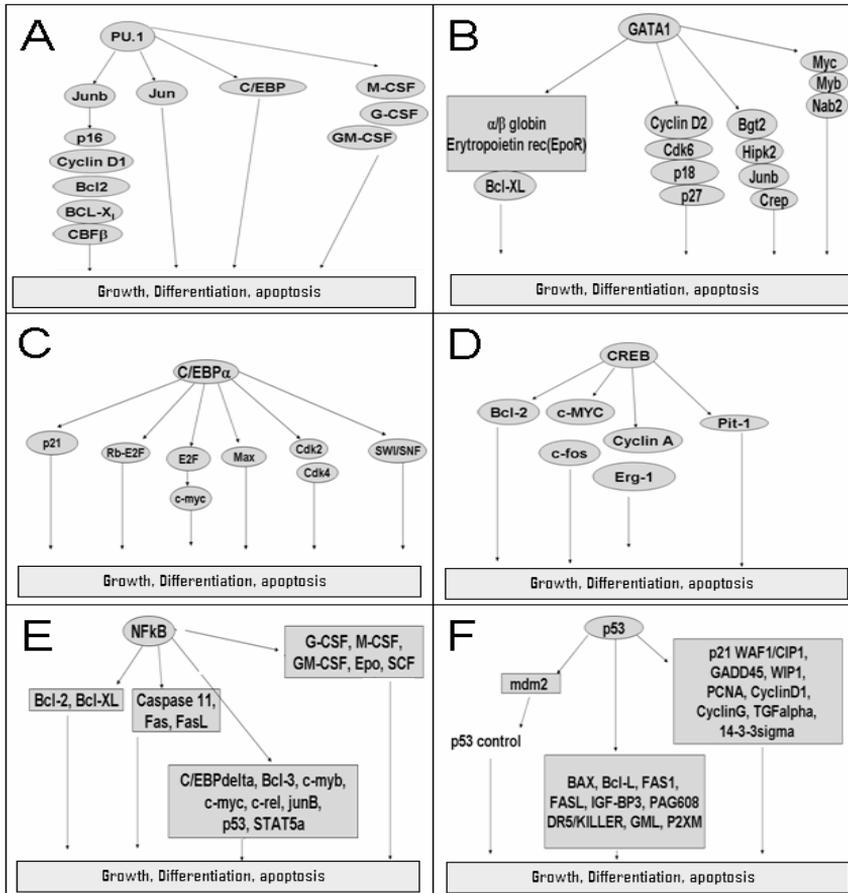


Fig.5. Schemes outlining the role of transcription factors, including PU.1 (A), GATA1 (B), C/EBP α (C), CREB (D), NF- κ B (E) and p53 (F) in the signal transduction pathways during proliferation and differentiation.

p53. The p53 protein product is a regulator of DNA transcription. It binds directly to DNA, recognizes DNA damage (single- or double-strand breaks), and mediates at least two important cellular events. It can induce cell cycle arrest in G1 or it can promote apoptosis. If cellular damage is "considered" repairable, p53-induced cell cycle arrest allows time for DNA repair. With more extensive damage, p53 moves the cell into the apoptotic pathway to prevent the cell with an impaired DNA sequence from proliferating as a defective or malignant clone. p53 exerts control of the cell cycle through up-regulation of p21, an inhibitor of the cyclin-dependent kinases (CDKs) responsible for carrying the cell through G1, and consequently, through the inhibition of cyclinD/CDK4 phosphorylation of Rb (Fig. 5, F) (Jin et al., 2008). In the event that DNA damage is more severe and non-repairable, p53 performs its alternate role of moving the cell into apoptosis through the Bax (Bcl2-associated X protein)/Bcl-2 pathway (Israels and Israels, 1999). In summary, p53 loss is relatively rare in leukemia. However, small sub-populations of leukemia cells may harbor these genetic changes, resulting in a relative resistance to cytotoxic regimes. p53-negative sub-populations may, therefore, survive drug treatment and initiate relapsed disease showing a more aggressive phenotype and increased drug resistance (Wickremasinghe and Hoffbrand, 1999).

NF- κ B (nuclear factor κ B). The eukaryotic NF- κ B plays an important role in inflammation, autoimmune response, cell proliferation, and apoptosis by regulating the expression of genes involved in all these processes (Fig.5, E). Five members of the NF- κ B family have been identified: NF- κ B1 (p50/p105), NF- κ B2 (p52/p100), RelA (p65), RelB, and c-Rel (Lerebours et al., 2008). They share a highly conserved Rel homology domain (RHD), which is responsible for DNA binding, dimerization, and interaction with I κ B. NF- κ B targets genes that promote tumor cell proliferation,

survival, metastasis, inflammation, invasion, and angiogenesis (Fig. 5, E) (Sethi et al., 2008).

The Rel/NF- κ B signal transduction pathway is misregulated in a variety of human cancers, especially in those of lymphoid cell origin. Several human lymphoid cancer cells have been reported to have mutations or amplifications of genes encoding NF- κ B transcription factors. In most cancer cells NF- κ B is constitutively active and resides in the nucleus. Experimental *in vitro* and *in vivo* studies have shown that down-regulation of NF- κ B activity by natural and synthetic NF- κ B inhibitors suppresses the development of carcinogen-induced tumors, inhibits the growth of cancer cells and induces apoptosis with alternation of gene expression which is critical for the control of carcinogenesis and cancer cell survival (Sarkar and Li, 2008).

Table 2. Frequency of mutations in AML.

Mutations	Frequency in AML
ras	18%
Flt3	32%
ras and Flt3	1%
ras or Flt3	49%
p53	10%
p53 and ras	1%
p53 and Flt3	1%
p53, ras aor Flt3	54%
none	46%

Sp (specificity protein). Sp protein family consists of four members: Sp1, Sp2, Sp3 and Sp4. Sp1 is a ubiquitous DNA-binding transcription activator that recognizes GC-rich sequences in the promoters of target genes. Sp1 is abundantly

expressed in myeloid cells and with cooperation with GABP (GA binding protein) transcription factor, Sp1 achieved high levels of myeloid-specific expression of the CD18 promoter; and cooperation with C/EBP leads to regulation of CD11b promoter activity by Sp1 (Khanna-Gupta et al., 2000). The Sp3 transcription factor can act as an activator as well as an inhibitor. In most cases, the increase of the Sp1/Sp3 ratio has been correlated with increased expression of response genes, suggesting that transcription is regulated via the co-operative action of both transcription factors (Bouwman and Philipsen, 2002). It was demonstrated that Sp1/Sp3 is involved in the activation of the GATA1 erythroid promoter in K562 cells that leads to differentiation (Hou et al., 2008). Sp1 interacts directly with proteins of the basal transcriptional machinery such as TFIID components, and with several sequence-specific activators including NF- κ B, GATA, YY1(Yin and yang 1), E2F1 and Rb (retinoblastoma) (Koutsodontis et al., 2002).

p21 (Cyclin-dependent kinase inhibitor 1A (p21, Waf1/Cip1)). The encoded protein binds to and inhibits the activity of cyclin-CDK2 or -CDK4 complexes, and thus functions as a regulator of cell cycle progression at G1 (Jin et al., 2008). The expression of this gene is tightly controlled by the tumor suppressor protein p53, through which this protein mediates the p53-dependent cell cycle G1 phase arrest in response to a variety of stress stimuli. Also there is another p53-independent regulation of p21 gene transcription. Gartel et al. (1999) showed that STAT1/2/3 (signal transducer and activator of transcription 1/2/3), p73, C/EBP α , RAR α , Sp1, Sp3, C/EBP β can bind to p21 promoter and activate transcription. The p21 protein can also interact with proliferating cell nuclear antigen (PCNA), a DNA polymerase accessory factor, and plays a regulatory role in S phase DNA replication and DNA damage repair. This protein was reported to be specifically cleaved by CASP3-like caspases, which thus leads to a dramatic activation of CDK2, and may be

instrumental in the execution of apoptosis following caspase activation. Two alternatively spliced variants, which encode an identical protein, have been reported (Fan et al., 2004; Jin et al., 2000).

2. Leukemia

2.1. Introduction to leukemia

Leukemia is one of the most common forms of cancer especially in children. Leukemia is characterized by unregulated proliferation of one cell type. It may involve any of the cell line or a stem cell common to several cell lines. Leukemias are classified into 2 major groups (Fig. 6):

- 1) chronic in which the onset is insidious, the disease is usually less aggressive;
- 2) acute in which the onset is usually rapid, the disease is very aggressive, and the cells involved are usually poorly differentiated with many blasts.

Acute leukemias represent a group of diseases, that includes both the myeloid (AML) and lymphoid (ALL) malignancies (Basso et al., 2007). AML is more common in adults, and ALL in children.

Causes of leukemia: high level radiation/toxin exposure, viruses, genes, chemicals, but mostly unknown.

Treatment: chemotherapy, immunotherapy, radiation, bone marrow transplantation.

Prognosis for AML: 1) Survival rates greatly improved over past 25 years; 2) Majority of patients still succumb to the disease; 3) Remission rates inversely related to age: 5-year survival in adults under 65 is 33% and over 65 is 4%; 4) Dependent

upon several factors, such as age, white blood cell count, presence of translocations in bone marrow (Iwakiri et al., 2002; Tabuchi, 2007).

Future treatment for AML can be: clinical trials; new drug treatments; vaccines; immunotherapy; leukemia type-specific therapy; gene therapy: block encoding instruction of an oncogene and target the oncogene; blood and marrow stem cell transplantation.

