VILNIUS UNIVERSITY

GIEDRĖ VALIULIENĖ

EPIGENETIC REGULATION AND LEUKAEMIA – RESEARCH OF NOVEL BIOLOGICAL, MOLECULAR AND THERAPEUTIC ASPECTS

Summary of doctoral dissertation
Physical Sciences, Biochemistry (04P)

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VILNIAUS UNIVERSITETAS

GIEDRĖ VALIULIENĖ

EPIGENETINIS REGULIAVIMAS IR LEUKEMIJA – NAUJŲ BIOLOGINIŲ, MOLEKULINIŲ IR TERAPINIŲ ASPEKTŲ TYRIMAI

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CONTENT

The materials presented in this dissertation are published in:

1. Valiulienė G, Stirblytė I, Jasnauskaitė M, Borutinskaitė V, Navakauskienė R. Anti-leukemic effects of HDACi Belinostat and HMTi 3-Deazaneplanocin A on human acute promyelocytic leukemia cells. Eur J Pharmacol. 2017; 799:143-53.

2. Valiulienė G, Treigytė G, Savickienė J, Matuzevičius D, Alksnė M, Jarašienė-Burinskaja R, Bukelskienė V, Navakauskas D, Navakauskienė R. Histone modifications patterns in tissues and tumours from acute promyelocytic leukemia xenograft model in response to combined epigenetic therapy. Biomed Pharmacother. 2016; 79:62-70.

3. Valiuliene G, Stirblyte I, Cicenaite D, Kaupinis A, Valius M, Navakauskiene R. Belinostat, a potent HDACi, exerts antileukaemic effect in human acute promyelocytic leukaemia cells via chromatin remodelling. J Cell Mol Med. 2015; 19:1742-55.

4. Savickiene J, Treigyte G, Stirblyte I, Valiuliene G, Navakauskiene R. Euchromatic histone methyltransferase 2 inhibitor, BIX-01294, sensitizes human promyelocytic leukemia HL-60 and NB4 cells to growth inhibition and differentiation. Leuk Res. 2014; 38:822-9.

5. Savickiene J, Treigyte G, Valiuliene G, Stirblyte I, Navakauskiene R. Epigenetic and molecular mechanisms underlying the antileukemic activity of the histone deacetylase inhibitor belinostat in human acute promyelocytic leukemia cells. Anticancer Drugs. 2014; 25:938-49.

ABBREVIATIONS

APL – acute promyelocytic leukaemia.

ATG5 – autophagy related 5.

ATO – arsenic trioxide.

BECN1 – Beclin 1.

Bel – belinostatat, (2E)-N-hydroxy-3-[3-(phenylsulfamoyl)phenyl]prop-2-enamide; *PubChem CID*: 6918638.

BIX – BIX-01294, N-(1-benzylpiperidin-4-yl)-6,7-dimethoxy-2-(4-methyl-1,4-diazepan-1-yl)quinazolin-4-amine; *PubChem CID*: 25150857).

CD – cluster of differentiation.

CDKN1B – cyclin dependent kinase inhibitor 1B.

CEBP – CCAAT/enhancer binding protein.

ChIP – chromatin immunoprecipitation.

DBR – treatment with 3-deazaneplanocin A, belinostat and retinoic acid.

DNMT – DNA nethyltransferase.

DZNep – 3-deazaneplanocin A, (1S,2R,5R)-5-(4-aminoimidazo[4,5-c]pyridin-1-yl)-3-(hydroxymethyl)cyclopent-3-ene-1,2-diol; *PubChem CID*: 73087).

EED –embryonic ectoderm development (subunit of PRC2 complex).

EHMT2 – euchromatic histone lysine methyltransferase 2.

EZH2 – enhancer of zeste 2 polycomb repressive complex 2 subunit.

H2A, **H2B**, **H3**, **H4**, **H1** – histones.

HAT – histone acetyltransferase.

HDAC – histone deacetylase.

HDACi – histone deacetylase inhibitor.

HMT – histone methyltransferase.

HMTi – histone methyltransferase inhibitor.

MS – mass spectrometry.

NBT – nitro blue tetrazolium.

NET – neutrophil extracellular traps.

NOG – imunodeficient mice.

NPM1 – nucleophosmin.

PARP – poly (ADP-ribose) polymerase.

PB – peripheral blood.

PCAF – histone acetyltransferase, p300/CREB binding protein associated factor.

PI – propidium iodide.

PML – promyelocytic leukaemia protein.

PPAR – peroxisome proliferator-activated receptors.

PRC2 – polycomb repressive complex 2.

RA – all trans retinoic acid.

RT-qPCR – quantitative reverse transcription polymerase chain reaction.

SUZ12 – suppressor of zeste 12 protein (subunit of PRC2 complex).

WT1 – Wilms tumour protein.

1. INTRODUCTION

Leukaemia is a disorder of haematopoietic cells, which disrupts the balance between cell proliferation, differentiation and apoptosis. Generally the term leukaemia (Greek *leukos* – white, *haima* – blood) describes cases of cancer, which disturb white blood cell (leucocyte) production and/or their functions. Leukaemia is classified into several main types: myeloid leukaemia and lymphoid leukaemia. Both of them could be of acute or chronic form. The term lymphoid describes that oncogenic changes take place in the cells of bone marrow, which later form lymphocytes. Myeloid leukaemia takes part in cells, which morph into granulocytes or monocytes (according to *National Cancer Institute*, 2014).

Several thousands of people worldwide are being diagnosed with acute promyelocytic leukaemia (APL; subtype of acute myeloid leukaemia) annually. Previously considered one of the most malignant forms of cancer, APL currently has become one of the most easily treatable forms (Lo-Coco and Cicconi, 2011). Usually APL patients are being treated with anthracyclines and RA (*all trans retinoic acid*) combination (Lo-Coco et al., 2013). It has been showed that RA is able to induce promyelocytic leukaemia cell differentiation into mature granulocytes (rev. Özpolat, 2008). Therefore, currently APL is being used as a standard model to study and evaluate the efficacy of differentiation therapy. However, despite the general success of RA therapy, RA treatment resistant cases still remain a serious issue (Fung and So, 2013; Tomita et al, 2013). According to Gallagher (2002), acquired secondary resistance to ATRA therapy is common in many patients treated with RA alone or a combination of RA with chemotherapy medication. It has been demonstrated that epigenetic remodelling could overcome APL resistance to RA (rev. Arteaga et al., 2015). Moreover, it is known that limited efficacy of RA treatment in other types of AML does also rely largely on epigenetic factors (rev. Schenk et al., 2014). That allows us to conclude that it is rational to complement leukaemia differentiation therapy with epigenetically active compounds.

In this study, we investigated the application of histone methyltransferase inhibitor (HMTi) BIX-01294, which specifically inhibits enzymatic activity of EHMT2, reduces H3K9me2 levels and significantly suppresses cancer cell growth (Chang et al., 2009; Huang et al., 2017). S-adenosylhomocysteine (AdoHcy) hydrolase and HMT inhibitor

3-deazaneplanocin A (DZNep) and hydroxamate-type HDACi belinostat (PXD101) were also investigated for leukaemia differentiation therapy. Belinostat was previously shown to inhibit class I and II of HDACs and enhance the acetylation level of histones and non-histone proteins. The activity of this drug caused cell cycle arrest, induction of apoptosis and inhibition of cell proliferation (Qian et al., 2006; Gravina et al., 2012). Belinostat is officially approved for relapsed or refractory peripheral T-cell lymphoma treatment (Lee et al., 2015) and is currently under evaluation of treatment in number of phase I/II clinical trials in haematological malignancies and solid tumours (Foss et al., 2015; according *ClinicalTrials.gov*). It should be noticed that, although DZNep primarily inhibits AdoHcy, the effects of DZNep on cancer cells were found to be relatively specific to EZH2 (catalytic sub-unit of PRC2). DZNep was found to inhibit formation of H3K27me3 and H4K20me3, as well as to induce apoptosis in cancer cells (Miranda et al., 2009; Girard et al., 2014).

Aim of this study – evaluate the impact of epigenetic regulation of human acute promyelocytic leukaemia cells induced to granulocytic differentiation.

Study objectives:

1. Determine the effect of HMTi BIX-01294, 3-deazaneplanocine A and HDACi belinostat on NB4 and HL-60 cell (APL) growth, viability and granulocytic differentiation.
2. Evaluate the gene and protein expression changes related to chromatin modification, cell cycle and differentiation in APL cells, treated with BIX-01294, 3-deazaneplanocine A, belinostat, retinoic acid and with various combinations of these agents.
3. Estimate the impact of used epigenetic modifiers on APL cell histone epigenetic profile.
4. Identify protein complexes associated to the hyperacetylated histone H4 after APL cell treatment.
5. Create a model of APL xenograftic NOG mice and evaluate the efficacy of used epigenetic therapy *in vivo.*

Scientific novelty

For the first time we have shown that HDACi belinostat (PXD101), HMTi BIX-01294 and 3-deazaneplanocine A facilitate RA-induced human acute promyelocytic leukaemia cell NB4 and HL-60 differentiation into mature granulocytes. Results obtained by us support the efficacy of APL epigenetic therapy *in vitro.*

Also we have pioneered in showing that in control NB4 cells hyperacetylated histone H4 is found together with DNA replication and transcription related proteins (e.g. POLA2, GCOM1, POLR2M, NELFE), while after the influence of belinostat it was found associated with proteins crucial for pro-apoptotic processes (e.g. S100A9, S100A8, LGALS7, GOLGA3), proteins important in defence against oxidative stress (TXNRD2) and cancer inhibitors (APC). These results expand the current knowledge of belinostat action mechanism in cancer cells.

In this study we have created a model of APL xenograftic NOG mice, characterised it and showed that it can be successfully used in evaluating efficacy of APL epigenetic therapy. Our studies have also demonstrated that the treatment combining belinostat, 3-deazaneplanocine A and RA prolongs APL xenograftic mice life span and protects them against tumour formation. Moreover, for the first time we have evaluated the effect of epigenetic therapy on histone modification changes in APL xenograftic mice tumours and tissues. Results obtained could be valuable for future APL epigenetic therapy clinical studies as well.

Statements to be defended:

1. HDAC and HMT inhibitors promote pro-differentiation effect of retinoic acid in the APL cells.
2. Combined action using HDACi and HMTi results in higher differentiation facilitating efficacy compared to action of single agents.
3. Combined treatments (HDACi and HMTi) change the epigenetic landscape of APL cells and up-regulate differentiation related genes (*CEBPE, PPARG*) expression, while inhibiting epigenetic modifiers (HDAC1, HDAC2 and PRC2 complex) protein expression.
4. Epigenetic therapy *in vivo* prolongs xenograftic mice lifespan, protects from tumour formation and changes epigenetic landscape of tissue cells.

2. METHODS

2.1. Cells and culture conditions

The human promyelocytic leukaemia NB4 and HL-60 cells (from DSMZ, GmbH, Germany) were cultured in RPMI 1640 medium supplemented with 10% foetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco, USA) at 37 ^{o}C in a humidified 5% CO_2 atmosphere. In each experiment, logarithmically growing cells were seeded at 5 x 10^5 cells per ml in 5 ml of medium. In this research we used HDACi belinostat (Selleck chemicals, USA), HMTis BIX-01294 (IBL Intern., Germany) and 3-deazaneplanocin A (DZNep; Cayman Chemical, USA), also retinoic acid (RA; Sigma, USA). Cells were treated with BIX-01294 (2-5 μM) alone and with 2 μM BIX-01294 in combination with 1 μM RA. Cells were also treated with 0.5 μM DZNep, alone or in combination with 1 μM RA. Belinostat alone was used in 0.2-2 μM concentration. 0.2 μM belinostat was used in combined treatment with 1 μM RA. In addition, combination 0.2 μM Bel + 0.5 μM DZNep + 1 μM RA was also used in our research. In pre-treatment experiments cells were exposed to 0.8 μM belinostat and 0.5 μM DZNep for 4 h following which the drug was washed out, cells were resuspended in fresh media, and incubated with the differentiation inducer 1 μM RA. Treated cells were cultured and harvested at the time-points indicated.

2.2. Cell proliferation, differentiation and viability assays

Cell proliferation was evaluated by the trypan blue exclusion test, as described previously (Valiulienė et al., 2017). The value of growth inhibition after 24 h and 48 h incubation was calculated, according to the formula: GI (%) = 100 - ((VC_T/VC_C) x 100) (where VC_T stands for viable cells from treated sample, VC_C -viable cells from control sample). The degree of granulocytic differentiation was assayed by the NBT test, as discussed earlier (Valiulienė et al., 2017).

2.3. Flow cytometric assessment of CD11b surface marker

NB4 and HL-60 cells (0.5×10^6 cells/sample) were washed twice with PBS (pH 7.4) and then exposed to mouse monoclonal anti-human CD11b (BD Pharmingen, USA). All other steps were performed as described earlier (Valiulienė et al., 2017).

2.4. Flow cytometric analysis for determination of apoptosis

The analysis was performed on a flow cytometer BD FACSCanto II (Beckton and Dickinson) with BD FACSDiva software. Early and late apoptosis were analysed by flow cytometer after dual staining with FITC-labelled Annexin-V and propidium iodide (PI) (Kit-AX; Xebio, Czech Republic), according to the manufacturer's instruction.

2.5. Flow cytometric analysis for determination of cell cycle distribution

Control and treated cells were harvested, washed twice with phosphate-buffered saline **(**PBS) and fixed in 70% ethanol overnight at -20 ^{o}C. Fixed cells were washed twice with PBS and stained in PBS containing propidium iodide (PI) (50 μg/ml) and RNAse (0.2 mg/ml) for 30 min at 37 ^{o}C. Cell cycle analysis was performed on a flow cytometer BD FACSCanto II.

2.6. Quantitative real-time PCR (RT-qPCR)

Total RNA from treated and control NB4 and HL-60 cells (3.0×10^6 cells/sample) were isolated using TRIzol® reagent (Life Technologies, Invitrogen, Belgium) according to the manufacturer's instructions. RNA samples further treated, as described previously (Valiuliene et al., 2015). The primers used in our research are indicated in Table 2.6.1.

Table 2.6.1. Primers used in gene expression analysis.