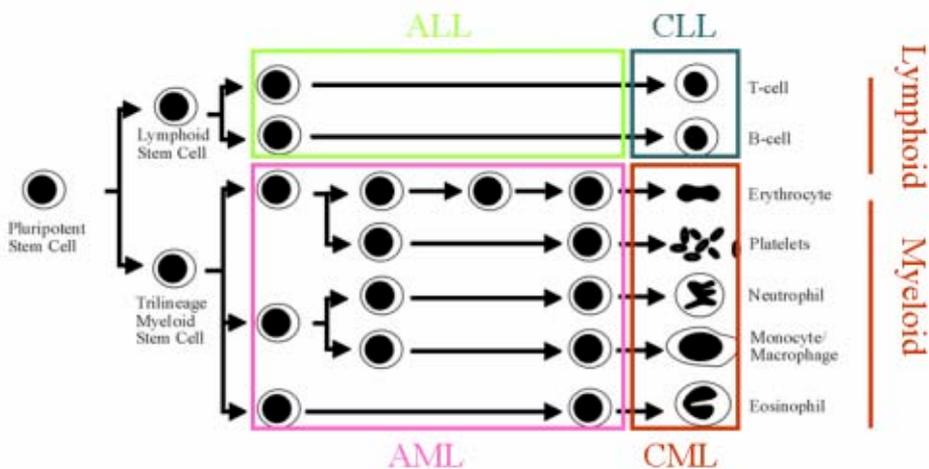


Fig.6. Scheme of hematopoiesis and possible leukemia development. Upon activation, the HSC are able to differentiate into clonal progenitors that can expand exponentially as well as continue the process of differentiating. Hematopoietic cells are broadly divided into "lymphoid" and "myeloid" cells. Lymphoid cells differentiate T cells, B cells, natural killers, and dendritic cells. Myeloid cells include red blood cells, platelets, monocytes/macrophages, and granulocytes. Different types of leukemia can occur during hematopoiesis: acute myeloid (AML), acute lymphoid (ALL), chronic myeloid (CML) and chronic lymphoid (CLL) leukemia.

2.2. Characterization of AML

AML is a very heterogeneous disease with regard to clinical features and acquired genetic alterations, both those detectable microscopically as structural and

numerical chromosome aberrations, and those detected as submicroscopic gene mutations and changes in gene expression (Steffen et al., 2005).

It has been shown that AML1/ETO or CBF β /MYH11 or TEL/AML1 alone does not cause leukemia. AML due to cooperation of at least two classes of mutations (Fig. 7):

- 1) Class I mutations: constitutively activated TPK or other signalling promoting growth and viability;
- 2) Class II mutations: repression of nuclear transcription via PML/RAR α , AML1/ETO, CBF/MYH11 and C/EBP α that block differentiation.

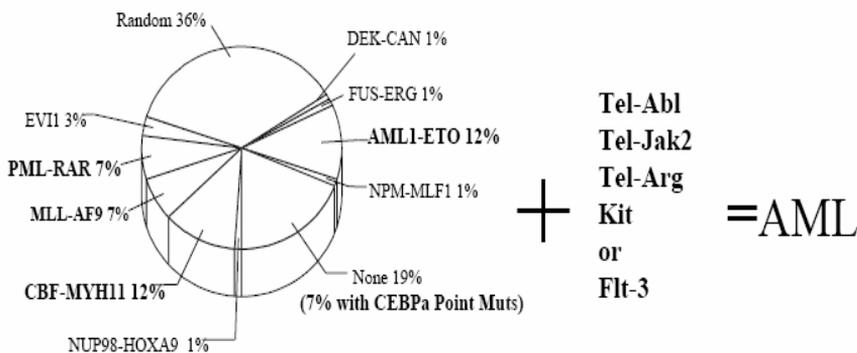


Fig. 7. Frequency of possible aberrations in AML. AML due to cooperation of at least two classes of mutations: mutations that affect proliferation and mutations that affect differentiation process.

Acute myeloid leukemia have been divided into 8 subtypes, M0 through to M7 under the FAB (French-American-British) classification system based on the type of cell from which the leukemia developed and degree of maturity (Kuriyama, 2003). This is done by examining the appearance of the malignant cells under light microscopy or cytogenetically by characterization of the underlying chromosomal

abnormality (Table 3). Each subtype is characterized by a particular pattern of chromosomal translocations and has varying prognoses and responses to therapy.

2.2.1. BCR-ABL1 fusion protein

t(9;22) translocation leads to fusion of BCR (Breakpoint Cluster Region homolog) with ABL1 (v-abl Abelson murine leukemia viral oncogene 1) and constitutive activation of ABL1 tyrosine kinase activity (Sindt et al., 2006), leading to Ras/MAP kinase pathway activation and proliferation, inhibition of apoptosis of cells (Fig. 8, A).

2.2.2. c-kit

Expression of c-kit has been found in about 85% of human AML cells and in many cases Kit/stem cell factor receptor (Kit/SCFR) was constitutively phosphorylated, thereby creating docking sites for cytoplasmic signalling molecules containing Src homology 2 domains. Several signal transduction molecules have been found to be phosphorylated by and in some cases bind to the activated Kit/SCFR, including PI3K, Vav, Grb2 and Shc, leading to activation Ras/MAP kinase pathway (Lennartsson et al., 1999).

2.2.3. AML1-ETO fusion protein

The most frequent translocation in AML is the t(8;21) translocation, found in 10-15% of adult patients with this disease. It causes the replacement of the C-terminus of the transcription activator AML1 (now known as Runx1) by the transcriptional repressor ETO (Eight-Twenty One oncoprotein), resulting in the fusion protein AML1-ETO. ETO binds to co-repressors, e.g. NCoR (nuclear receptor co-repressor 1), SMRT (silencing mediator for retinoid and thyroid hormone receptor) and mSin3, and histone deacetylases (HDAC). The recruitment of HDACs changes the DNA conformation in a way that makes it less accessible for the basal transcription machinery and results in a repression of AML1 target genes (Tabe et al., 2007). AML1-ETO influences the expression or the function of various

transcription factors, such as MEF (myeloid Elf-1-like factor), C/EBP α , AP1 (Activator protein 1) and PU.1, as well as GM-CSF, M-CSF, IL-3 and p14 (Fig. 8, C) (Peterson et al., 2007; Vangala et al., 2003). This serves to induce not only a differentiation block, which has been shown to occur in myeloid cell lines forced to overexpress AML1-ETO, but it has also an important influence on apoptosis, survival, self-renewal and proliferation of hematopoietic progenitors cells in a cell specific and differentiation-stage specific manner.

Table 3. The French-American-British (FAB) classification of AML and associated genetic abnormalities (Bennett et al., 1976).

FAB subtype	Common name and % of case	Associated translocations and rearrangements	Genes involved
M0	Acute myeloblastic leukemia with minimal differentiation (3%)	t(9,22)(q34,q11), Del(5q), del(7q), +8, +13, t(12;13)(p13;q14)	ABL, BCR, EGR1,IRF1,CSF1, CDK6 ETV6, TTL
M1	Acute myeloblastic leukemia without maturation (15-20%)	+6 (or trisomy 6), +4	
M2	Acute myeloblastic leukemia with maturation (25-30%)	+4 t(8;21)(q22;q22), t(6;9)(p23;q34) t(7;11)(p15;p15)	AML1, ETO, DEK, CAN(NUP214) HOXA9, NUP98
M3	Acute promyelocytic	t(15,17)(q22,q12)	PML,RARa,

	leukemia (5-10%)	t(11,17)(q23,q12) t(11,17)(q13,q12) t(5,17)(q23,q12)	PLZF,RARa, NuMa,RARa, NPM1,RARa
M4	Acute myelomonocytic leukemia (25-30%)	+22, +4, t(6;9)(p23;q34) Inv(16)(p13,q22) t(10,11)(p11.2,q23) t(10,11)(p12,q23) t(3;7)(q26;q21)	DEK, CAN MYH11,CBFb, ABI1,MLL, AF10,MLL EVI1, CDK6
M5	Acute monocytic leukemia (2-9%)	t(9;11)(p22,q23) t(10,11)(p11.2,q23) t(10,11)(p12,q23)	AF9,MLL ABI1,MLL, AF10,MLL
M6	Erythroleukemia (3-5%)	Del(5q), Del(7q)	EGR1,IRF1,CSF1R, ASNS,EPO,ACHE,MET
M7	Acute megakaryocytic leukemia (3-12%)	Del(5q), Del(7q), t(1,22)(p13,q13) t(11,12)(p15,p13)	EGR1,IRF1,CSF1R, ASNS,EPO,ACHE,MET, OTT,MAL, NUP98,JARID1A

ABL1- v-abl Abelson murine leukemia viral oncogene homolog 1; BCR- breakpoint cluster region homolog; EGR1- early growth response 1; IRF1- interferon regulatory factor 1; CSF1R- CSF1 receptor; CDK6- cyclin-dependent kinase 6; TTL- twelve-thirteen translocation leukemia; AML1- acute myeloid leukemia; ETO- eight twenty one; DEK- DEK protein ; CAN(NUP214)- nucleoporin 214 kDa; HOXA9- homeobox A9; NUP98- nucleoporin 98 kDa; MYH11- myosin heavy chain 11; CBFb- Core-binding factor subunit beta; MLL- mixed lineage leukemia; AF9- ALL1 fused gene from chromosome 9; ABI1- Abelson interactor 1; AF10- ALL1 fused gene from chromosome 10; EVI1- Ecotropic Viral Integration Site 1; ASNS- asparagines synthetase; EPO- erythropoietin; ACHE- acetyl cholinesterase; MET- hepatocyte growth factor receptor; OTT- one twenty-two; MAL- megakaryocytic leukemia; JARID1A- Jumonji AT rich interactive domain 1A; RAR α - retinoic receptor alpha; PML- promyelocytic leukemia; NUMA1- nuclear mitotic apparatus protein1; NPM1- nucleophosmin; PLZF- promyelocytic leukemia zinc finger.

2.2.4. CBF β -MYH11 fusion protein

The two other leukemia-associated translocations in AML are inv(16) and t(16;16). They fuse CBF β (Core Binding Factor beta) to MYH11 (Myosin heavy chain 11), and fusion CBF β -MYH11 protein can bind to AML1 protein, thereby contacts DNA and act as a repressor of AML1 function (Fig. 8, C) that leads to block of differentiation and unchecked proliferation (Claxton et al., 1994). Inv(16) is strongly associated with AML-M4eo and characterized by myeloblastic/monoblastic infiltration of the bone marrow, an elevated monocytic count in the peripheral blood and presence of atypical eosinophils. Inv(16) is often a sole aberration- secondary cytogenic changes are trisomy 8 and 22 (Castilla et al., 1996).

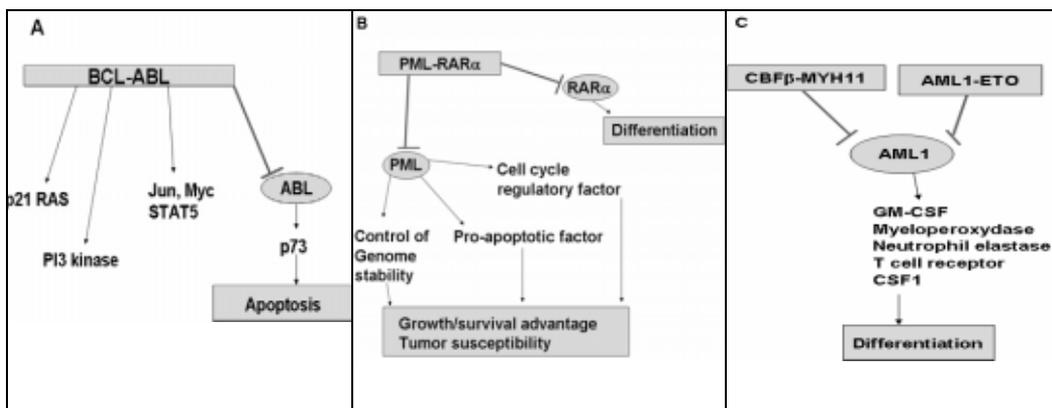


Fig. 8. Possible fusion proteins in AML and their functions. (A) BCL-ABL fusion protein block ABL function that can induce apoptosis and also fusion proteins can affect signalling pathways including PI3K and Ras. (B) PML-RAR α fusion protein block differentiation and apoptosis and induce proliferation of cells. (C) AML1-ETO and CBF β -MYH11 fusion proteins block differentiation by inhibition of AML1 target genes.

2.2.5. RAR α fusion proteins

The acute promyelocytic leukemia (APL) belongs to AML FAB M3 group (Table 2) and accounts for 10% of all AML cases.

2.2.5.1. t(15;17) translocation is detected in as many as 90% of APL patients and has become the definitive marker for the disease (Borrow et al., 1994). Translocation generates the PML-RAR α fusion protein. PML/RAR α fusion might cause APL by inhibiting the wild-type RAR α receptor (Hodges et al., 1998). Expression of the PML-RAR α chimeric protein causes delocalization of PML and other components of nuclear bodies (NBs) to a micro-speckled nuclear structure and differentiation of APL cells with ATRA results in restoration of their normal localization in NBs (Kastner et al., 1992; Koken et al., 1994; Weis et al., 1994) (Fig. 8, B).

It has been shown that PML-RAR α also can suppress PU.1 expression and reduce neutrophil differentiation (Mueller et al., 2006).

2.2.5.2. t(11,17)(q23,q12). The translocation t(11;17)(q23;q21) leading to a PLZF/RAR α rearrangement has been described in a very small number of cases and has been associated with a poor response to ATRA and an adverse prognosis (Culligan et al., 1998). PLZF/RAR α proteins heterodimerize with RXR and form repressor complex that block target genes expression in a fashion unresponsive to physiological retinoid levels. However, the POZ domain of the PLZF portion of the fusion protein independently recruits the SMRT and NCoR nuclear repressors. Thus, the presence of a second ATRA-unresponsive histone deacetylase complex formed by the PLZF fusion partner provides an explanation for the lack of sensitivity of leukemias harboring this fusion protein to treatment with a ligand that specifically overcomes repression mediated through the RAR α moiety of the fusion protein.

2.2.5.3. t(11,17)(q13,q12) ; t(5,17)(q23,q12). Two additional very rare chromosomal alterations involving the RAR α gene have also been described in APL patients. The first is the t(5;17)(q35;q21) translocation involving the nucleophosmin (NPM) gene, the second is the t(11;17)(q13;q21) translocation involving the nuclear

mitotic apparatus (NuMA) protein gene. APL patients with the NPM-RAR α translocation and the NuMA-RAR α translocation are sensitive to ATRA treatment (Redner et al., 2000; Redner et al., 1996).

2.2.6. MLL fusion proteins

Translocations involving MLL (Mixed-Lineage Leukemia) gene occur in 3% of all adult AML. More than 60 translocations involving the region 11q23 have been described, and over 30 fusion partners of MLL have already been defined. Most of the known MLL fusion partners, e.g. ENL, AF9, AF4 contain transcriptional activation domains that are necessary for immortalization of hematopoietic cells and differentiation inhibition (Steffen et al., 2005).

2.2.7. Other transcription factor and gene mutations in AML

PU.1 mutations have recently been described in 7% of AML patients. So far it is unknown, how these mutations contribute to AML pathogenesis (Pabst and Mueller, 2007).

C/EBP α mutations occur predominantly in 10% of AML, mostly including AML FAB M1 or M2 subtypes. These mutations disrupt DNA binding as well as dimerization of the protein or lead to complete loss of C/EBP α function (Steffen, et al., 2005).

The **p53** tumor-suppressor gene, located at chromosome 17p13, is commonly mutated in a wide variety of human cancers, including AMLs. The normal p53 gene encodes for a nuclear phosphoprotein that binds to DNA and can influence the expression of other genes involved in cell proliferation and apoptosis (Zolota et al., 2007). p53 mutations were identified in all morphologic types of AMLs, except FAB-M3, and are more common in cases with chromosome 17 complex translocations or monosomy (Herzog et al., 2005).

The **c-H-ras**, **c-K-ras**, and **c-N-ras** proto-oncogenes encode homologous 21 kDa proteins that bind and hydrolyze GTP and are involved in signal transduction

and cellular proliferation. Mutations of the c-ras genes represent one of the more frequent molecular abnormalities identified in AML (up to 25% cases) (Steffen, et al., 2005).

The **Rb1** tumor-suppressor gene, located at chromosome 13q14, encodes protein that can bind DNA or form complexes with other proteins that leads to the transition the cell cycle from the G1 to S phase. Rb1 abnormalities have been identified in a set of AML (26% of FAB M4 and M5) (Furukawa et al., 1991; Hillion et al., 1991; Melo et al., 1998).

2.3. AML cell lines

ME-1 cell line. This human acute myeloid leukemia cell line was established from the peripheral blood of a 40-year-old Japanese man with AML FAB M4eo at second relapse in 1988; cells were described to carry the inv(16)(p13q22) leading to the fusion gene CBF β -MYH11. Most of ME-1 cells had a blastic appearance, but small percentage of the cells was macrophage-like cells, eosinophils, or basophils. IL-3, IL-4, GM-CSF induced the differentiation of ME-1 mostly into macrophage-like cells (Yanagisawa et al., 1994; Yanagisawa et al., 1996).

THP-1 cell line. The THP-1 cell line was established from the peripheral blood of a 1-year old boy with acute monocytic leukemia (AML-M5). These cells carry the translocation t(9;11) that leads to expression of MLL-AF9 fusion protein (Tsuchiya et al., 1980). The cell line can differentiate into macrophage-like cells (phagocytic) and can be used for induction of differentiation studies. When stimulated with PMA cells have been reported to differentiate into a monocytic pathway and release arachidonic acid and prostanoids (Traore et al., 2005).

K562 cell line. The myeloid leukemia-derived Epstein-Barr virus (EBV)-negative human lymphoid cell line (K562) cell line was established from the pleural

effusion of a 53-year-old female with chronic myelogenous leukemia in terminal blast crises (Lozzio and Lozzio, 1975). These cells have a primary aberration t(9;22) that leads to expression of fusion BCR-ABL1 protein. The cell population has been characterized as highly undifferentiated and of the granulocytic series (Lozzio and Lozzio, 1979; Lozzio et al., 1979). Recent studies have shown the K562 blasts are multipotential, hematopoietic malignant cells that spontaneously differentiate into recognizable progenitors of the erythrocyte, granulocyte and monocytic series. Other cell line characteristics: hemoglobin production, Bcl-2 positive, HSP70/72 positive, cytokine production (PDGF, TGFbeta), inducibility to differentiation with hemin to erythrocyte and with TPA to megacaryocyte (Villeval et al., 1983).

KASUMI-1 cell line. Human acute myeloid leukemia cell line established from the peripheral blood of a 7-year-old Japanese man with acute myeloid leukemia (AML FAB M2) (in 2nd relapse after bone marrow transplantation) in 1989; cells carry the t(8;21) ETO-AML1 fusion gene (Asou et al., 1991). This cell line is an intensively investigated model of the functional consequences of the AML1-ETO fusion oncogene on myeloid differentiation. Second class of mutations that confer a proliferative and/or survival advantage to hematopoietic progenitors an activating mutation in the tyrosine kinase domain of the c-kit gene was identified in the AML1/ETO expressing Kasumi-1 cell line (Larizza et al., 2005).