Gene	Primers
ATG5	5' CTTTGCATCACCTCTGCTTTC 3' 5' TAGGCCAAAGGTTTCAGCTTC 3'
BECN1	5' CTCCCGAGGTGAAGAGCATC 3' 5' GCTGTTGGCACTTTCTGTGG 3'
CEBPA	5' GCTCGCCATGCCGGGAGAACT 3' 5' TGCAGGTGGCTGCTCATCGG 3'
CEBPE	5' CAGCCGAGGCAGCTACAATC 3' 5' AGCCGGTACTCAAGGCTATCT 3'
EED	5' GTGACGAGAACAGCAATC 3' 5' TATCAGGGCGTTCAGTGTTTG 3'
EZH2	5' GTGGAGAGATTATTTCTCAAGATG 3' 5' CCGACATACTTCAGGGCATCAGCC 3'
EHMT2	5' CTCCGCTGATTTTCGAGTGTAA 3' 5' GTCGAAGAGGTAAGAATCATCC 3'
GAPDH	5' AACTCTGGTAAAGTGGATATTG 3' 5' GGTGGAATCATATTGGAACA 3'
CSF3	5' GCTGCTTGAGCCAACTCCATA 3' 5' GAACGCGGTACGACACCTC 3'
CSFR	5' CTTGTGGCCTATAACTCAGCC 3' 5' CCCACTCAATCACATAGCCCT 3'
HDAC1	5' CAAGCTCCACATCAGTCCTCCC 3' 5' TGCGGCAGCATTCTAAGGTT 3'
HDAC2	5' AGTCAAGGAGGCGGCAAAA 3' 5' TGCGGATTCTATGAGGCTTCA 3'
CDKN1B	5' TAATTGGGGCTCCGGCTAACT 3' 5' TGCAGGTCGCTTCCTTATTCC 3'
PCAF	5' GGCCGAGGAGTCTTGTAAAT 3' 5' AGTGAAGACCGAGCGAAGCA 3'
PML-RARA	5' CAGTGTACGCCTTCTCCATCA 3' 5' GCTTGTAGATGCGGGGTAGA 3'
PPARG	5' GCTCTAGAATGACCATGGTTGAC 3' 5' ATAAGGTGGAGATGCAGGCTC 3'
SUZ12	5' AGGCTGACCACGAGCTTTTC 3' 5' GGTGCTATGAGATTCCGAGTTC 3'
WT1	5' GGCATCTGAGACCAGTGAGAA 3' 5' GAGAGTCAGACTTGAAAGCAGT 3'

2.7. Chromatin immunoprecipitation for RT-qPCR analysis

ChIP assay was performed as described earlier (Valiulienė et al., 2017). The primer sets for the tested genes were as denoted in Table 2.7.1. For data evaluation, the

percentage input was calculated, according to the formula: $100 \times 2^{(\text{Adjusted input} - \text{Ct (IP)})}$. Then data were represented as a fold change in percentage input (compared to untreated control).

Table 2.7.1. Gene primers used for *ChIP* - qPCR analysis.

Gene	Primers
CEBPA	5' GTGCAGCCTCGGGATACTC 3' 5' CTCCTCCTGCCTGCCCTA 3'
CEBPE	5' GCTAACCGGAATATGCTAATCAG 3' 5' CCTTTCAGAGACACCTGCTC 3'
PPARG	5' CAGCACCACCGATCAGAAGA 3' 5' TCCCATTTCCGAGGAGGGAT 3'

2.8. Chromatin immunoprecipitation for mass spectrometry analysis

All ChIP procedures were carried out as discussed in the previous section. Protein A/G PLUS-Agarose - Antibody - Protein complexes were denatured in 7 M Urea, 2 M Thiourea, 40 mM DTT solution, with continuous rotation at 500 rpm in the temperature controlled shaker for 0.5 hour at 20 °C. Complexes were centrifuged (166 x *g*, 7 min, 20 °C) and extraction repeated additional 3 times. All four extracted fractions were combined and subjected to further MS analysis.

2.9. Mass spectrometry and data analysis

Extracted proteins were applied on Amicon Ultra-0.5 mL 30 kDa centrifugal filter unit (Sigma-Aldrich, USA). Trypsin digestion was done according to a modified FASP protocol as described by Wisniewski and colleagues (2009). Liquid chromatography (LC) separation of trypsin cleaved peptides and mass spectrometric analysis were performed as described earlier (Simoliūnas et al., 2013). Raw data files were processed as discussed previously (Valiulienė et al., 2015).

2.10. Total protein isolation and western blot analysis

All procedures were done as described previously (Valiulienė et al., 2017). Immunoblotting was performed using antibodies against GAPDH and PCAF (Abcam, UK), EZH2, SUZ12, HDAC1, HDAC2 (Cell signaling, USA), survivin, p27, PARP-1,

DNMT1 (Santa Cruz Biotechnology, Germany), against acetylated H4K16, against di- and trimethylated H3K9 (Upstate Biotechnology, USA), against trimethylated H3K27, trimethylated H3K4, against hyperacetylated H4, cycline E1, caspaze-3 (Millipore, USA), WT-1 and EED proteins (Thermo Fisher Scientific, EU). HRP conjugated secondary antibodies were purchased from (Dako Cytomation, USA). Blots were scanned and optical density evaluated using ImageJ software.

2.11. In vivo analysis

Male immunodeficient NOG mice at the age of 9-12 weeks were purchased from Taconic (Ry, Denmark). Animals were housed and maintained in specific pathogen free conditions. Studies were conducted in accordance with the guidelines of "Law on the Care, Welfare and Use of Animals" of the Republic of Lithuania. License for the use of laboratory animals in this research (No. 3; 2013-11-13) was obtained from the Lithuanian Food and Veterinary Office. Xenograft model of APL was established by intravenous (i.v.) inoculation of exponentially growing NB4 cells (5×10^6/mouse), suspended in 20 μl serum-free cell cultivation medium, in immunodeficient NOG mice. Three mice per treatment group were employed. APL xenograft mice (denoted as APL) were not treated and used as a control. Xenograft mice began to receive a treatment after the appearance of first typical illness symptoms and specific cell surface markers detection in the peripheral blood cells (PB). APL xenograft mice were treated with the combination of Bel (20 mg/kg) and DZNep (1 mg/kg) via intraperitoneal (i.p.) route (5 times/week; 5 injections) and further treated orally with RA (0.45 mg/d, 5 times/week, 20 times) (denoted as APL-DBR).

PB counts, assessment of human-origin PB cell surface markers, quantitative real time PCR and western blot analysis were performed as described previously (Valiulienė et al., 2016).

2.12. Statistical analysis

Unless otherwise specified, all experiments were repeated at least three times. Data were expressed as mean values with SDs. For statistical analysis two-sample Student's t-test was used. Significance value was set at $p \leq 0.05$.

3. RESULTS

3.1. BIX-01294 effect on human APL cells

3.1.1. BIX-01294 effect on APL cell growth and survival

The anti-leukaemic activity of BIX-01294 was investigated on two human acute promyelocytic leukaemia cell lines HL-60 and NB4 (Savickiene et al., 2014a). BIX-01294 was shown to inhibit the growth of both cell lines concentration-dependently. Also it notably decreased HL-60 and NB4 cell viability, when used at higher (4-5 μM) concentrations (Fig. 3.1.1.1A). The analysis of cell cycle upon 48 h treatment (Fig. 3.1.1.1B) demonstrated the shift in the proportion of cells in G0/G1 phase and a concentration-dependent reduction (about 1.6-2-fold) in G2/M phase.

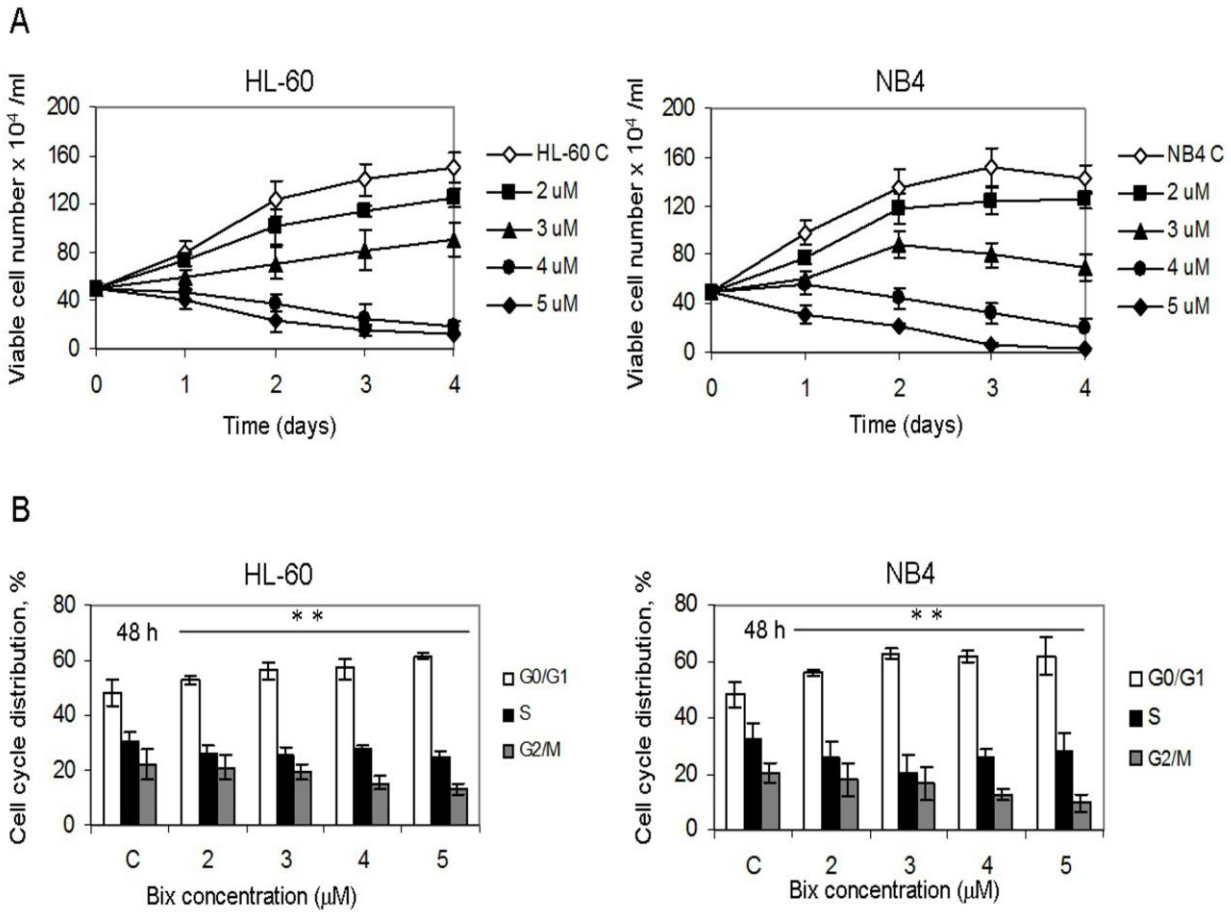

Fig. 3.1.1.1. Concentration-dependent anti-proliferative effects of BIX-01294 in HL-60 and NB4 cells. Data are averaged, ±SD (n = 3), $p \leq 0.001$ (**) (Savickiene et al., 2014a).

Flow cytometric examination after 48 h treatment (Fig. 3.1.1.2) revealed that depending on the concentration, the population of PI-stained cells increased, whereas no significant increase of Annexin-V-positive cells were detected. High BIX-01294 concentrations (4-5 μM) were cytotoxic to NB4 and HL-60 cells (42%-49% dead cells were registered). Only small number of early apoptotic cells (about 10%) were detected, which indicates mostly non-apoptotic manner of cell death.

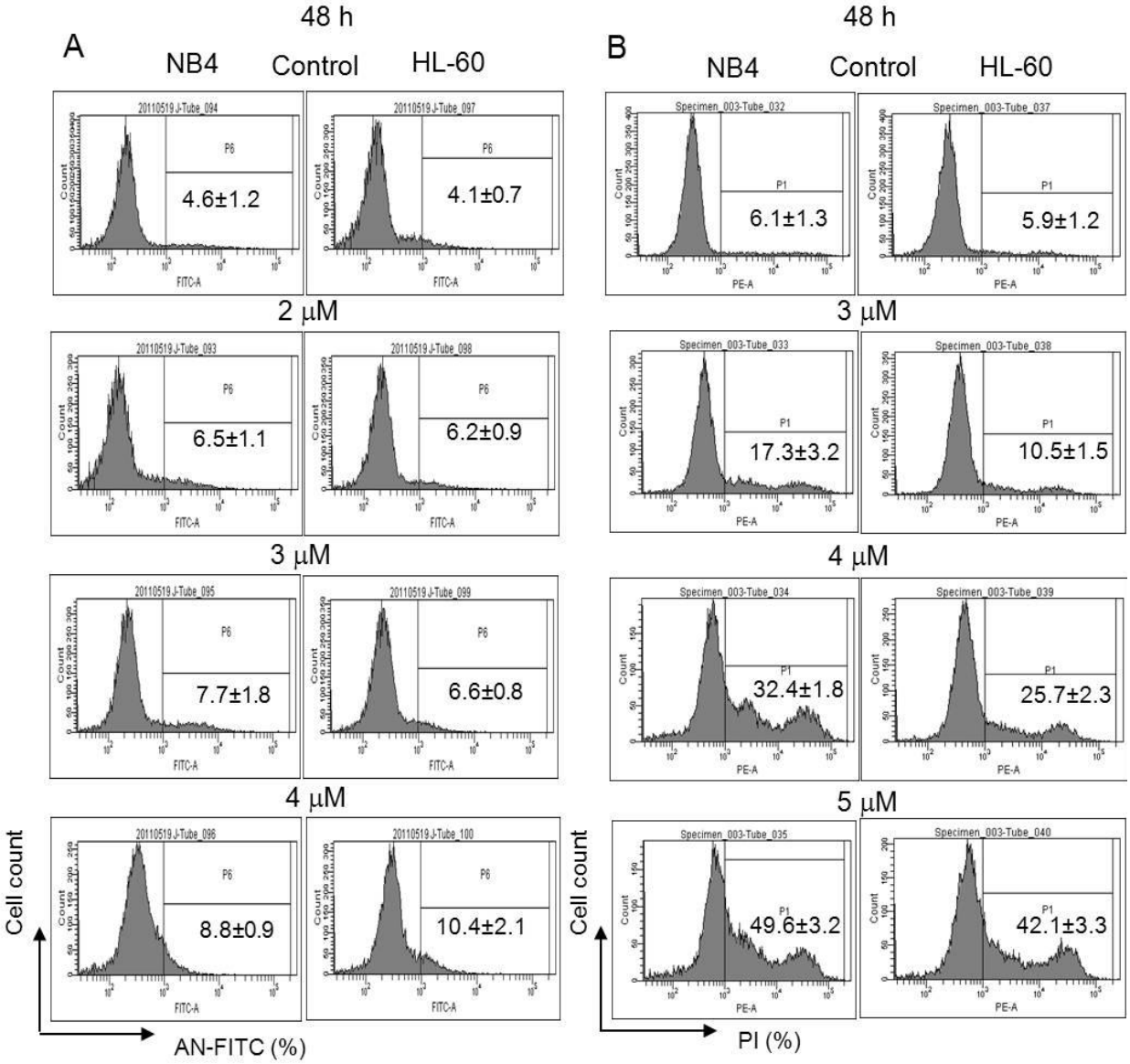

Fig. 3.1.1.2. BIX-01294 induces concentration-dependent cytotoxic effects in HL-60 and NB4 cells (Savickiene et al., 2014a).

3.1.2 BIX-01294 effect on RA-induced differentiation of APL cells

HL-60 and NB4 cells were treated with 2 μM BIX-01294 and 1 μM RA for 4 days. NBT test demonstrated that such treatment increases both cell lines' granulocytic

differentiation (Fig. 3.1.2.1A). Comparable tendencies were observed in the sequential assay, when cells for 24 h were pre-treated with 2 μM BIX-01294 and then further cultivated in the medium supplemented with RA.

The amount of granulocytic differentiation upon 48 h sequential treatment was approximately 1.4-fold higher than in RA alone treated cells (Fig. 3.1.2.1B). Regarding all combined treatments, the activity of NBT-reduction gained the greatest value of 80-85%, compared to 60-70% in RA treated cells. It should be stated that BIX-01294 alone did not induce HL-60 and NB4 cell differentiation, as demonstrated by NBT-test and the absence of CD11b expression (data not shown).

Flow cytometric examination of CD11b expression (Fig. 3.1.2.1C) revealed that BIX-01294 in combination with RA (when presented in simultaneous or sequential manner) enhances RA pro-differentiation effect in NB4 and HL-60 cells (1.41 and 1.37-fold and 1.59- and 1.69-fold, respectively).

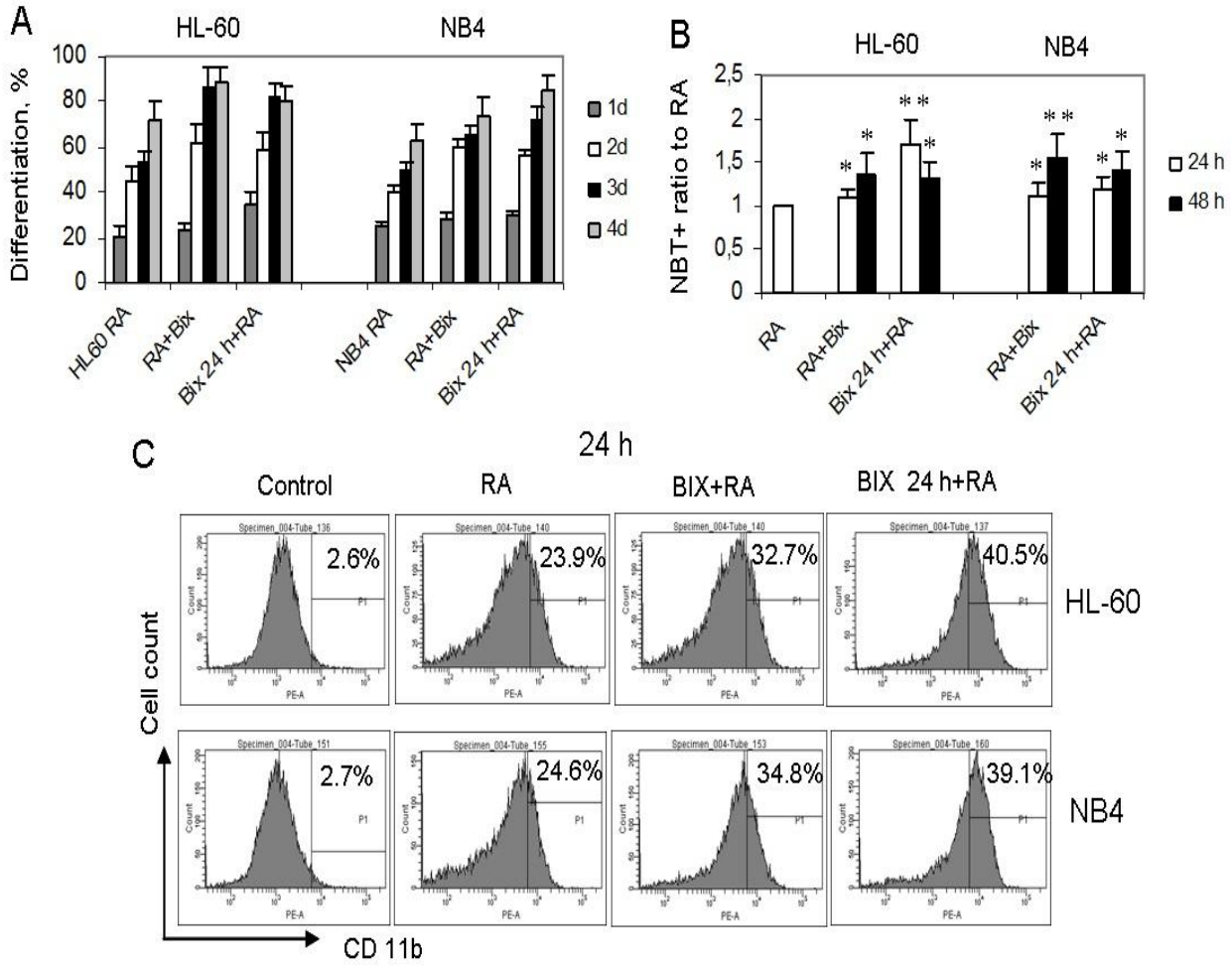

Fig. 3.1.2.1. BIX-01249 accelerates and increases RA-induced granulocytic differentiation of HL-60 and NB4 cells. Data are averaged, ±SD (n = 3), $p \leq 0.05$ (∗), $p \leq 0.001$ (∗∗) (Savickiene et al., 2014a).

3.1.3. BIX-01249 effect on protein level regulation

Western blot analysis (Fig. 3.1.3.1A) supported results obtained by Annexin-PI test (Fig. 3.1.1.2), as the absence of PARP-1 cleavage was observed (only a weak signal in NB4 cells at 5 μM concentration). These results proposed the non-apoptotic manner of APL cell death upon treatment with BIX-01294. It should be noticed that treatment with BIX-01294 resulted in cell type-specific and also concentration-dependent orchestrated alterations in H3K9me2, survivin and cyclin E1 protein levels (Fig. 3.1.3.1A).

In both cell lines, 48 h exposure with intermediate BIX-01294 concentration (3 μM) resulted in the H3K9me2 reduction. Simultaneously, similar changes in p27, survivin and chromatin modifying enzymes, HDAC1 and DNMT1 were detected (Fig. 3.1.3.1B).

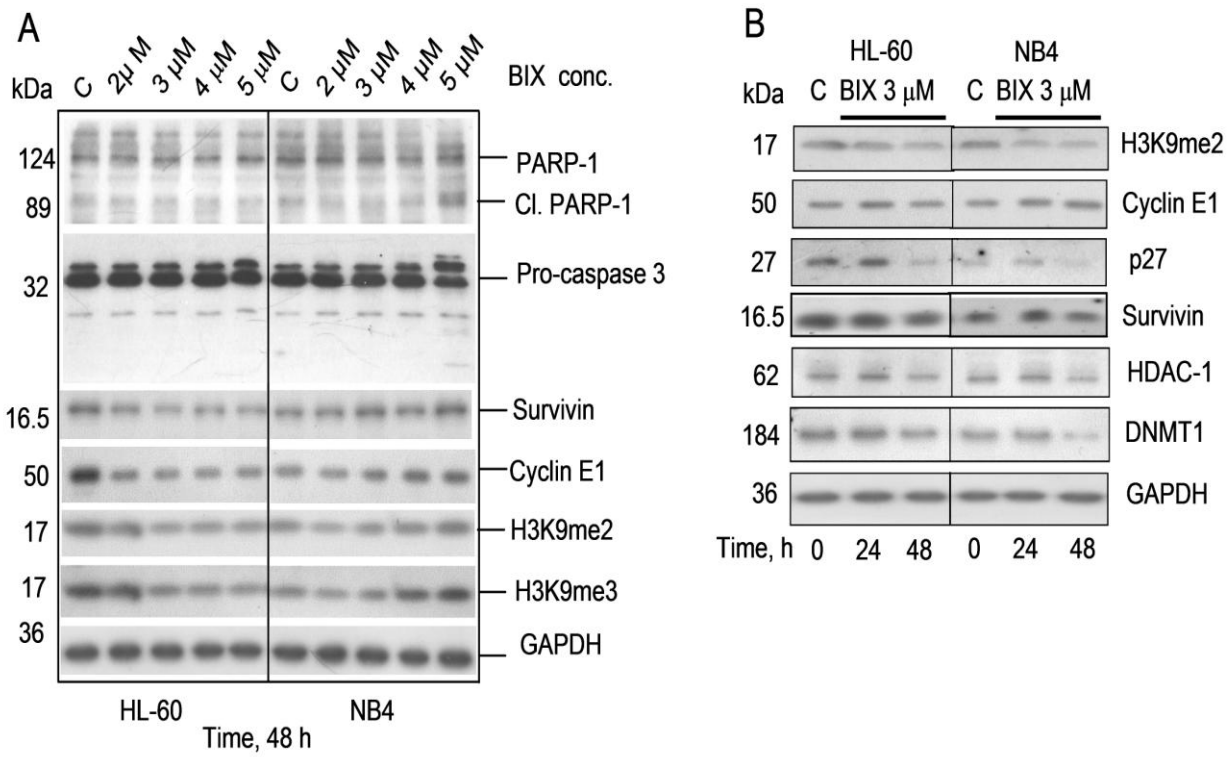

Fig. 3.1.3.1. BIX-01294-induced changes in cell cycle- or survival regulating protein expression and histone H3K9 modification state (A, B) (Savickiene et al., 2014a).

3.1.4. Discussion

It is worth noting that the exact mechanism of how BIX-01294 causes APL cell death still remains elusive. Previous studies (Kondo et al., 2008) have showed that RNA interference based *EHMT2* gene expression inhibition causes notable morphological

changes in cancer cells, increases β-galactosidase expression and inhibits telomerase activity. Our results of *ATG5* and *PPARG* gene expression analysis, demonstrating an obvious expression increase of these genes after the treatment with BIX-01294 (Savickiene et al., 2014a), may suppose that BIX-01294 triggers cellular senescence and mitotic catastrophe pathways in NB4 and HL-60 cells. It is a known fact that PPARγ activation plays an important role in regulation of senescence signalling pathways (Altucci et al., 2001; Lu et al., 2005). Very important notion is that PPARγ, together with enhanced expression of survivine, suppresses *TRAIL*-induced apoptosis (Li et al., 1998), which is the most common pathway of NB4 cell death (Castedo et al., 2004). ATG5, on it's own, is vital for mitotic catastrophe independent of autophagy, which becomes apparent in cancer cells after application of sub-lethal doses of DNA damaging drugs and anti-mitotic agents (Han et al., 2010). It has been demonstrated that ATG5, found in cytosol, plays a role in regulation of signalling pathways of autophagy, while ATG5 protein, present in the nucleus, is able to induce mitotic catastrophe transformations (due to ATG5 interaction with survivine orderly alignment of chromosomes is being disturbed, rendering the chromosomes in segregation defects (Han et al., 2010)). It is known that survivine not only protects the cells from apoptosis by intrinsic (mitochondrial) pathway, but also regulates cell division and participates in mitotic spindle checkpoint (Chang et al., 1999; Dohi et al., 2004; Vernier et al., 2011). Our study has also revealed that after application of BIX-01294 on NB4 cells and following cell inability to proceed into G2/M cell cycle phase (Fig. 3.1.1.1B), the quantity of survivine protein rises notably (Fig. 3.1.3.1). These results have positively correlated with an increased amount of constitutive heterochromatin mark H3K9me3 (Fig. 3.1.3.1). It is important to note that different HL-60 and NB4 cell responses to lethal BIX-01294 concentrations could depend on p53 protein – this protein is found in NB4 cells. However, it is not expressed in HL-60 cells, which results in latter cells being less sensitive to senescence triggers (Lekstrom-Himes, 2001). Moreover, PML protein through PML/Rb/E2F signalling pathway (Truong et al., 2003) may participate in initiation and maintenance of cellular senescence pathways in NB4 cells.

Talking about BIX-01294-induced cancer cell death in generally, it was demonstrated that, e.g. in lung adenocarcinoma cells BIX-01294 evokes apoptosis by

activating mitochondrial pathway (Wan et al., 2017). However, in breast and colon cancer cells, BIX-01294 was demonstrated to induce autophagy-dependent cell death via EHMT2 dysfunction and intracellular ROS accumulation (Kim et al., 2013).

Summing all previous results, it can be stated that suppression of EHMT2 activity can be considered an important additional mechanism, describing HL-60 and NB4 cell growth inhibition and differentiation enhancement. Further we studied the effect HDACi belinostat has on NB4 and HL-60 cells.