NB4 cell line. NB4 cell line, the first ever isolated human APL line, with the typical t(15;17) chromosomal balance translocation, that leads to expression of the PML-RAR α fusion protein (Mozziconacci et al., 2002). PML-RAR α contributes to leukemogenesis of APL by blocking the differentiation and promoting the survival of myeloid precursor cells (Grignani et al., 1993). The disruption of normal differentiation can be overcome by treatment with ATRA, which induces differentiation of NB4 cells into mature granulocytes that have specific phenotype (CD11b, CD11c, CD13, and CD33) (Zang et al., 2000)

HL-60 cell line. Human leukemia cell line, HL60, is p53 null and extremely sensitive to a variety of apoptotic stimuli including DNA damage (Kim et al., 2007). HL-60 cells also have high level of c-myc (Mangano et al., 1998). HL-60 cell line provides a unique *in vitro* model system for studying the cellular and molecular events, especially differentiation process. Proliferation of HL-60 cells occurs through the transferrin and insulin receptors, which are expressed on cell surface. The requirement for insulin and transferrin is absolute, as HL-60 proliferation immediately ceases if either of these compounds is removed from serum-free culture media (Breitman et al., 1980). Spontaneous differentiation to mature granulocytes can be induced by compounds such as dimethyl sulfoxide (DMSO), or retinoic acid (Collins et al., 1977; Collins et al., 1978). Other compounds like 1,25-dihydroxyvitamin D₃, 12-O-tetradecanoylphorbol-13-acetate (TPA) and GM-CSF can induce HL-60 to differentiate to monocytic, macrophage-like and eosinophil phenotypes, respectively (Mangelsdorf et al., 1984; Olsson et al., 1983).

3. Differentiation therapy in AML

Local remodeling of chromatin is a key step in the transcriptional activation of genes. Dynamic changes in the nucleosomal packaging of DNA must occur to allow transcriptional proteins to contact with the DNA template. The realization that proteins, which regulate the modification of chromatin, participate in many leukemia chromosomal rearrangements has generated new excitement in the study of chromatin structure. Recent reports have kindled the hope that pharmacological manipulation of chromatin remodeling might develop into a potent and specific strategy for the treatment of leukemias (Fig. 9) (Advani et al., 1999; Slack and

Rusiniak, 2000). The best understood mechanism by which cells regulate chromatin structure is posttranslational modification of histones by acetylation (Walia et al., 1998). Acetylation of histones disrupts nucleosomes and allows the DNA to become accessible to transcriptional machinery. Removal of the acetyl groups causes the maintaining transcriptionally repressed chromatin architecture. However, understanding of the regulation of gene-specific histone acetylation is still limited (Bruserud et al., 2006).

Differentiation therapies are broadly defined as those that induce malignant reversion, i.e. the malignant phenotype becomes benign. Clinically, these therapies have been most successful for acute promyelocytic leukemia, with the use of ATRA. This treatment has changed a cancer with a previously dismal outcome into one of the most treatable forms of leukemia. The exact mechanisms of differentiation are unknown — it is unclear if it occurs by inducing terminal differentiation (G0 arrest), by inducing differentiation ‘backwards’ to the non-malignant form of the cell, or by triggering apoptosis. It is likely that it involves all of these pathways (Spira and Carducci, 2003).

Although there are probably mechanistic differences in how the various agents lead to differentiation, the overall process is likely to allow malignant tumor cells to revert to a more benign form, in which their replication rates are lower compared with malignant forms, leading to a decreased tumor burden. They might also have a decreased tendency for distant metastatic spread, and the process may also restore traditional apoptotic pathways, all of which could improve a patient’s prognosis.

3.1. Retinoids and their mechanism of action

Retinoids have been extensively used and studied in cancer therapy over the years and the best example is almost certainly represented by the successful use of

ATRA in the treatment of acute promyelocytic leukemia. 13-*cis*-RA is clinically effective against juvenile chronic myelogenous leukemia and mycosis fungoides (cutaneous T-cell lymphoma). The combination of 13-*cis*-RA with interferon- α -2a (IFN α) has been shown to be effective against squamous cell carcinomas of skin and cervix. However, natural retinoids have displayed limited efficacy in most solid malignancies. Furthermore, the reversible effect of retinoids requires prolonged treatments that are often associated with toxicity and the development of retinoid resistance. Many of the factors influencing resistance are, however not well understood (Ortiz et al., 2002).

Retinoids also have shown enhanced antitumor activity when combined with modulators of other nuclear hormone receptors, such as the Vitamin D Receptors (VDR) and steroid receptors. This leads to cell growth inhibition and induce apoptosis in breast, prostate, lung and ovarian cancer cells and are effective in reducing breast tumor mass in nude mice (Koshizuka et al., 1999).

Combinations of ATRA with specific HDACIs achieve synergistic activity *in vitro*. Phenylbutyrate is clinically used in humans and the availability of novel HDACIs warrants pursuing of preclinical and clinical studies in combination with retinoids. Arsenicals induce apoptosis in APL cells and combination with ATRA *in vitro* and in animal studies have revealed synergistic effects that justify further studies with selective retinoids (Chen et al., 1999; Coffey et al., 2001; Demary et al., 2001).

Once within the cell, ATRA can enter the nucleus and directly modulate gene expression via binding to high affinity retinoid receptors. These receptors are transcription factors that control expression of specific genes in a ligand dependent manner.

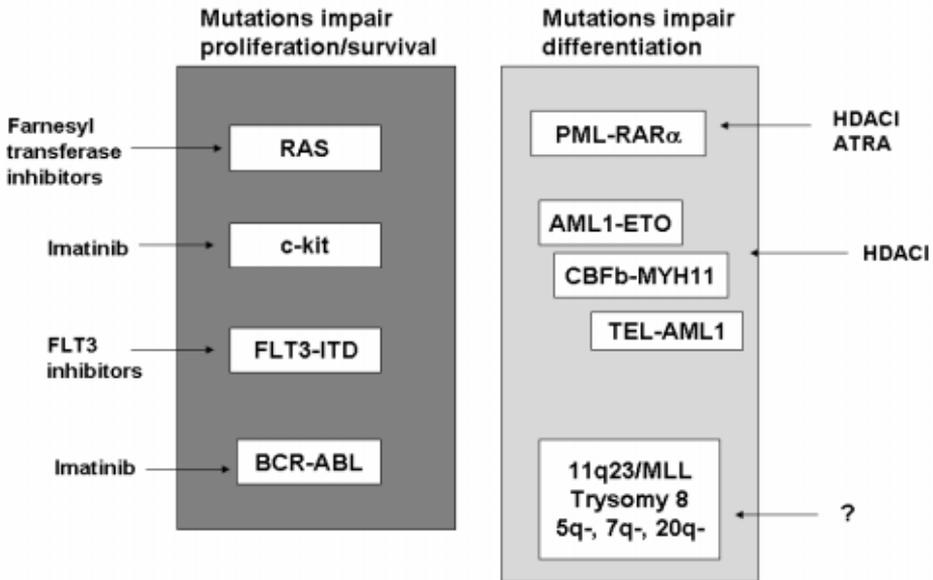


Fig. 9. Pathogenesis and treatment of acute leukemias. There are two classes of cooperating mutations in acute leukemia, those that confer proliferation and/or survival and those that impair hematopoietic differentiation. Also different chemical agents are available for AML treatment.

There are several receptors ATRA binding to:

- 1) Retinoic Acid receptors (RAR), for with three separate genes (α , β and γ) have been identified and cloned. All 3 forms of RAR share common structure and have two zinc finger motifs, that can bind to DNA;
- 2) Retinoid X receptors (RXR). Three genes for RXR have also been identified and share a similar structure (Melnick and Licht, 1999).

The retinoid response is mediated, at least in part, by direct binding of RAR-RXR heterodimers to specific retinoic acid response elements (RARE) within the promoters of target genes, as c-myc, C/EBP γ , p-21, NF κ B, Bcl-2 family members and others, that can be involved in many processes. Also RXRs can form homodimers with themselves and bind to RXRE elements (Fig. 10).