3.2. Belinostat effect on human APL cells

3.2.1. Effect of belinostat on APL cell growth and survival

The anti-proliferative effect of belinostat on APL cells, using range of different concentrations (0.2-0.5-1 μM) was evaluated (Savickiene et al., 2014b; Valiuliene et al., 2015). Belinostat inhibited NB4 and HL-60 cell proliferation in a concentration- and time-dependent manner with the most prevalent effect in NB4 cells (Fig. 3.2.1.1A). The growth of both cell lines was inhibited up to 90% after 24 and 48 h treatment with 1 μM belinostat (Fig. 3.2.1.1B).

We also evaluated the belinostat activity towards cell cycle progression. Flow cytometric analysis after 24 h treatment with belinostat revealed concentration-dependent changes (Fig. 3.2.1.1C). Low concentrations (0.2-0.5 μM) of belinostat resulted in G0/G1-phase increase (by 8-12%) with a subsequent decrease in S-phase. Worth noting, higher concentrations (1-2 μM) caused evident cell accumulation in S-phase (to 56-63%) and decrease in both G0/G1 and G2/M-phases (to 12-16%), which was the most evident (9-3%) after longer 48 h incubation (data not shown), indicating that cells were arrested in S-phase transition.

We demonstrated that cytotoxicity of belinostat was also concentration-dependent (Fig. 3.2.1.2A). Exposure to belinostat dose dependently induced apoptosis as well, as determined by augmentation of apoptotic cell population in the subG1 phase (Fig. 3.2.1.2B) and by two-color staining with Annexin-V-FITC and PI (Fig. 3.2.1.2C).

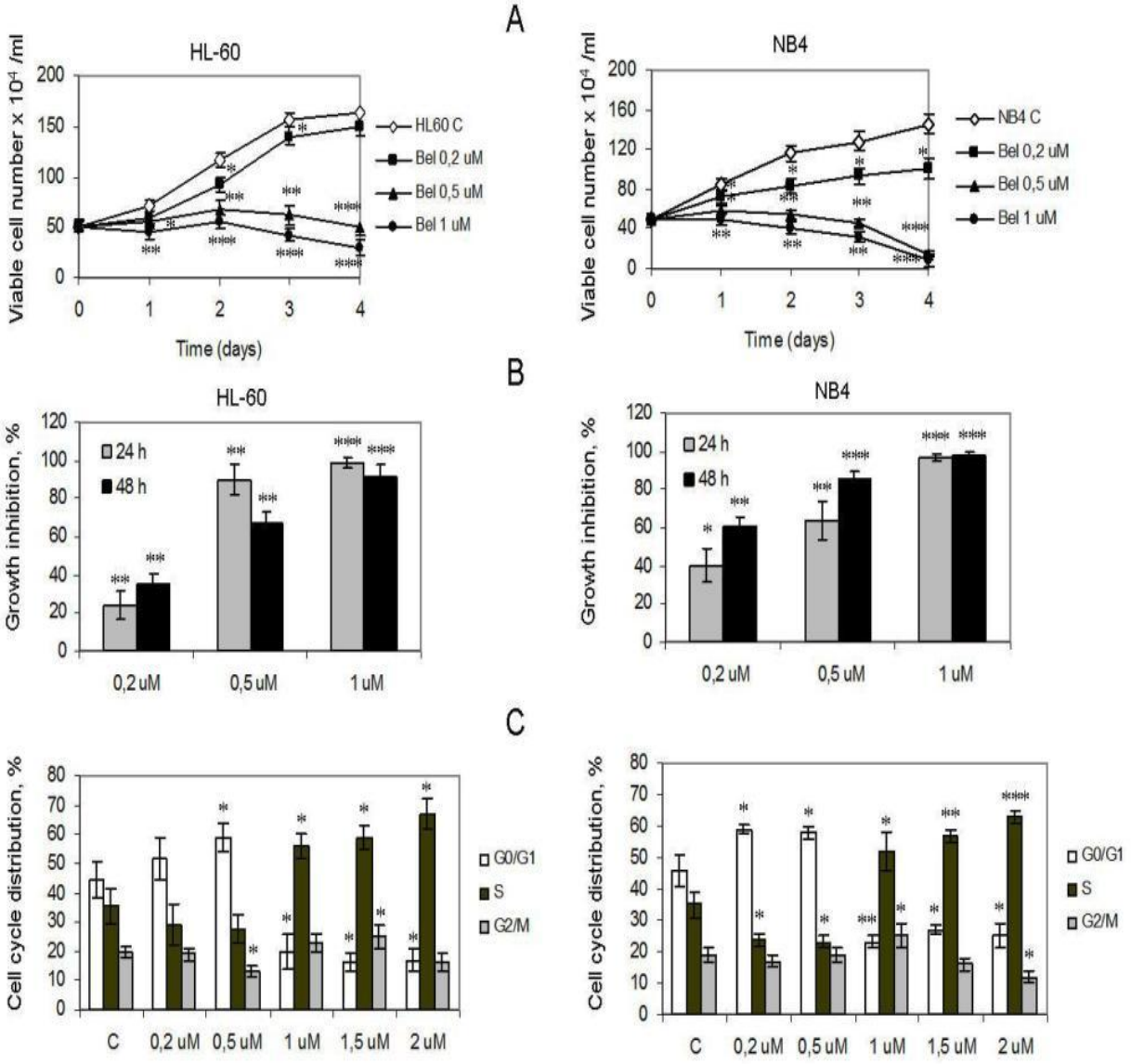

Fig. 3.2.1.1. Concentration-dependent anti-proliferative effects of belinostat in HL-60 and NB4 cells. Data are averaged, ±SD (n = 3), $p \leq 0.05$ (*), $p \leq 0.001$ (**) and $p \leq 0.0001$ (Savickiene et al., 2014b).

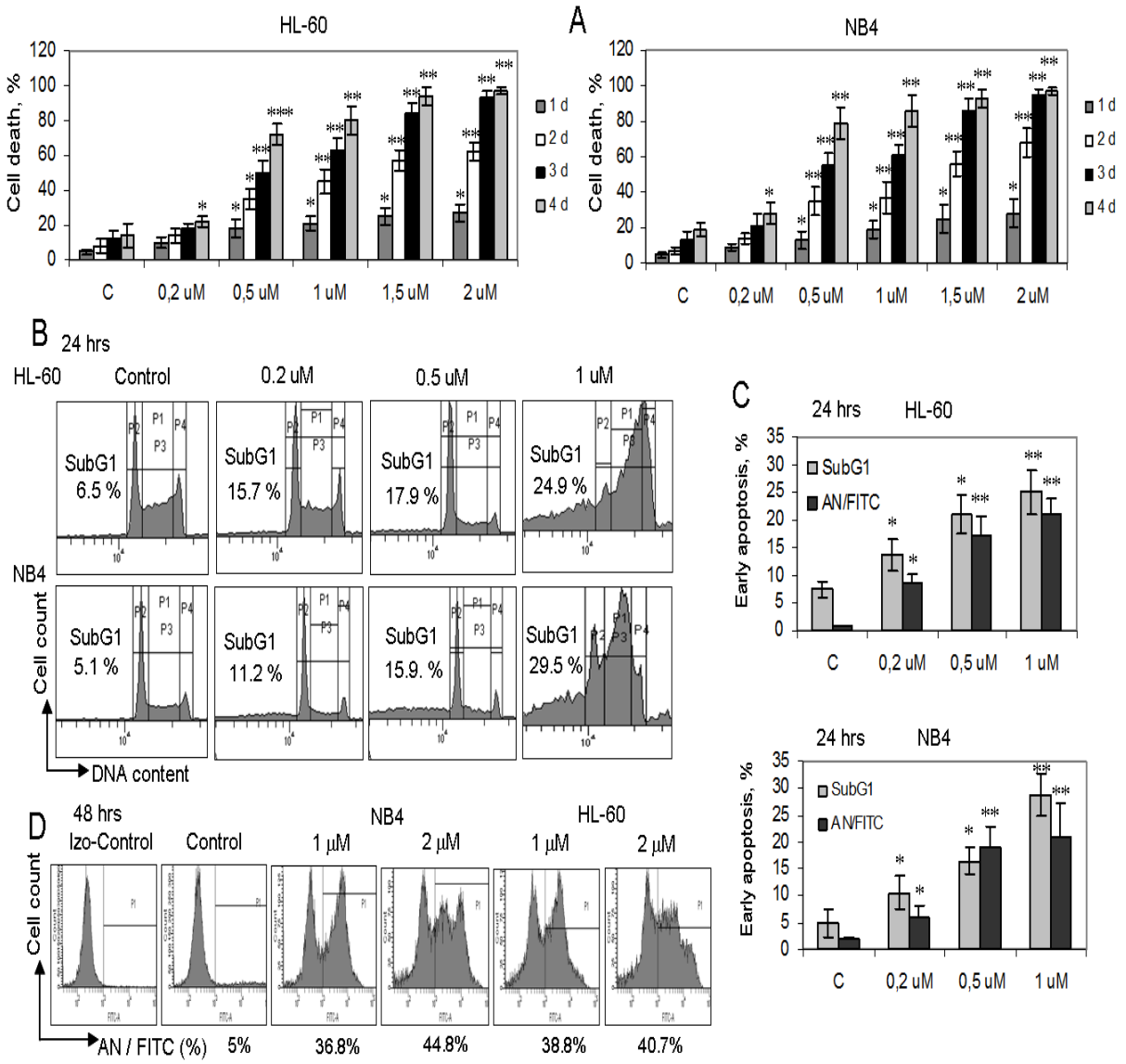

Fig. 3.2.1.2. Belinostat induces concentration-dependent cytotoxic effects in HL-60 and NB4 cells. Data are averaged, ±SD (n = 3), $p \leq 0.05$ (*), $p \leq 0.001$ (**) and $p \leq 0.0001$ (Savickiene et al., 2014b).

3.2.2. Effect of belinostat on APL cell granulocytic differentiation

In order to evaluate granulocytic differentiation, cells were treated with 1 μM RA, 0.2 μM Bel alone and with their combination (1 μM RA + 0.2 μM Bel). NBT test revealed that belinostat alone is not sufficient to induce NB4 and HL-60 cells differentiation (data not shown). However, we showed that it enhances and accelerates RA-induced granulocytic differentiation (Fig. 3.2.2.1A). Combined treatment increased differentiation 1.5-1.8-fold, compared to treatment with RA alone (Fig. 3.2.2.1B). Flow cytometric analysis after 48 h of combined treatment clearly indicated high expression of

an early granulocytic marker CD11b (77.4% and 59.3% in HL-60 and NB4 cells, respectively) (Fig. 3.2.2.1C).

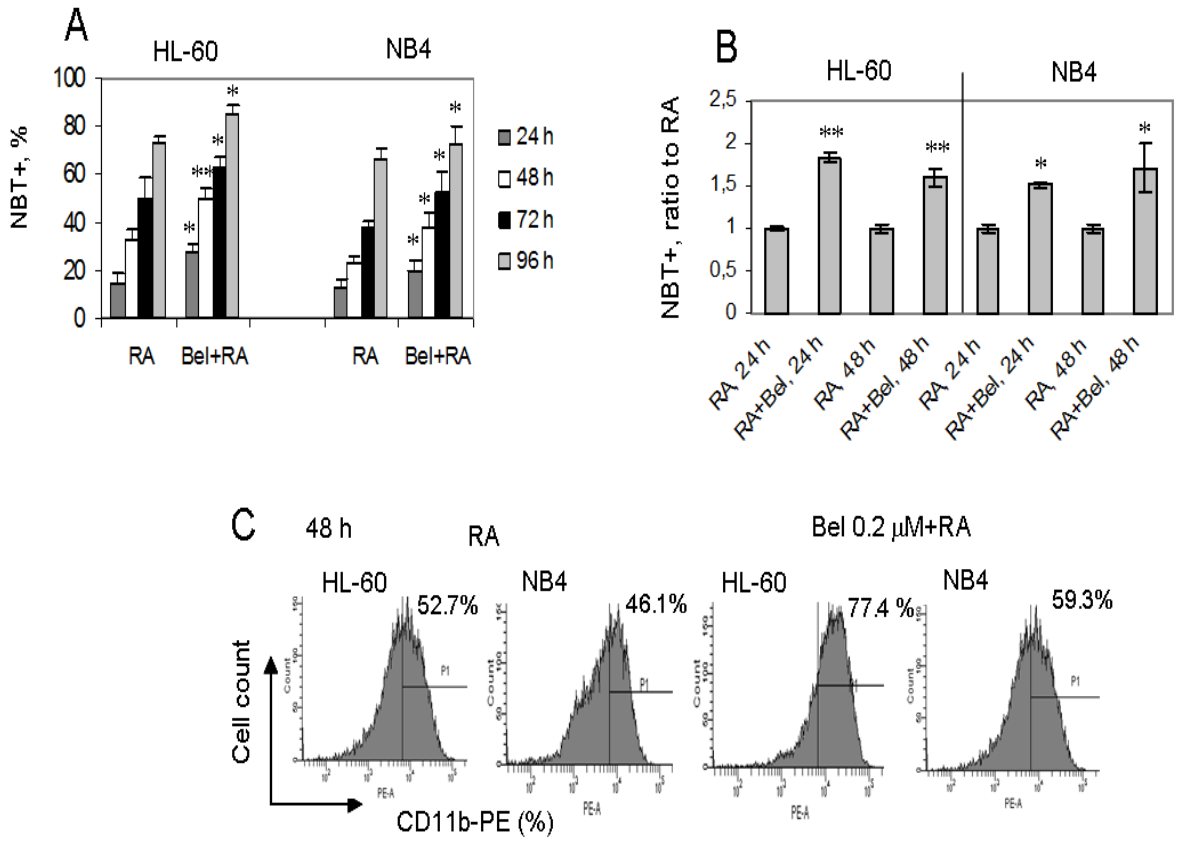

Fig. 3.2.2.1. Belinostat enhances granulocytic differentiation upon continuous treatment with RA. Data are averaged, ±SD (n = 3), $p \leq 0.05$ (*), $p \leq 0.001$ (**) (Savickiene et al., 2014b).

3.2.3. Effect of belinostat on APL cell protein level modulation

In order to evaluate molecular mechanisms that belinostat modulates in greater detail, we investigated the effect of 0.2 μM Bel treatment (alone or in combination with 1 μM RA) on histone H4 hyperacetylation level, as well as HDAC1 and HDAC2 protein level regulation (Fig. 3.2.3.1).

The most efficient increase in NB4 cells' histone H4 hyperacetylation level was observed after combined treatment 0.2 μM Bel + 1 μM RA. The effect was rapid (evident after 6 h incubation) and highly pronounced (histone H4 hyperacetylation level increased 21-fold compared to control cells). Combined treatment of Bel + RA also had the most obvious effect on HDAC1 protein level down-regulation. From the presented data it is evident that RA alone is insufficient to reduce HDAC1 protein level; however, it is capable to enhance belinostat effect on HDAC1 protein level down-regulation. In

contrast to HDAC1, HDAC2 protein level was shown to be up-regulated immediately upon treatment with belinostat, RA or their combination. Later HDAC2 was restored to its previous level (except after treatment with belinostat).

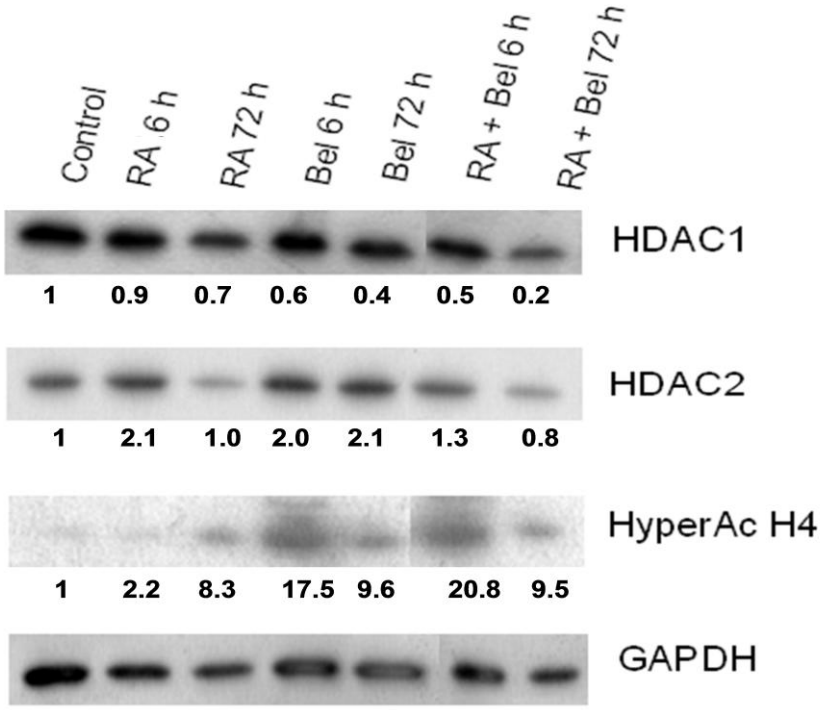

Fig. 3.2.3.1. Effects of Bel, RA and their combined treatments on NB4 cells protein level regulation (Valiuliene et al., 2015).

3.2.4. Belinostat modulates protein complexes associated with hyperacetylated histone H4

We were interested, if the increase in basal histone H4 hyperacetylation level after NB4 cells treatment with belinostat is accompanied by composition changes in protein complexes that are found in association with this epigenetic mark. Therefore, we used Co-IP and subsequent MS analysis that helped us to reveal proteins that are associated with hyperacetylated histone H4 in control and belinostat treated NB4 cells. Quantitative changes of identified proteins in control and belinostat treated cells are presented in Table 3.2.4.1 as C/Bel ratio (the mark "C" denotes that protein was only detected in control cells, whereas mark "Bel" indicates that protein was seen only in treated cells).

In untreated NB4 cells hyperacetylated histone H4 was found to associate with 45 different proteins (Table 3.2.4.1). Only in control cells hyperacetylated histone H4 was found associated with proteins that are involved in DNA replication (POLA2),

transcription (GCOM1, POLR2M, NELFE, NCL), translation (RPL7) and RNA splicing (SCNM1). Also, hyperacetylated histone H4 was identified, associated with proto-oncogene SPECC1, regulator of apoptosis ADAMTSL4, as well as with proteins involved in different signalling cascades: NF-kappaB, JAK2/STAT4, Ras and Hedgehog signal transduction pathways. Interestingly, hyperacetylated histone H4 in control NB4 cells was found to be associated with Nucleophosmin (NPM), a protein, which regulates tumour suppressors TP53/p53 and ARF and is shown to be over-expressed in actively proliferating cells, like various cancer and stem cells (Lim and Wang, 2006). It is worth mentioning that NPM was found in complexes with hyperacetylated histone H4 after treatment with 2 μM Bel as well, but to a much lesser extent.

After 6 h treatment with 2 μM belinostat (Table 3.2.4.1) hyperacetylated histone H4 was identified to be associated with proteins that are pro-apoptotic and necessary for apoptotic response (S100A9, S100A8, LGALS7, GOLGA3, PPT1). Tumour suppressor APC was found in immunoprecipitated complexes as well. It is important to underline that hyperacetylated histone H4 has been also found to be associated with proteins that are involved in the defense against oxidative stress (TXNRD2) and access of retinoic acid to the nuclear retinoic acid receptors regulation (CRABP1).

Table 3.2.4.1. Summary of identified NB4 cells proteins found in complexes with hyperacetylated histone H4 in control and Bel treated cells (Valiuliene et al., 2015).

No. (1)	Accession (2)	Gene name (3)	Score (4)	C/Bel ratio (5)*	Function (6)
1	Q5QNW6	HIST1H2AH	9505,53	0,77105	Core component of nucleosome.
2	Q99878	HIST1H2AJ	8305,59	0,59452	Core component of nucleosome.
3	P33778	HIST1H2BB	42815,45	1	Core component of nucleosome.
4	P58876	HIST1H2BD	10401,13	0,51171	Core component of nucleosome.
5	P57053	HIST2H2BF	45639,36	Bel	Core component of nucleosome.
6	P84243	H3F3A	10974,41	1,10517	Core component of nucleosome.
7	Q6NXT2	H3F3C	828,6	0,69768	Core component of nucleosome.
8	P62805	HIST1H4A	17505,7	0,84366	Core component of nucleosome.
9	P16401	HIST1H1B	615,33	1,1853	Nucleosomal condensation.
10	P16403	HIST1H1C	2448,72	1,05127	Nucleosomal condensation.
11	Q71UI9	H2AFV	5543,68	1,23368	Replaces conventional H2A in a subset of nucleosomes.
12	P57053	H2BFS	10401,13	0,5886	Replaces conventional H2A in a subset of nucleosomes.
13	P0C0S5	H2AFZ	7646,1	Bel	Replaces conventional H2A in a subset of nucleosomes.
14	Q14181	POLA2	152,92	C	DNA replication.
15	Q9BZD3	GCOM1	224,06	C	Component of Pol II(G) complex.
16	P0CAP2	POLR2M	284,39	C	Component of Pol II(G) complex.
17	P18615	NELFE	266,18	C	Represses RNA polymerase II transcript elongation.
18	P51504	ZNF80	444,63	Bel	Transcriptional regulation.
19	P18124	RPL7	235,63	C	Translation aparatus regulation.
20	P47914	RPL29	622,4	0,92312	Translation apparatus regulation.
21	Q9BWG6	SCNM1	620,47	C	RNA splicing.
22	Q8WXA9	SREK1	148,46	Bel	Regulation of alternative splicing.
23	P19338	NCL	132,66	C	Pre-rRNA transcription and ribosome assembly.
24	P02788	LTF	275,73	C	Antimicrobial and anti-inflammatory activity.
25	P61626	LYZ	751,9	3,56085	Bacteriolysis.
26	P06702	S100A9	2362,67	Bel	Antimicrobial activity. Phagocyte migration promotion. Apoptosis.
27	P05109	S100A8	1869,7	Bel	Antimicrobial activity. Phagocyte migration promotion. Apoptosis.
28	P60709	ACTB	3806,68	1,82212	Cell motility.
29	P63261	ACTG1	1713,96	0,34301	Cell motility.
30	Q562R1	ACTBL2	664,65	Bel	Cell motility.
31	A6NHL2	TUBAL3	110,36	C	Microtubule element.
32	Q71U36	TUBA1A	452,48	C	Microtubule element.

(1)	(2)	(3)	(4)	(5)	(6)
33	P07437	TUBB	620,75	2,2034	Microtubule element.
34	Q9BQS8	FYCO1	61,54	C	May mediate microtubule plus end-directed vesicle transport.
35	Q13326	SGCG	315,83	C	Component of sarcoglycan complex.
36	Q9NY65	TUBA8	123,57	Bel	Microtubule element.
37	Q9BQE3	TUBA1C	54,26	Bel	Microtubule element.
38	O15144	ARPC2	666,79	Bel	Regulation of actin polimerization.
39	Q96A32	MYLPF	737,84	Bel	Myosin light chain.
40	Q6UY14	ADAMTSL4	247,27	C	Positive regulation of apoptosis.
41	P47929	LGALS7	392,7	Bel	Apoptosis regulation. Pro-apoptotic.
42	Q08378	GOLGA3	7,44	Bel	Golgi str. maitanace. Cleavage product necessary for apoptotic response.
43	P50897	PPT1	124,76	Bel	Lysosomal degradation. DNA fragmentation during apoptosis.
44	Q5M775	SPECC1	380,22	C	Proto-oncogene.
45	P25054	APC	30,36	Bel	Tumour supressor.
46	Q04760	GLO1	253,31	C	Involved in the regulation of TNF-induced transcriptional activity of NF-kappa-B.
47	Q5T200	ZC3H13	22,03	C	Downregulation of NF-kappaB pathway.
48	O95989	NUDT3	215,22	C	Signal transduction. Negatively regulates ERK1/2 pathway.
49	Q99665	IL12RB2	269,83	C	Signalling component coupling to the JAK2/STAT4 pathway. Promotes the proliferation of T-cells as well as NK cells.
50	Q8IV04	TBC1D10C	834,75	0,8781	Ras signalling pathway inhibition.
51	Q96NH3	C6orf170	1777,55	C	Controls ciliary morphology. Involved in Hedgehog signal transduction.
52	P06748	NPM1	612,64	1,85893	Regulates tumour supressors TP53/p53 and ARF. Chaperone.
53	Q9NNW7	TXNRD2	158,36	Bel	Implication in the defenses against oxidative stress.
54	P29762	CRABP1	133,35	Bel	Regulates access of retinoic acid to the nuclear retinoic acid receptors.
55	P17066	HSPA6	121,58	Bel	Chaperone.
56	P48741	HSPA7	78,01	Bel	Chaperone.
57	P11142	HSPA8	144,69	Bel	Chaperone. Repressor of transcriptional activation.
58	P55735	SEC13	122	C	May be involved in protein transport.
59	P62987	UBA52	755,27	0,77105	Proteosomal degradation, chromatin structure maintenance, gene expression regulation and stress response.
60	P0CG47	UBB	241,72	C	Proteosomal degradation, chromatin structure maintenance, gene expression regulation and stress response.
61	Q6ZMR5	TMPRSS11A	860,74	Bel	Pobable serine protease.
62	P00738	HP	1190,67	Bel	Makes haemoglobin accessible to degradative anzymes.
63	Q6S8J3	POTEE	346,61	C	Protein and ATP binding.
64	A5A3E0	POTEF	369,2	2,13828	Protein and ATP binding.
65	P0CG39	POTEJ	107,66	1,46228	Protein and ATP binding.
66	Q9BTF0	THUMPD2	238,14	C	RNA binding. Methyltransferase activity.

(1)	(2)	(3)	(4)	(5)	(6)
67	Q68CQ7	GLT8D1	206,26	C	Glycosyltransferase.
68	A6NIV6	LRRIQ4	145,7	Bel	Leucine-rich repeats and IQ motif containing.

***** **"C"** denotes that protein is only seen in control. "**Bel**" - only detected in treated cells.

3.2.5. Discussion

In this study we have demonstrated that belinostat, dependent on concentration (0.2-2 μM), is able to suppress NB4 and HL-60 cell line proliferation and induce apoptosis (Fig. 3.2.1.1A and Fig. 3.2.1.2C). Previous studies (Stapnes et al., 2007) have displayed a similar, concentration dependent, HDAC inhibitors (including belinostat) effect on primary AML cells, derived from patient blood. It is important to note that high concentrations of medication have resulted in proliferation arrest and pro-apoptotic effect, while low and moderate concentrations (e.g. 5-80 nM of belinostat) had an opposite activity, not restricting cell proliferation or even facilitating it. Our research has indicated that 0.2-0.5 μM concentrations of belinostat block cell cycle in the G0/G1 cycle phase, while higher belinostat concentrations (1-2 μM) block cell cycle in the S phase (Fig. 3.2.1.1C). Due to belinostat notably up-regulating histone acetylation (Fig. 3.2.3.1), chromosome condensation processes could be disturbed, which in turn can lead cells further towards an apoptotic death (Castedo et al., 2004). Our previous study (Savickiene et al., 2014b) has showed that cytotoxic concentrations of belinostat activates caspase-3 and induces PARP-1 cleavage, which is typical of endogenous (mitochondrial) apoptosis pathway.

We have also evaluated the ability of belinostat to enhance RA-induced APL cell granulocytic differentiation. We have demonstrated that belinostat combined together with RA increases RA-induced differentiation 1.5-1.8-fold (Fig. 3.2.2.1B). It is worth noting that combination Bel + RA was more potent in inducing APL cell granulocytic differentiation, compared to treatment with Bix + RA (Fig. 3.1.2.1A-B). Combination with HMTi BIX-01294 and RA increased RA-induced differentiation ~1.4-fold (Fig. 3.1.2.1A). Talking in general, it should be stated that belinostat induced granulocytic differentiation enhancement is found to be of similar magnitude as the rest of HDACis used for epigenetic leukaemia therapy. Our team has previously showed that application of HDACi phenylbutyrate, BML-210 or FK228 also strengthens RA-induced differentiation on very similar level (Savickiene et al., 2006a; Savickiene et al., 2006b; Savickiene et al., 2012).