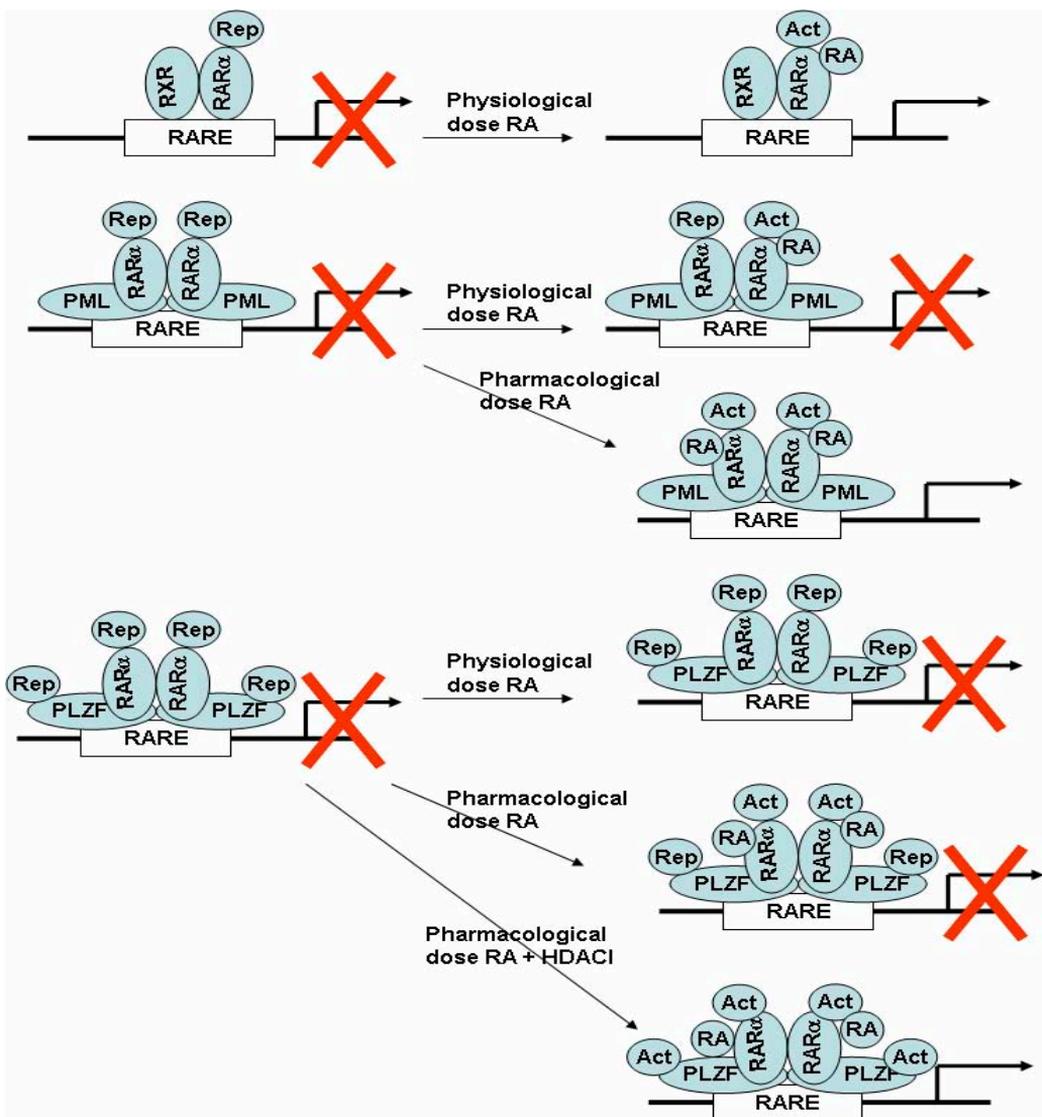


Fig. 10. RAR α fusion proteins and their response to retinoic acid treatment. Binding of physiological dose of retinoic acid (RA) to RXR-RAR α or pharmacological dose to PML-RAR α leads to the dissociation of co-repressor complex (Rep) and the recruitment of co-activator complexes (Act) that contain enzymatic activities required for chromatin remodeling, specific histone modifications and recruitment of RNA polymerase II, for example nucleosome remodeling activity (NRA), histone acetyltransferase (HAT) activity and histone methyltransferase (HMT) activity. PLZF-RAR α after treatment with combination of pharmacological doses of RA and HDACi also can initiate transcription of RA target genes.

RAR can form heterodimers with other nuclear receptors, like thyroid hormone receptor, vitamin D3 receptor and peroxisome proliferators' activator receptor (PPAR). All these heterodimers can recognize a similar consensus RARE (Kastner et al., 2001).

In the absence of ligand, the RARs are able to heterodimerize with RXRs, bind to RARE element and also associate with transcriptional repression complex. This co-repressor complex acts in a combinatorial manner to antagonize actions of co-activator complexes and by mediating covalent modifications, that serve as marks for recruitment of additional factors involved in transcriptional repression (Fig. 10). NCoR (nuclear-receptor co-repressor) and SMRT (silencing mediator of retinoic-acid and thyroid-hormone receptors) nucleate a core co-repressor complex that contains HDAC3, TBL1 (transducin (beta)-like 1), TBLR1, and GPS2 (G protein pathway suppressor 2), with additional weakly interacting factors (e.g., Sin3 complexes) forming a functional holocomplex.

When ATRA binds to RAR-RXR receptor, conformational change is induced that leads to co-repressor complex dissociation and co-activator complex association (Collins, 1998) (Fig. 10).

So, RA presumably affects transcriptional activity of target genes via switching a closed, inactive chromatin structure to an open, active one, that provides access for other transcription factors to induce expression.

It has been reported that RAR α is directly involved in differentiation of HL-60 leukemia cells, and that the dominant inhibitory form of RAR α can prevent this differentiation process. Similar results have been also observed in differentiation of a multipotent murine hematopoietic cell line. Thus, there is increasing evidence that RARs are involved in many important biological processes.

It is well known that one anticancer effect of retinoids is mediated through their anti-AP-1 activity. AP-1 activity can be induced by growth factors, cytokines,

oncogene products, and tumor promoters, resulting in the proliferation and transformation of cells, and the stimulation of tumor progression. Retinoid receptors are involved in anti-AP-1 activity via binding directly of RXR to components of AP-1, such as c-jun, resulting in the inhibition of AP-1 DNA binding. The mechanisms by which ATRA affects AP-1 activity in gastric cancer cells and others, where retinoid receptors are involved, are largely unknown, although, effects of ATRA on growth inhibition and anti-AP-1 activity has been detected (Wu et al., 2000).

3.2. HDACIs as agents for therapy of AML

HDAC inhibition was empirically discovered as a novel form of cancer therapy. The biology of the various HDAC isoforms and their relationship to tumorigenesis are just beginning to be elucidated and the studies are largely driven by the perceived clinical potential of HDACIs. It remains to be seen whether a more detailed understanding of the specific roles played by various HDAC isoforms during human tumorigenesis will lead not only to development of isoform-specific inhibitors but also to more effective or less toxic antitumor therapeutics, as compared to the multiclass HDACIs that are currently undergoing clinical evaluation. Rationally designed combinations of HDACIs with various other types of approved or investigational anticancer agents are showing promise in tumor cell culture systems, but then must yet be proven in clinical trials (Villar-Garea and Esteller, 2004).

Different HDACIs are able to change transcription, both positively and negatively, for about 2% of all human genes. While this number seems small, the genes affected by these inhibitors are known to exert critical, common function in controlling cell cycle and apoptosis or differentiation process.

Some HDACIs may also comprise histone acetylation-independent mechanisms involving non-histone HDAC substrate, such as NF- κ B, STAT3, p53 and HSP90 (Kulp et al., 2006). They are furthermore capable to facilitate Akt (also called PKB for protein kinase B) dephosphorylation by causing the disruption of HDAC-protein phosphatase 1 complexes.

There are many known inhibitors that block access to the active site of HDAC. The most potent one discovered so far is Trichostatin A (TSA). It is a fermentation product of *Streptomyces*, originally used as an anti-fungal agent, but later discovered to have potent proliferation-inhibitory effects cancer cells. TSA belongs to the group of hydroxamic acids and is effective *in vitro* at nanomolar concentrations (Yoshida et al., 1990).

Some of the compounds, such as butyrate, phenylbutyrate, depsipeptide (FK228), pyroxamide, suberoyl anilide bishydroxamide (SAHA), valproic acid, CI-994 and oxamflatin have entered into clinical trials (Fig. 11). The proposed mechanisms comprise re-expression of silenced genes and/or the silencing of downstream genes due to regained access of their promoters to other modulatory factors (Nemeth, et al., 2007).

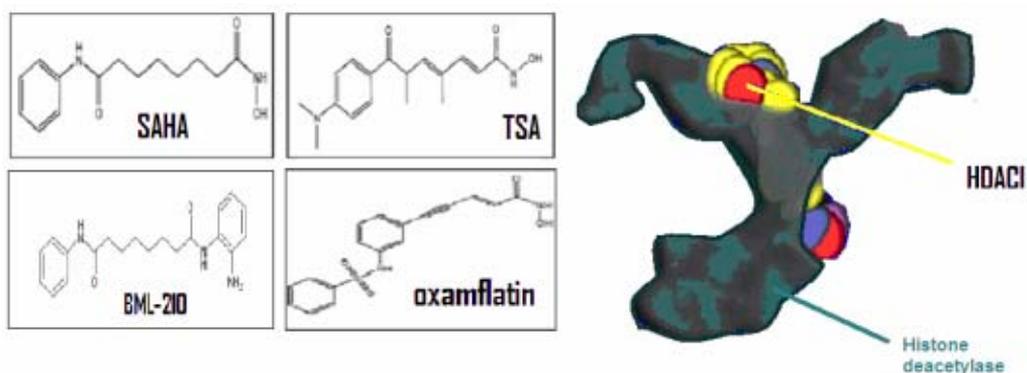


Fig.11. Structures of few HDACIs. HDACI fits into HDAC catalytic pocket and inhibits its activity.

AIMS

Today, cancer is understood to be an epigenetic as well as a genetic disease. Major epigenetic hallmarks of the cancer cell are DNA methylation and histone modifications. The latter changes may be an optimal target for novel anticancer agents. The main goal of using histone deacetylase inhibitors (HDACIs) would be restoration of gene expression of those tumor-suppressor genes that have been transcriptionally silenced by promoter-associated histone deacetylation.

The specific aims of this thesis were therefore to:

- elucidate the main effects of novel HDACIs, primarily BML-210, alone or in combination with other chemical agents, viz. *all-trans* retinoic acid (ATRA), on leukemia and other cancer cell growth, apoptosis and differentiation;
- identify new proteins that play important roles in the effects seen during treatment with HDACIs;
- elucidate the role and protein interactions of α -dystrobrevin during cancer cell proliferation and differentiation.

MATERIALS AND METHODS

Cell cultures

The human leukemia cell lines (NB4, HL-60, K562, THP-1) were cultured in RPMI media (Gibco Invitrogen, Lidingö, Sweden) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco), and 20 mM glutamine at 37 °C in humidified air and 5% CO₂. Cultures were seeded at a density of 4x10⁵ cells/ml and were (at maximum 1.5x10⁶ cells/ml) subsequently transferred to a fresh medium.

Human epithelial cervical cancer cells (HeLa) were cultured in DMEM media (Gibco) containing 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco) at 37 °C in humidified air and 5% CO₂.

Cell viability and growth

Cell viability was assayed by exclusion of 0.2% trypan blue. Cell number was determined by counting cells in suspension in a haemocytometer. 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).

Chemicals and antibodies

ATRA were purchased from Sigma Chemical Co. (St.Louis, MO) and added to the medium from solution of 500 µM to a final concentration of 1 µM or 10 µM. ATRA was dissolved in ethanol. BML-210 (Biomol, Plymouth Meeting, PA) was dissolved in DMSO (stock solution 10 mM) and added to the medium at a 5 –

30 μ M final concentration. The final DMSO concentration in the medium did not exceed 0.001%. FK228 was obtained from Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan), phenyl butyrate and pifithrin- α from Calbiochem (Mannheim, Germany).

Polyclonal antibodies against alpha-dystrobrevin were provided by Kulyte et al. (2002). Antibodies to phosphotyrosine (IgG2bk, Upstate Biotechnology, NY, USA), horseradish peroxidase-conjugated secondary antibodies (DAKO Denmark A/S, Glostrup, Denmark) were used.