Enhancement of histone acetylation is one of the main markers, describing HDACi application efficacy. As it is known, histone acetylation creates favorable conditions for

activating gene expression, while histone deacetylation, on the contrary, leads to inhibition of gene expression (Verdone et al., 2005). It has also been showed that APL cell differentiation into granulocytes is accompanied by the increase in acetylation of histone H4 (Nouzova et al., 2004). Considering the effect of belinostat on epigenetic modulators, we have demonstrated that after the application of belinostat on NB4 cells *HDAC1* and *HDAC2* gene expression is being considerably suppressed (Valiulienė et al., 2015). We have also noticed that belinostat significantly lowers HDAC1 protein level and notably enhances histone H4 hyperacetylation (Fig. 3.2.3.1). Our research has also showed that belinostat facilitates acetylation of H3K9 as well (Savickiene et al., 2014b). Studies by other scientific groups (Fraga et al., 2005; Wada et al., 2009) have revealed that the expression of HDAC proteins in AML cells, especially HDAC1, is being considerably enhanced, which results in histones being hypoacetylated. It has also been showed that during the granulocytic differentiation of leukaemia cells HDAC1 protein expression is being suppressed along with a restoration of acetylation levels, common for healthy phenotype. Results obtained in our study coincides with data published by other groups. Worth noting is that HDAC gene and protein expression data, obtained after the treatment with belinostat support the efficacy of used epigenetic therapy.

Summarising, our findings, regarding belinostat's effect on cell growth, differentiation, gene and protein expression, as well as on epigenetic modifications, confirmed potential value belinostat has in APL therapy. For that reason we have conducted an additional study, examining the effect of combined treatment with belinostat, HMT inhibitor 3-deazaneplanocine A and RA. For combined treatment 3-deazaneplanocine A was chosen because of this agent's activity *in vitro,* showing the inhibition of EZH2 (PRC2 complex catalytic sub-unit) protein expression (Girard et al., 2014). In addition, it was demonstrated that combined inhibition of EZH2 and HDAC has a synergistic anti-proliferative effect in human cancer cells (Takashina et al., 2016).

3.3. Belinostat and 3-deazaneplanocin A (DZNep) effect on human APL cells

3.3.1. Effect of belinostat and DZNep on APL cell growth and survival

The effects of belinostat and DZNep on cell growth inhibition and cell death were evaluated during 2 days of treatment (Valiulienė et al., 2017). Belinostat concentration was 0.2 μM, DZNep 0.5 μM alone or in combination with 1 μM RA. We also used a 4 h pre-treatment with belinostat and DZNep before the treatment with RA − 0.8 μM belinostat + 0.5 μM DZNep (4 h) → 1 μM RA – to evaluate whether it had the anti-proliferative and death inducing effect.

It was demonstrated that combined pre-treatment with belinostat and 3-deazaneplanocin A before the treatment with RA did not produce greater growth inhibitory effect compared with the treatment with RA alone (Fig. 3.3.1.1A). In addition, either of the last-mentioned treatments induced comparable amount of cell death (Fig. 3.3.1.1B). In contrast, the combined treatment of RA with belinostat and DZNep had the biggest influence on diminished growth in both NB4 and HL-60 cells (Fig. 3.3.1.1A). The same tendency was observed after evaluation of cell death (Fig. 3.3.1.1B). Cell death in both cell lines was the highest after the combined treatment of RA + Bel + DZNep. Our study also revealed that this combined treatment caused the highest increase in APL cell apoptosis (Valiulienė et al., 2017). Western blot analysis has demonstrated that after the treatments with belinostat, DZNep and combined application of RA + Bel + DZNep, PARP-1 protein is being cut into fragments (Valiulienė et al., 2017).

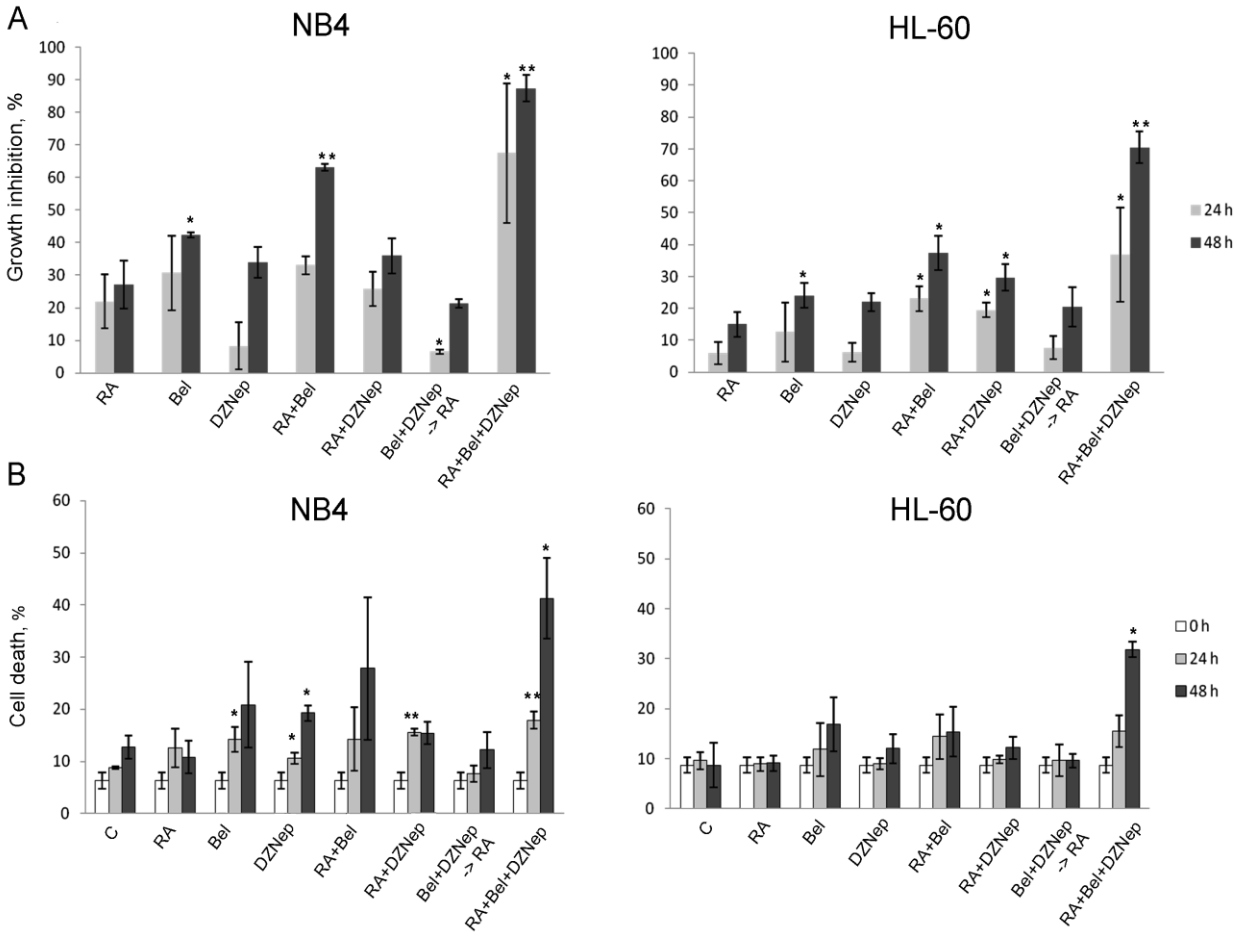

Fig. 3.3.1.1. Leukaemia cell growth inhibition and death. Data are averaged, ±SD (n = 3), $p \leq 0.05$ (∗), $p \leq 0.001$ (∗∗) (Valiulienė et al., 2017).

3.3.2. Effect of belinostat and DZNep on APL cell induced granulocytic differentiation

The differentiation analysis using NBT assay was performed and it demonstrated that the highest leukaemic cell differentiation was reached after the treatment with 0.8 μM Bel + 0.5 μM DZNep + 1 μM RA (Fig. 3.3.2.1A). This tendency was observed in both NB4 and HL-60 cell lines: after 72 h treatment approximately 86% and 83% of differentiated cells were detected, respectively. Similar results were observed after treatment with RA alone or in Bel + DZNep → RA treatment (Fig. 3.3.2.1A). The same tendency was confirmed by the flow cytometric assessment of CD11b cell surface marker (Fig. 3.3.2.1B). Treatment Bel + DZNep → RA increased RA-induced NB4 cells granulocytic differentiation nearly by 10% after 48 h treatment. We showed that the treatments with HDACi and HMTi alone did not induce leukaemia cell differentiation (Fig. 3.3.2.1A).

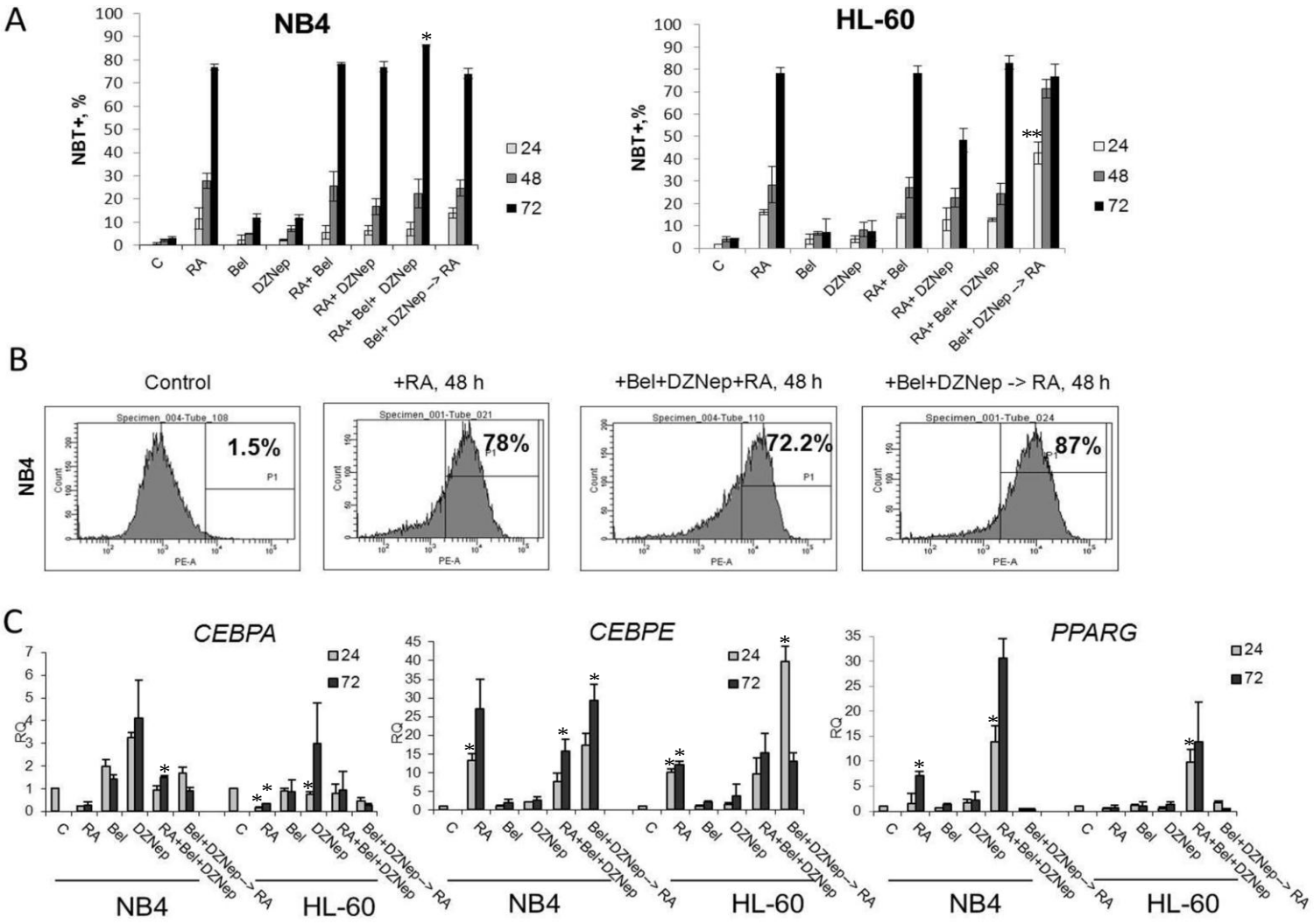

Fig. 3.3.2.1. Granulocytic cell differentiation and gene expression of differentiation associated genes (*CEBPA*, *CEBPE* and *PPARG*) in NB4 and HL-60 cells. Data are averaged, ±SD (n = 3), $p \leq 0.05$ (∗) (Valiulienė et al., 2017).

In addition, we analysed the effects of RA, belinostat, DZNep and the combined treatment on the gene expression of transcription factors PPARγ, C/EBPε, and C/EBPα (Fig. 3.3.2.1C).

It should be noticed that PPARγ and C/EBP transcription factors are crucial for granulocytic differentiation process. The effect of C/EBPα is very important in early differentiation stages and during the process of terminal granulocytic differentiation the expression of *CEBPA* decreases (Gery et al., 2004; Scott et al., 1992). Our study confirmed the tendency that the expression level of *CEBPA* is down-regulated along the process of leukaemic cell granulocytic differentiation. *CEBPA* expression level decreased the most after the treatment with 1 μM RA.

We determined that the expression level of another transcription factor, *CEBPE*, did increase along the process of cell differentiation. The *CEBPE* mRNA level was up-regulated after the treatment with RA alone and by the combinations: Bel + DZNep + RA and Bel + DZNep → RA. Treatment with Bel + DZNep → RA caused the highest augmentation in HL-60 cells (at 24 h to about 40-fold), whereas after 72 h combined treatment in NB4 cells *CEBPE* mRNA level was up-regulated 29-fold. Belinostat and DZNep alone did not cause a high increase in *CEBPE* mRNA level. In general, the enhanced *CEBPE* expression was observed when leukaemia cells were induced to differentiate by treating them with RA and RA combinations with HDACi and/or HMTi.

In addition, we evaluated the expression of *PPARG* gene. This gene is known to be associated with cell differentiation and apoptosis (Yasugi et al., 2006). In our study the expression of *PPARG* increased along the process of induced granulocytic differentiation after the treatment with RA as a single agent (in NB4 cells after 72 h treatment the expression was augmented app.7-fold) and after the 72 h treatment with Bel + DZNep → RA (augmentation was detected in both cell lines; in NB4 cells it was app. 31-fold, whereas in HL-60 cells it reached app. a half of this quantity).

3.3.3. Effect of belinostat and DZNep on APL cells' epigenetic modifiers' gene expression and protein level modulation

We determined that in NB4 and HL-60 cell lines 24 h combined treatment with RA + Bel + DZNep diminished *EZH2* gene expression more effectively compared to treatments with belinostat, DZNep or RA alone (Fig. 3.3.3.1A). Decrease in *SUZ12* gene expression (Fig. 3.3.3.1B) was observed in NB4 cells upon treatment with belinostat as a single agent and also after treatment Bel + DZNep → RA. No pronounced changes of *EED* gene expression were detected in HL-60 cells, whereas in NB4 cells the up-regulation of *EED* gene was evident upon 72 h treatment with RA and RA in combination with belinostat and DZNep (Valiulienė et al., 2017). Combined treatment with these agents also had the biggest effect on PRC2 protein level down-regulation (Fig. 3.3.3.1C). For instance, the EZH2 protein after 72 h combined treatment was completely undetectable, whereas, SUZ12 and EED (except in NB4 cells) proteins were barely detectable.

We have also evaluated the changes in the methylation of histone H3 on K27me3 (inhibition of EZH2) and K9me2 (Fig. 3.3.3.1C). After the analysis of histone methylation (H3K27me3 and H3K9me2) we did not observe any pronounced effect on the reduced H3 methylation, though the strong decrease in EZH2 protein quantity was detected.

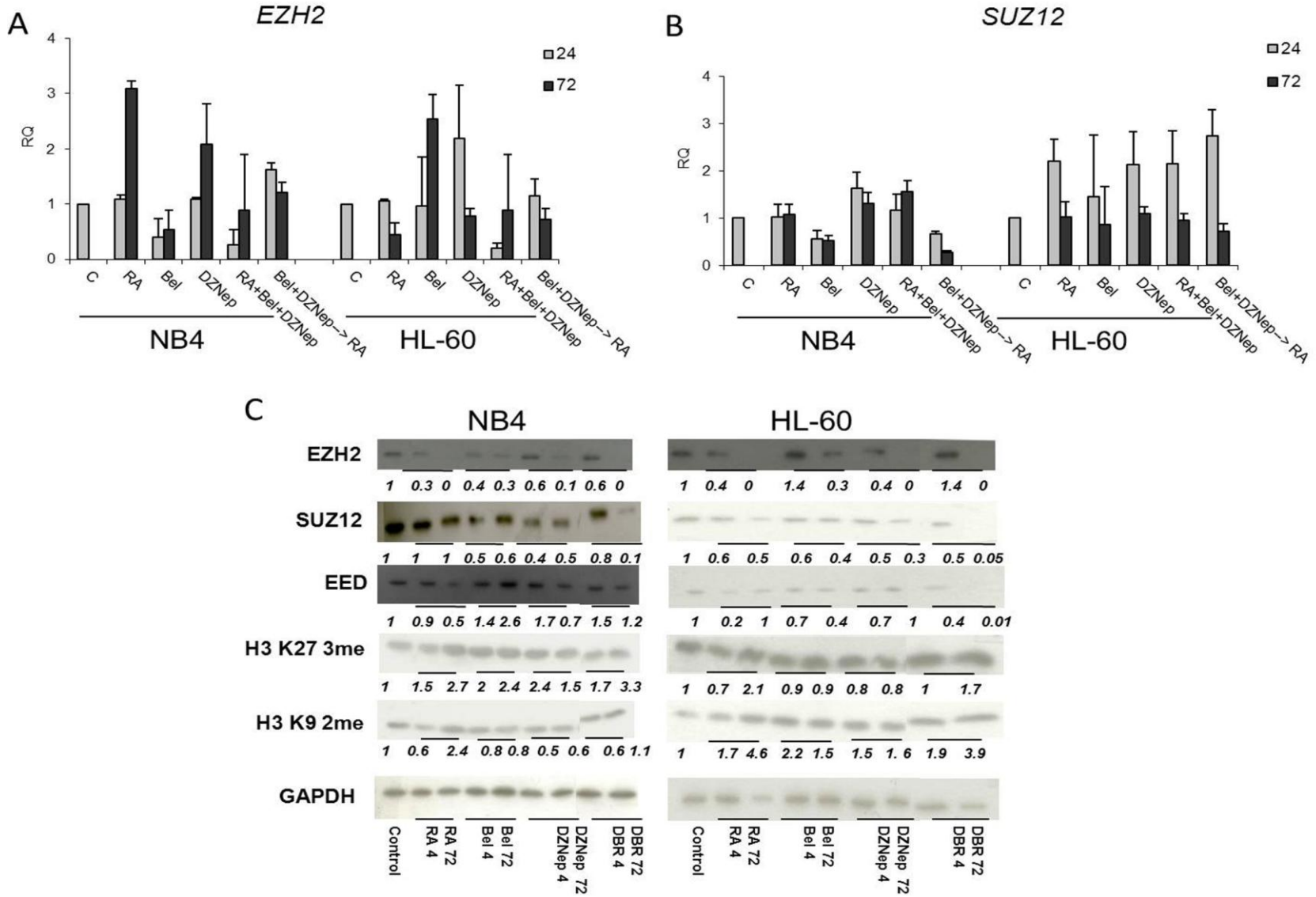

Fig. 3.3.3.1. Changes in gene and protein expression of the PRC2 complex components Data (A-B) are averaged, ±SD (n = 3) (Valiulienė et al., 2017).

In this study we determined a pronounced up-regulation of *HDAC1* gene expression in NB4 cell line after 24 h treatment with DZNep alone and in HL-60 cells after 72 h treatments with DZNep and with combination RA + Bel + DZNep (Fig. 3.3.3.2A). However, western blot analysis demonstrated the reduction of HDAC1 protein quantity in NB4 cells after 4 and 72 h treatments with RA, belinostat and DZNep alone and with the combined treatment (Fig. 3.3.3.2C). The highest decrease was detected after 72 h of treatment with the combination of belinostat, DZNep and RA (decreased app. 70%) and belinostat alone (decreased by app. 60%). The changes of HDAC1 protein quantity in HL-60 cell line had a slightly different pattern. We could observe that treatments with belinostat and DZNep alone caused the depletion of HDAC1 protein quantity after 4 and 7 h of treatment, while the treatment with RA or RA combined with epigenetic modifiers did not cause such an evident depletion (Fig. 3.3.3.2C). The analysis of *HDAC2* gene expression changes (Fig. 3.3.3.2B) showed that in NB4 and HL-60 cells the most obvious reduction in *HDAC2* gene expression was detected upon combined treatments with belinostat, DZNep and RA. The highest decrease in HDAC2 protein level was also detected upon combined treatments (Fig. 3.3.3.2C).

Our study also showed that histone H4 acetylation (Fig. 3.3.3.2C) increased in both cell lines mostly after the combined treatment with DZNep, belinostat and RA (14-22-fold). We could presume that the up-regulation of histone H4 acetylation in NB4 cells was reached mainly due to the activity of HDACi belinostat because there was no notable increase after the treatments with RA or DZNep. However in HL-60 cells upon 72 h treatment, RA as a single agent induced the highest augmentation in histone H4 acetylation, though increase was more rapid (see 4 h treatment) upon combination with DZNep, belinostat and RA.

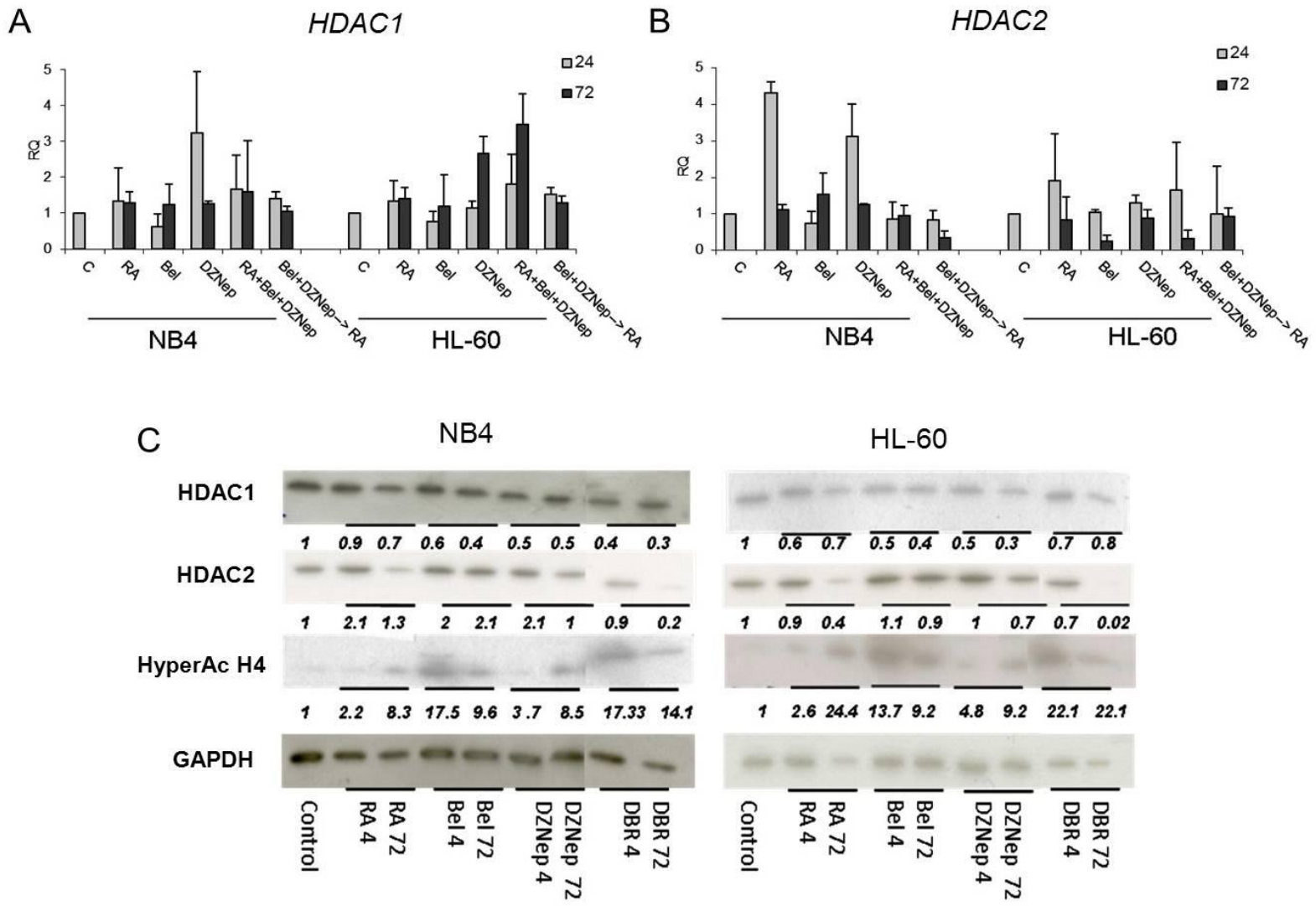

Fig. 3.3.3.2. Changes in HDAC1 and HDAC2 gene and protein expression and hyperacetylation of histone H4. Data are averaged, ±SD (n = 3) (Valiulienė et al., 2017).

3.3.4. Discussion

During this study we have showed that combination of RA, HDACi belinostat and HMTi 3-deazaneplanocine A is more effective at inhibiting NB4 and HL-60 cell proliferation than treatments with single RA, belinostat or DZNep (Fig. 3.3.1.1A). The most substantially APL cell apoptosis was induced when using combination of RA + Bel + DZNep (Valiulienė et al., 2017).

Treatment with combined RA + Bel + DZNep has suppressed HDAC1 and HDAC2 protein expression and facilitated acetylation of histone H4 (Fig. 3.3.3.2). Fiskus et al. (2009) have demonstrated that another pan-HDACi panabinostat (LBH589) decreased the number of PRC2 sub-units in AML cells and in turn suppressed modification H3K27me3. We have showed that belinostat diminishes the expression of EZH2, SUZ12 and EED proteins in NB4 and HL-60 cells (Fig. 3.3.3.1). However, the strongest effect was observed when belinostat acted in combination of DZNep + Bel + RA, which resulted in complete absence of EZH2 protein after 72 h. Although surprising, H3K27me3 quantity after combined treatment with DZNep + Bel + RA has not decreased.

On the ground of the data presented in this 3.3. chapter, we have also performed an *in vivo* pre-clinical study (Valiulienė et al., 2016), which confirmed the efficacy of combined epigenetic APL therapy we used. We will further discuss this investigation in more detail.

3.4. Belinostat and 3-deazaneplanocin A (DZNep) effect on APL xenograftic NOG mice

Xenograft models are suitable for *in vivo* study of leukaemia's pathogenesis and the pre-clinical development of anti-leukaemia agents. However, the notion of epigenetic regulatory mechanisms linking to adult cell functions in pathological conditions during different *in vivo* treatments remains elusive. In this study, for the first time we characterised epigenetic chromatin modifications in tissues and tumours from murine xenograft model generated using NB4 cells engrafted in immunodeficient NOG mice. Xenografts were subjected to combined epigenetic treatment by HDACi belinostat, HMTi 3-deazaneaplanocin A and all-*trans* retinoic acid based on *in vitro* model, where

such combination inhibited NB4 cell growth and enhanced retinoic acid-induced differentiation to granulocytes.

3.4.1. Characterisation of APL xenograft model

To study *in vivo* the picture of epigenetic changes in different tissues of malignant organism, murine xenograft model was developed and characterised by few parameters. The results of PB cell counts of untreated APL xenograft mice (APL) or treated with epigenetic drugs in combination DZNep, Bel and RA (APL-DBR) are presented in Table 3.4.1.1. The most prominent increase in the white blood cell (WBC) counts was evident for untreated APL xenograft as compared to control NOG mice. This can be associated with the hyperleucocytosis as was shown in some cases of APL (Testi et al., 2005). All engrafted mice showed increased counts of neutrophils (NEU) and decreased ones of eosinophils (EOS) as compared to control NOG mice. It should also be emphasized that epigenetic treatment prolonged APL mice survival (46 d ± 5 vs. 41 d ± 1 of untreated xenograft).