Anti-Bcl-2, -p21, -p53, -Sp1, NF- κ B p65, NF- κ B p50, pro-caspase- 3, pro-caspase- 8, pro-caspase- 9 and FasL were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and anti-phospho-p38 MAP Kinase (Thr 180/Tyr 182), anti-p38 MAP Kinase from Cell signalling technology, Inc. (Beverly, MA). Polyclonal anti-acetylated histone H4 antibody and polyclonal anti-phosphorylated histone H3 at serine 10 antibody were from Upstate Biotechnology Inc.

Oligonucleotides were synthesized by MWG-Biotech AG (Ebersberg, Germany).

Hydrophilic and hydrophobic protein fraction preparation

Hydrophilic and hydrophobic protein fractions were isolated with MEM-PER Eukaryotic Membrane Protein Extraction Reagent Kit according to the manufacturer's instructions (Pierce, Rockford, IL). The hydrophobic fraction contains the integral membrane and with membrane associated proteins. The hydrophilic fraction contains the soluble proteins.

Isolation of cytosolic and nuclear proteins

The cells (5×10^6 to 10^7) were harvested by centrifugation at $500 \times g$ for 6 min, washed twice in ice cold PBS and suspended in Nuclei EZ lysis buffer (Sigma-Aldrich, Stockholm, Sweden) for 5 min on ice. The cell homogenates were then centrifuged at $1500 \times g$ for 5 min. The supernatant, corresponding to the cytosolic fraction, was clarified by centrifuging at $15,000 \times g$ for 15 min, 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 $500 \times g$ for 5 min, and completely resuspended in Nuclei EZ storage buffer (Sigma) and frozen at -76°C .

For SDS/PAGE analysis of total nuclear proteins, nuclei were resuspended (approx. $5 \times 10^7/\text{ml}$) in two volumes (v/v) of 2 x lysis solution (100 mM Tris, pH 7.4, 5 mM MgCl_2 , 200 mM DTT and 4% SDS), then 3 volumes of 1x lysis solution and benzonase (Pure Grade, Merck Chemicals Inc., Darmstadt, Germany) were added to give a final benzonase concentration of 2.5 units/ml. The lysates were incubated for 1 h at 0°C and then centrifuged at $15,000 \times g$ for 30 min. The supernatants were immediately subjected to electrophoresis or frozen at -76°C .

Isolation of neutrophils from human blood

The neutrophils were isolated essentially according to Boyum (1968) as described by Kulyte et al. (2002).

Cell cycle analysis

Untreated cells and cells treated with different chemical agents 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 $500 \times g$ for 5 min, cells were suspended in phosphate-buffered saline (PBS) containing propidium iodide (PI) ($50 \mu\text{g}/\text{ml}$) and

RNase (0.2 mg/ml) and incubated at room temperature for 30 min. The tubes were then taken at 4 °C in dark until analysis by Becton-Dickinson FACSCalibur flow cytometer (BD Bioscience, San Jose, CA). 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.

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 (DAKO Denmark A/S) in the dark at 4 °C for 30 min. Cells were washed with PBS containing 2% bovine serum albumine (BSA), fixed in 4% paraformaldehyde for 15-30 min on ice and pellet was resuspended in PBS. Eighth thousand events were analyzed for each sample by immunofluorescence using flow cytometry. Proliferating cells with and without CD11b antibodies were used as a control.

Electrophoretic mobility shift assay (EMSA)

The probes used were synthetic oligonucleotides (MWG-Biotech AG) representing binding sites: (5'-AGTTGAGGGGACTTTCCCAGGC-3') consensus NF κ B; (5'- AAGCCTGGGCAACATAGAAAGTCCCCATCTG TACAAAA-3') NF κ B from the FasL promoter; (5'-ATTCGATCGGGGC GGGGCGAGC-3') consensus Sp1; (5'-ATTCGATCGGTTTCGGGGCGA GC-3') mutated consensus Sp1; (5'-GGCCGAGCGCGGGT CCCGCCTCC TTGAGGCGGG-3') Sp1-3 (element 3) from the p21 promoter; (5'-ATCA GAAAATTGTGGGCGGAACTTCCAGG-3') Sp1 from the FasL promoter; (5'-CTGCGCGGGGCGGGCGCCGC-3') Sp1 from the p65 promoter; (5'-CTGCGCGGGTTGGG CGCCGC-3') mutated Sp1 from the p65 promoter; (5'-ATCAGGAACATGTCCCAA CATGTTGAGCTCT-3') p53 from the p21

promoter; (5'-ATCAGGAATTCGCTCCCAACATGTTG AGCTCT-3') mutated p53 from the p21 promoter; (5'-GCCCTGTGCCA GGGGAGAGGAAGTGGAGGG-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 x Tris-borate buffer. After electrophoresis, gels were dried and then exposed on X-ray films.

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) and phosphorylated histone H3 antibodies (Upstate) according to the instructions of manufacturer.

Immunoprecipitation

Immunoprecipitation with a rabbit polyclonal antibodies against alpha-Dystrobrevin or antibodies against phosphorylated tyrosine (anti-pY) were

performed as follows. Fifty μl of prepared Protein A/G slurry (Santa Cruz Biotech.) washed with PBS was added to the cell fraction and incubated with rotation to remove endogenous IgG at 4°C 30 min. Beads were pull down by centrifugation at $10,000 \times g$ for 10 min at 4°C and the supernatant was transferred to a fresh Eppendorf tube. Then 12.5 μg of primary antibodies, i.e. anti-dystrobrevin, anti-pY or irrelevant IgG, were added to the eppendorf tube containing the cold precleared lysate and incubated at 4°C for 4 h. One hundred μl of Protein A/G was added to the lysate and rotated at 4°C overnight. Beads were pull down by centrifugation at $10,000 \times g$ for 10 min at 4°C , the supernatant was carefully removed and beads were washed 3-5 times with 500 μl of 1x RIPA buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 10 mM EDTA, pH 8.0, 0.1%SDS, 1% NP-40, 1% DOC).

2-D electrophoresis

For 2DE, the immunoprecipitated protein complexes were actively solubilised in 80 μL of IEF buffer (8M urea, 2M thiourea, 4% CHAPS, 1% DTT (dithiothreitol), 0.002 % Bromphenol blue, 0.8% Pharmalyte 3-10) and in 120 μL of rehydration buffer (8 M urea, 2 M thiourea, 2% CHAPS, 0.2% DTT, 0.002 % Bromphenol blue, 0.5% Pharmalyte, pH range 4-7) and then Immobiline DryStrips strips pH 4-7 (Amersham Biosciences, Uppsala, Sweden) were hydrated with the rehydration solution in a strip holder for 16 h at room temperature. The first dimension of isoelectric focusing was performed with a 2DE Multiphor unit (Amersham Biosciences) by steps of increased voltage up to ~ 70 kVh.

Focusing Immobiline DryStrips were incubated for 15 min at room temperature in electrophoresis buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% (w/v) glycerol, 2% (w/v) SDS) with 1% (w/v) DTT, followed by 15 min in electrophoresis buffer with 2% (w/v) iodoacetamide.

The second dimension was performed in a gradient gel (8–18% ExcelGel SDS polyacrylamide). 2-DE gels were silver stained as described previously (Shevchenko et al., 1996). Spots were selected by visual inspection and gel slices were excised by scalpel.

MALDI-TOF (Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight) mass spectrum analysis

Areas of the gel that were deemed to be of interest were cut out and subjected to in-gel tryptic digestion as described previously (Shevchenko et al., 2002). For MALDI-TOF-MS analysis, samples were desalted and purified by using C18zipTips (Millipore) following the manufacturer's instructions (Millipore). After that 1 μ l of each sample mixed with 1 μ l α -cyano-4-hydroxycinnamic acid matrix (in 70% acetonitrile with 0.3% (v/v) trifluoroacetic acid) and were spotted on the target plate. Samples were analyzed with a MALDI-TOF MS using a Voyager-DETM Pro (Applied Biosystems, Framingham, MA). Positive ionization, an acceleration voltage of 20 kV, grid voltage 75%, guide wire 0.02 and the extraction delay time 200 ns were used to collect spectra in the mass range of 700-4000 Da. Reflector mass spectra were acquired and calibrated either externally or internally, using trypsin autolysis peptides (m/z 842.5200, 1045.5642, 2211.1046). Data processing of the spectra was performed in Data ExplorerTM Version 4.0 (Applied Biosystems). Mass spectrometry data were searched against a human protein database (a subset of proteins from the NCBI non-redundant protein database) using the software search algorithm MASCOT (Matrix Science Ltd, London, UK). Restrictions were passed on mass tolerance (\pm 50 ppm), maximum missed cleavages by trypsin (up to 1), and cysteine modification by carbamidomethylation.

ESI (ElectroSpray Ionization) tandem mass spectrum analysis

The experiments were performed on a hybrid mass spectrometer, API Q-STAR *Pulsar i* (Applied Biosystems) equipped with a nanoelectrospray ion source (MDS Protana, Odense, Denmark). Samples were desalted and purified on a C18ZipTip (Millipore) according to the manufacturer's instructions and 2 μ l of eluted peptide solutions (50% acetonitrile in water with 1% formic acid) were then loaded onto the silver-coated glass nanoelectrospray capillaries. Mass spectra and spectra of peptide fragments after CID (collision-induced dissociation) of selected peptide ions were acquired with instrument settings recommended by Applied Biosystems. Data processing was performed with Analyst QS Software (Applied Biosystems). Mass spectrometry data were searched against a human protein database (a subset of proteins from the NCBI non-redundant protein database) using the software search algorithms MASCOT (Matrix Science Ltd.).

Immunofluorescence labeling of cells

NB4 and HeLa cells were treated with varying concentrations of ATRA and BML-210 as described above. After 24-96 h of treatment cells were rinsed in ice-cold phosphate saline buffer (PBS: 10 mM Na_2HPO_4 , 1.8 mM KH_2PO_4 and 150 mM NaCl, pH 7.6).

Cover-slips with the captured cells were rinsed three times in PBS and fixed for 15 min in phosphate buffer supplemented with 3.3% (w/v) paraformaldehyde. Then cells were rinsed three times in PBS, pH 7.6 and permeabilized with 3.3% Triton X-100 for 15 min. The cells were blocked with phosphate buffer containing 5% (v/v) goat serum (DAKO) for 60 min at room temperature. Then, cover-slips were rinsed and incubated with the indicated primary antibodies for 90 min at 37 °C and three times rinsed with PBS, pH 7.6. Finally, cover-slips were incubated with secondary antibodies, i.e. Alexa 564-coupled goat anti-rabbit or Alexa 488-coupled goat anti-

rabbit Fab fragments (Molecular Probes, Eugene, OR) at a concentration 15 µg/ml for α -DB visualization, or Alexa 488-coupled goat anti-mouse Fab fragments for visualization of HSP90. Also Alexa 488-phalloidin was used for F-actin visualization. Slides were subsequently examined with a confocal laser scanning microscopy.

Confocal laser scanning microscopy

A Sarastro 2000 confocal laser scanning microscope (Molecular Dynamics, Sunnyvale, CA, USA) was used for visualization of human α -DB in both proliferating and differentiated NB4 cells, as well as in HeLa cells. Fluorescent markers, filters, and thresholds were combined to minimize bleeding between the red and green channels of the microscope. The microscope was equipped with an Argon ion laser operated at 10–15 mW with 30% efficiency. A wavelength of 488 nm was selected with an interfluorescence filter, the dichroic beam splitter was adjusted to 565 nm (B/S 565), and the second barrier filter was set at 510 nm (EFLP 510). Red and green fluorescinated probes were respectively detected with a 600 EFLP and a 545 DF 30 detector filter. ImageSpace Software (Molecular Dynamics) installed on Iris Indigo and O2 workstations (Silicon Graphics, Mountain View, CA) were used to run the microscope and to collect data.

For confocal imaging we also used a Bio-Rad Radiance 2100 and Radiance 2000MP (Carl Zeiss, Jena, Germany) to assess dual labelling of either filamentous actin (F-actin) and Dystrobrevin (DB), or heat-shock protein 90 (HSP90) and DB. Images were taken in sequence after inserting the signal enhancing lenses by activating channel 1 (blue), not used: Mai-Tai laser (815 nm), with dichroic beam-splitter 500DCLPXR, blocking filter BGG22 and emission filter D488/10; channel 2 (green): Argon laser (488 nm), no blocking filter and emission filter HQ545/40 and channel 3 (red): Argon laser (488 and 514 nm), no blocking filter and emission filter

E600 LP. The microscope was a Nikon Eclipse TE2000U (Tokyo, Japan), equipped with PlanApo Dich x60 oil immersion objective (NA 1.40).

RNA interference experiments

HeLa cells were transfected using Lipofectamine™ 2000 Reagent (Invitrogen Life Technologies, Carlsbad, CA) with three different DTNA Validated pre-designed RNAi Duplex sequences (Ambion, Huntington, UK) according to the manufacturer's recommendations (Ambion). For control transfection, a Nonsilencing Control siRNA was used (Qiagen Inc., Valencia, CA) and for positive control was used Hs/Mm_MAPK1 Control siRNA (Qiagen). After 96 h after transfection, cells were prepared for confocal microscopy analysis and flow cytometry analysis for CD11b evaluation as described above.

RESULTS AND DISCUSSION

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

Local remodeling of chromatin is a key step in the transcriptional activation of genes. Dynamic changes in the nucleosomal packaging of DNA must occur to allow transcriptional proteins contact with the DNA template. The realization that the proteins, which regulate the modification of chromatin, participate in many leukemia chromosomal rearrangements has generated new excitement in the study of chromatin structure. Recent reports have kindled the hope that pharmacological manipulation of chromatin remodeling might develop into a potent and specific strategy for the treatment of leukemia (Advani, et al., 1999; Slack and Rusiniak, 2000). The best understood mechanism by which cells regulate chromatin structure is posttranslational modification of histones by acetylation (Walia, et al., 1998). Acetylation of histones disrupts nucleosomes and allows the DNA to become accessible to transcriptional machinery. Removal of the acetyl groups causes the maintaining transcriptionally repressed chromatin architecture. However, understanding of the regulation of gene-specific histone acetylation is still limited.

In our study, we have investigated the biological effects of the novel HDAC inhibitor BML-210 on leukemia cell (HL-60, NB4, THP1, K562) proliferation, viability, differentiation and transcriptional regulation of the cell cycle- and apoptosis-regulating genes by transcription factors Sp1, NF- κ B and p53.

BML-210 is a newly synthesized compound (N-(2-Aminophenyl)-N'-phenyloctanediamide) and has similar structure as many other HDACI (like suberoylanilide hydroxamic acid).

We found that BML-210 inhibits the growth of all examined 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 potentiates it. Expression of early granulocytic differentiation marker CD11b showed that HL-60 cells were induced to differentiation after 24 h of treatment with BML-210, and in contrast, BML-210 alone did not induce differentiation in NB4 cells. These cellular responses were associated with the increased accumulation of the cells in G1 and subG1 phases of the cell cycle. BML-210 alone induced histone H4 hyper-acetylation and its combination with retinoic acid induced a more rapid appearance of modification of histone H4. It is known that histone modification play critical role in transcription activation of different genes involved in cell cycle control and apoptosis. We have demonstrated that BML-210 affected transcription factor NF- κ B, Sp1, p53 binding activity to their consensus or specific promoter sequences and influenced expression of Sp1, p53, NF- κ B, 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.

II. Apoptotic effects of the novel histone deacetylase inhibitor BML-210 on HeLa cells.

The histone deacetylase inhibitors (HDACIs) have been shown to inhibit cancer cell proliferation, induce cell cycle arrest, and stimulate apoptosis. To date, the key apoptotic proteins and pathways necessary for the anti-tumor activities of HDACI remain less well-defined, and the specific genes regulated by HDACIs to mediate these effects have not been fully dissected. We have now addressed how novel HDACIs induce apoptosis, and how tumor cells can circumvent HDACI-mediated cell death. In the present study, we have investigated the antiproliferative effects of the novel HDACI, BML-210, and its combination with all-trans retinoic acid (ATRA) on human cervical cancer cells (HeLa).

Cell cycle analysis indicated that HeLa cell treatment with BML-210 alone at 10 μ M, and at 5 μ M in combination with 1 μ M ATRA decreased the proportion of cells in G₂/M phase, increased the number of cells in G₀/G₁ and caused an accumulation in subG₁, indicating the cells are undergoing apoptosis. These changes occurred concomitantly with an increased level of some proteins related to a malignant phenotype.

Since caspase plays major roles in apoptotic process, the involvement of caspase cascades has been examined in BML-210-induced HeLa cell apoptosis. We observed activation of caspases - 3, - 8, - 9 in HeLa cells after treatment with BML-210 and ATRA alone, or in combination. There are at least two major pathways, that initiate caspase cascades: one is mediated by cell-surface death receptors and another centers on the disruption of mitochondrial functions. In this study we found up-regulated expression of FasL after BML-210 treatment, suggesting that Fas/FasL pathway was activated during BML-210-induced cell apoptosis. Furthermore, we observed an increased amount of the transcription factor NF- κ B in the HeLa cell

nucleus at 24-h treatment with BML-210 or ATRA alone and in combination. Electrophoretic mobility shift assay (EMSA) experiments revealed enhanced binding activity of NF- κ B and transcription factor p53 to the p21 promoter immediately after treatment with BML-210.

In conclusion, these results suggest that BML-210 and its combination with ATRA are effective in inhibiting growth and inducing apoptosis in HeLa cells *in vitro*. The findings also raise the possibility that BML-210 may prove particularly effective in cancer therapy.

ADDITIONAL RESULTS RELATED TO THESE FINDINGS

A. V.V. Borutinskaite, R. Navakauskiene and K.-E. Magnusson. 2006.

Retinoic acid and histone deacetylase inhibitor BML-210 inhibit proliferation of human cervical cancer HeLa cells. *Ann. NY Acad. Sci.* 1091: 346-355.

Carcinoma of the uterine cervix is the second most common cancer in women worldwide and the first in developing countries. Infection with human papillomavirus (HPV)-types 16 and 18 has been associated with the development of cervical cancer. It is known, that E6 and E7 proteins, produced by HPV, are involved in abnormal cell proliferation. E7 protein can uncouple differentiation and proliferation by promoting cell cycle progression via p21 and disruption of the repressor complex formed between the retinoblastoma protein (Rb) and E2F transcription factor (Jones et al., 1997). E7 can indirectly bind to HDAC via the bridge-protein mi2b and repress transcription of target genes (Brehm et al., 1999).

In this study we have examined the antiproliferative effect of ATRA and HDACI BML-210 on human cervical cancer (HeLa) cells. To examine the molecular mechanism of the growth suppression by ATRA and BML-210, we analyzed the expression of some proteins (p21, p53, Sp1, p38, Bcl-2) that are involved in processes such as the inhibition of proliferation and the induction of apoptosis.

Our data suggest that the combination of ATRA and BML-210 leads to HeLa cell growth inhibition with subsequent apoptosis in a treatment time-dependent manner. We confirmed that BML-210 alone or in combination with ATRA causes a marked increase in the level of p21. p21 protein is important in cell cycle regulation and can be regulated by at least two other proteins: p53 or Sp1. We obtained an increased p53 level after treatment with BML-210 or its combination with ATRA. The changes in p53 level are under p38 phosphorylation influence. We also discovered that the HDACI BML-210 causes increased levels of anti-apoptotic protein Bcl-2 and phosphorylated p38 MAPK; the latter link in cell cycle arrest with response to extracellular stimuli. Our results suggest that ATRA and BML-210 are involved in different signalling pathways that regulate cell cycle arrest and lead to apoptosis of HeLa cells. Figure 12 presents scheme of possible protein involvement in HeLa cell signalling pathway leading to growth inhibition.

The finding that inhibition of histone deacetylation can bypass the transforming potential of high risk HPV oncoproteins by inducing a block in G1 to S transition and subsequent apoptosis may have important implications for the treatment of HPV mediated diseases like cervical cancer. Since the HDAC inhibitors are not toxic for normal cells, it is apparent that these inhibitors are useful for the treatment of diseases associated with an HPV infection.

Our results suggest that BML-210 is effective in inhibiting growth of HeLa cells *in vitro*. The findings raise the possibility that BML-210 may prove particularly effective in treatment of cervical cancers.

For the full contribution to *The New York Academy of Sciences* please see Appendix A.

B. J. Savickiene, G. Treigyte, V.V. Borutinskaite, R. Navakauskiene and K.-E. Magnusson. 2006. The histone deacetylase inhibitor FK228 distinctly sensitizes the human leukemia cells to retinoic acid-induced differentiation. *Ann. NY Acad. Sci.* 1091: 368-384.

Some histone-deacetylase inhibitors have successfully entered clinical trials, but the basis of their antitumor activity is not clear. The histone deacetylases regulate transcription by altering chromatin structure and can also modify individual protein function. Their activity is frequently altered in human tumors. The best-characterized example is evident in myeloid leukemia cells, where the oncogenic, chromosomal translocation fusion protein products PML-RAR or AML1-ETO function to silence genes and transform cells by interacting with HDACs (Nebbio et al., 2005).

We have studied *in vitro* effects of FK228 – a novel histone deacetylase inhibitor, on human leukemia cell lines, NB4 and HL-60. NB4 cells have a chromosomal translocation t(15;17) that causes formation of the PML-RAR α fusion protein that represses differentiation. In contrast, HL-60 cells have negative p53, amplified c-myc, and truncated GM-CSF that leads to cell differentiation block. In the present study, we showed that FK228 alone (0.2-1 ng/ml) inhibited leukemia cell

growth in a dose-dependent manner and induced cell death by apoptosis. FK228 had selective differentiating effects on two cell lines when cells were treated with FK228 for 6 h before induction of granulocytic differentiation by RA or in combination with RA. These effects were accompanied by changes in histone H4 and H3 modifications (hyper-acetylation and phosphorylation) and alterations in NF- κ B binding activity to specific DNA sequences associated with cell differentiation and death.

We have used a pifithrin- α (PFT), an inhibitor of p53 transcriptional activity. As a result of treatment, only NB4 cells with functional p53 were protected from FK228-induced apoptosis and this did not interfere with antiproliferative activity in p53-negative HL-60 cells. In NB4 cells, PFT inhibited p53 binding to the p21 (Waf1/Cip1) promoter and induced NF- κ B binding to DNA specific sequences leading to enhanced cell survival. Thus, beneficial effects of FK228 on human promyelocytic leukemia may be exerted through histone modification and selective involvement of transcription factors, like NF- κ B and p53, that leads to the induction of differentiation or apoptosis.

In summary, our data have provided evidence for myeloid cell line-specific, differential activity of FK228 in the enhancement of RA-mediated differentiation that is associated with the regulation of gene expression mediated through chromatin remodeling.

*For the full contribution to *The New York Academy of Sciences* please see Appendix B.*

III. Effects of retinoic acid and histone deacetylase inhibitor Bml-210 on protein expression in NB4 cells

Several undifferentiated leukemia cell lines can be induced to differentiate into granulocytes by treatment with all-trans retinoic acid, or its isomers (Fenaux et al., 2001). Due to this property retinoids have been proposed as a perspective radical therapeutic approach for acute promyelocytic leukemia (Koshizuka, et al., 1999). Acute promyelocytic leukaemia is associated with chromosomal translocation, which produces specific fusion proteins that bind to and repress promoters of ATRA target genes via aberrant recruitment of histone deacetylases. Histone deacetylation leads to changes in chromatin structure and transcriptional deregulation of genes that are involved in leukomogenesis. Thus, the chromatin remodeling using HDACIs provides a rationale for the treatment of acute promyelocytic leukemia (Villar-Garea and Esteller, 2004).

Our research is concentrated on identification of proteins that can be involved in cell proliferation and differentiation in acute promyelocytic cells, NB4 cells. The growth inhibition and/or differentiation were induced with retinoic acid (RA) and HDACI BML-210. Insoluble proteins of NB4 cells showed biggest differences in expression during treatments with RA and HDACI BML-210. Using mass spectrometry, we identified proteins that are known to be present in cancerous cells and are membrane or membrane-associated proteins. We found that after RA and BML-210 stimulation of NB4 cells, the expression of Rab2B in insoluble fraction is greatly decreased after 8 h of treatment, concomitant with the increasing in total soluble protein fraction during the same time of treatment, indicating that Rab2B expression may be associated with granulocytic differentiation of NB4 cells. We have also identified actin and several actin-associated proteins such as calpain,

nesprin-2, actin-binding LIM protein, vimentin and caldesmon. All these proteins are important for cytoskeletal reorganization during cell growth and differentiation.

Moreover, we identified the dystrobrevin protein that is important as a component of the dystrophin-associated protein complex, that links the cytoskeleton to the extracellular matrix. We found a few isoforms of α -dystrobrevin protein with molecular masses of approximately 84 kDa, 75 kDa, 65 kDa and 58 kDa bands. We further demonstrated changes in the expression of α -dystrobrevin in total soluble and insoluble protein fractions of NB4 cells treated with RA or BML-210 alone, or in combination.

In summary, we identified proteins in the insoluble fraction of NB4 cells, the expression level of which changed in the very beginning of NB4 cell differentiation. These changes could be related to early changes of chromatin structure during cell treatment with BML-210 with following granulocytic differentiation induced by RA. Identification of new proteins involved in differentiation can be useful in finding new ways of APL treatment.

IV. Multiple roles of alpha-dystrobrevin in human cancer cells during proliferation and differentiation processes.

Dystrobrevin (DB), the mammalian orthologue of the Torpedo 87 kDa postsynaptic protein, is a member of the dystrophin gene family with homology to the cystein rich carboxy-terminal domain of dystrophin (Blake et al., 1996).

The precise role of DBs in relation to the dystrophin-associated protein complex at the sarcolemma is unknown, but they are also proposed to play a role in intracellular signal transduction. The presence of the muscle-specific kinase at the neuromuscular junction suggests that DB could be a downstream substrate for

tyrosine phosphorylation. Earlier our group has shown that DB is tyrosine phosphorylated after leukemia cell treatment for granulocytic differentiation with ATRA (Kulyte et al., 2002). It is also possible that the src family kinases could phosphorylate dystrobrevin, since this protein family has also been implicated in signal transduction at the maturing neuromuscular junction. Senter et al. (1995) have shown that dystrophin is a substrate for a variety of protein kinases, such as CaM kinase, casein kinase II, and protein kinase c, both *in vitro* and *in vivo*.

The two distinct classes of DB, α and β , bind directly to dystrophin and are prominent components of the DAPC, that link the cytoskeleton to the extracellular matrix. Furthermore, the DAPC has been recognized to be molecularly heterogeneous. It is present in numerous tissues and is involved in brain development, synapse formation and plasticity, as well as in water and ion homeostasis. There is still not enough data concerning the roles of DB in other cell types.

We have shown that α -DB isoforms are expressed in NB4 cells and the level of α -DB isoforms expression differs in various cell fractions: cytoplasm, nucleus and hydrophobic cell and nucleus compartments. In our experiments we observed that in proliferating and differentiating NB4 cells only the DB-gamma isoform underwent tyrosine phosphorylation. Our findings confirm an observation, that the phosphorylation state of DB-gamma in the nucleus is changed during differentiation process after promyelocytic leukemic cell line treatment with ATRA.

To identify proteins interacting with α -DB in NB4 cells we immunoprecipitated hydrophilic cell proteins with polyclonal anti- α -DB antibodies. The proteins were identified using MALDI-TOF-MS and ESI-MS-MS analysis. Identified proteins could be divided into groups depending on their function: the first group containing β -Actin, α/β -tubulin, tropomyosin, gelsolin, desmin, stathmin are

cytoskeleton proteins and are involved in cell growth and/or maintenance processes; the second group comprises proteins responsible for signal transduction and cell communication processes, e.g. as prohibitin, STAG1, RIBA and AnnexinA4; the third group includes HSP90, HSP70, chaperonin-60 and BiP, that can be involved in protein metabolism, signal transduction and gene transcription regulation.

Using RNA interference (RNAi) method we displayed that HeLa cells lacking α -DB showed less expression of F-actin, supporting the idea that α -DB is involved in cytoskeleton reorganization in cells. Moreover, NB4 cells with repressed α -DB showed decreased proliferation that could have caused the suppression of actin expression.

The subcellular distribution of α -DB in NB4 and HeLa cells was also assessed in this study. Our data clearly show that α -DB changed its sub-localization during granulocytic differentiation of NB4 cells and growth suppression of HeLa cells.

In summary, we have identified a novel ensemble of α -DB interacting proteins in promyelocytic leukemia cells. We demonstrated that these proteins are important in cytoskeleton reorganization and signal transduction. We also revealed that α -DB changes in sub-cellular compartments after treatment with chemical agents (ATRA, BML-210). Altogether, our data suggest that α -DB may work as an important structural and signalling protein during proliferation and differentiation processes of human cancer cells.

CONCLUSIONS

- For the first time we demonstrate that BML-210, a novel histone deacetylase inhibitor (HDACI), can block both leukemic and epithelial cancer cell growth and induce an apoptotic process through gene expression changes caused by chromatin remodeling. We think that BML-210 can be a promising agent for cancer treatment.
- We also provide evidence for myeloid cell line-specific, differential activity of HDACIs enhancing ATRA-mediated differentiation, that is associated with the regulation of specific gene and protein expressions.
- We describe a novel ensemble of α -DB interacting proteins in promyelocytic leukemia cells. These proteins are important in cytoskeleton reorganization and signal transduction. Our data suggest that α -DB may work as an important structural and signalling protein during proliferation and differentiation processes in human cancer cells.

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