Leukaemia cell infiltration in NOG mice was also evaluated by the expression of certain genes and cell surface markers in PB of engrafted mice using RT-qPCR method. The results are presented in Table 3.4.1.1. as a relative quantity to APL xenograft (APL) control. It is widely accepted that *WT1* gene expression in the PB or bone marrow of healthy individuals is very low or even undetectable, but *WT1* mRNA is highly observable in the case of acute lymphoid or acute myeloid leukaemia and may serve as a prognostic marker (Lindstedt et al., 2014). In our study, *WT1* gene expression, as expected, was undetectable in PB of the wild type (N) and control NOG mice (C). *WT1* mRNA was found in untreated engrafted mice (APL) and upon epigenetic drug treatment (APL-DBR). In this case, mRNA levels of *PML-RARA* (the characteristic mark of APL) were undetectable. APL-DBR mice were characterised by high transcript levels (5.3-fold vs. APL) of colony-stimulating factor (*CSF3*) that is one of the regulators of neutrophil production during haematopoiesis. Considering the results of *PML-RARA* and *CSF3* gene expression after APL mice treatment with DZNep, Bel and RA, the possible therapeutic effect of these epigenetic agents could be proposed.

Flow cytometry analysis of cell surface markers CD33 and CD45 of myeloid origin and CD15 (shown for APL cells differentiation upon stimulation with RA) (Namikawa

et al., 1993; Majeti et al., 2009) demonstrated a significant increase in human CD33+ and CD45+ populations in PB upon xenotransplantation. In addition, our data correlated with the increase of WBC in PB of xenograft mice. Reduction in human CD33+ and CD45+ populations upon treatment with inhibitors of epigenetic modifiers were demonstrated (Table 3.4.1.1). Worth noting, increase in human CD15+ population in xenograft mice PB were also detected upon treatment.

In our xenograft model, tumour formation (in neck, bladder and abdominal areas), gut ulceration and the reduction of one of the kidneys were detected in control APL xenografts. Finally, anti-tumour efficacy was observed upon treatment with epigenetic drugs combination (APL-DBR), where tumours formation was prevented (only gut ulceration was observed).

Table 3.4.1.1. Disease response of APL xenografted mice to combined epigenetic treatment.

Variables		Treatment group			
		N	C	APL	APL - DBR
Survival data (days post exposure)				41 (±1)	46 (±5)
Tumour formation		-	-	Tumours in neck, bladder and abdominal areas; gut ulceration	Gut ulceration
Peripheral blood counts (relative to control)	WBC	0.7	1	4.1 (±0.3)	2.3 (±0.1)
	LYM	0.7	1	0.6 (±0.2)	0.8 (±0.1)
	MON	0.6	1	1.3 (±0.2)	1.1 (±0.2)
	NEU	1.2	1	1.1 (±0.1)	1.1 (±0.1)
	EOS	0.8	1	0.2 (±0.0)	0.8 (±0.2)
	HGB	0.8	1	0.7 (±0.0)	0.9 (±0.1)
	HCT	0.9	1	0.8 (±0.0)	0.9 (±0.1)
	RBC	0.8	1	0.7 (±0.1)	0.9 (±0.1)
	MCV	1.1	1	1 (±0.1)	1 (±0.0)
	PLT	0.4	1	1.3 (±0.3)	1.3 (±0.2)
Gene expression (relative to APL)	*WT1*	-	-	1	0.4 (±0.1)
	PML-RARA	-	-	1	-
	CSF3	-	-	1	5.3 (±0.6)
	G-CSFR	-	-	1	0.5 (±0.2)
Flow cytometric analysis, %	CD15	2.2	3.1	4 (±0.0)	8.3 (±5.4)
	CD33	1.6	1.2	12.2 (±4.6)	3.8 (±2.8)
	CD45	2.8	2.9	16.8 (±6.7)	5.1 (±2.9)

Peripheral blood counts are presented relatively to untreated control (NOG mice) on days 25-35 after APL xenografted mice (APL) treatment with DZNep, Bel and RA (APL-DBR). The number of animals per treatment group was three. Results are presented as means ±SD of samples from three animals (Valiulienė et al., 2016).

3.4.2. WT1 protein expression in APL xenograft mice tumours and tissues

In order to verify the applicability of WT1 as a marker for the disease detection and monitoring, we compared the expression of WT1 in developed tumours and tissues from untreated (APL) and treated with DZNep, Bel and RA (APL-DBR) xenograft mice. Untreated NB4 cells were used as a control. Results of western blots, illustrating WT1 protein expression and relative WT1 values (Fig. 3.4.2.1), indicated the over-expression of WT1 in untreated NB4 cells (C) and a significant (4.87-fold) decrease in NB4 cells treated with epigenetic agents (NB4-DBR) for 48 h. WT1 protein expression was detected in all tumours of APL xenograft mice with more pronounced levels in tumours near bladder and abdominal areas but to a lesser extent as compared to NB4 control. Similar level of WT1 expression was found in ulcerated guts and control NB4 cells with the apparent decrease after DBR treatment.

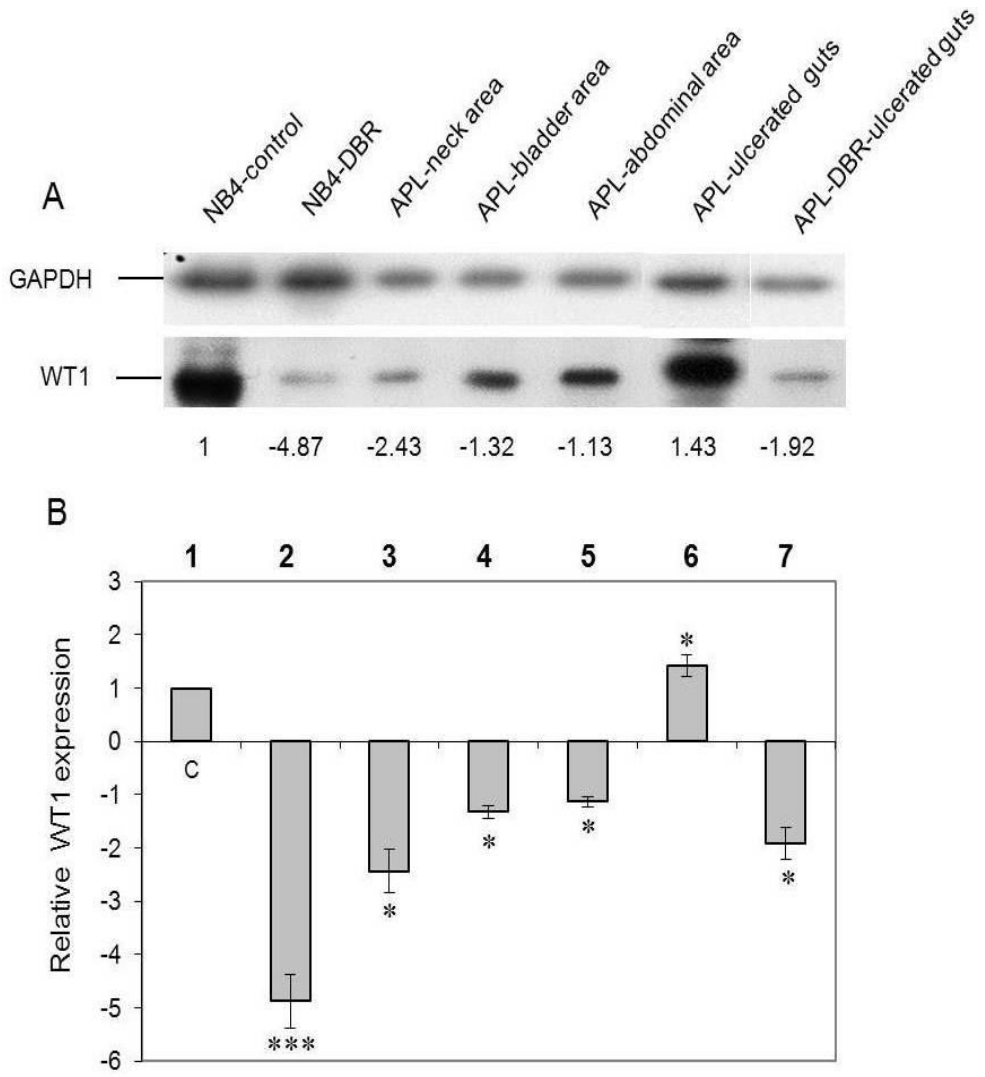

Fig. 3.4.2.1. WT1 protein expression in tumours of APL xenografs. Results are mean ±SD of samples from three animals, $p \leq 0.05$ (*) and $p \leq 0.0001$ (***). (Valiulienė et al., 2016).

Tissue-specific differences in WT1 protein abundance were also detected (Fig. 3.4.2.2). APL xenograft mice contained more WT1 protein in lungs and brain (2.02- and 1.87-fold vs. control (C-NOG), namely, in liver (4.70-fold vs. C) with some decrease after DBR treatment in brain (1.24-fold vs. C) and liver (3.74-fold vs. C). Reduced WT1 expression was detected in untreated APL mice spleen and kidneys (1.8- and 1.59-fold of C) and upon DBR treatment (1.02- and 1.36-fold of C). Since myeloid leukaemia and tumour cells show consistently augmented levels of WT1 protein, the presence of WT1 marker let to discriminate between normal and disease affected tissues. In our experiment, lungs, brain and liver represent more affected tissues in APL xenograft mice.

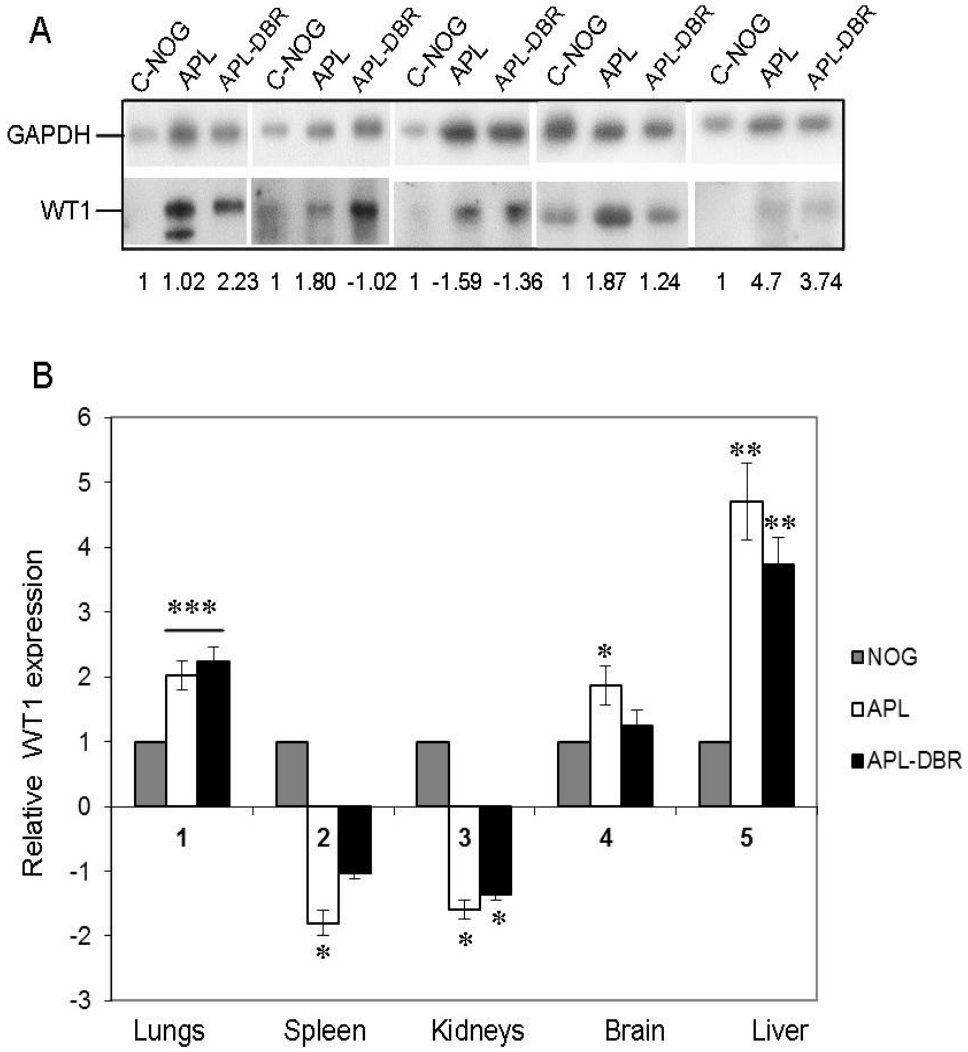

Fig. 3.4.2.2. WT1 protein expression in tissues of APL xenografs. Results are mean ±SD of samples from three animals, $p \leq 0.05$ (∗), $p \leq 0.001$ (∗∗) and $p \leq 0.0001$ (∗∗∗) (Valiulienė et al., 2016).

3.4.3. Histone modifications patterns in tissues of APL xenograft mice

The heterogeneous nature and function of different tissues may exhibit differential epigenetic responses in malignant organism. Next we determined the expression of chromatin epigenetic marks (euchromatic marks - acetylated histone H4 (H4Ac) and trimethylated histone H3 at K4 (H3K4me3), as well as heterochromatic marks - trimethylated histone H3 at K9 (H3K9me3) and trimethylated histone H3 at K27 (H3K27me3)) in untreated APL xenografts and upon treatment with DBR combination.

Epigenetic changes in xenograft mice lungs, spleen and kidneys were not significant (Valiulienė et al., 2016). The most profound differences in changes of epigenetic marks expression were observed in brain and liver (Fig. 3.4.3.1). APL xenograft mice brain and liver exhibited lower levels of H4ac (1.92-fold and 1.45-fold of control, respectively) with the higher level in DBR treated mice. Upon DBR treatment, H3K4me3, H3K9me3 and H3K27me3 expression levels were higher than in control NOG mice and untreated xenograft with the significant elevation of H3K9me3 (up to 3-fold vs. control) in both tissues and H3K27me3 (2.99-fold vs. control) in liver.

Collectively, our observations indicate that APL xenograft mice somatic tissues are subjected to specific histone modification changes dependently on their malignancy and functional activity during the epigenetic treatment.

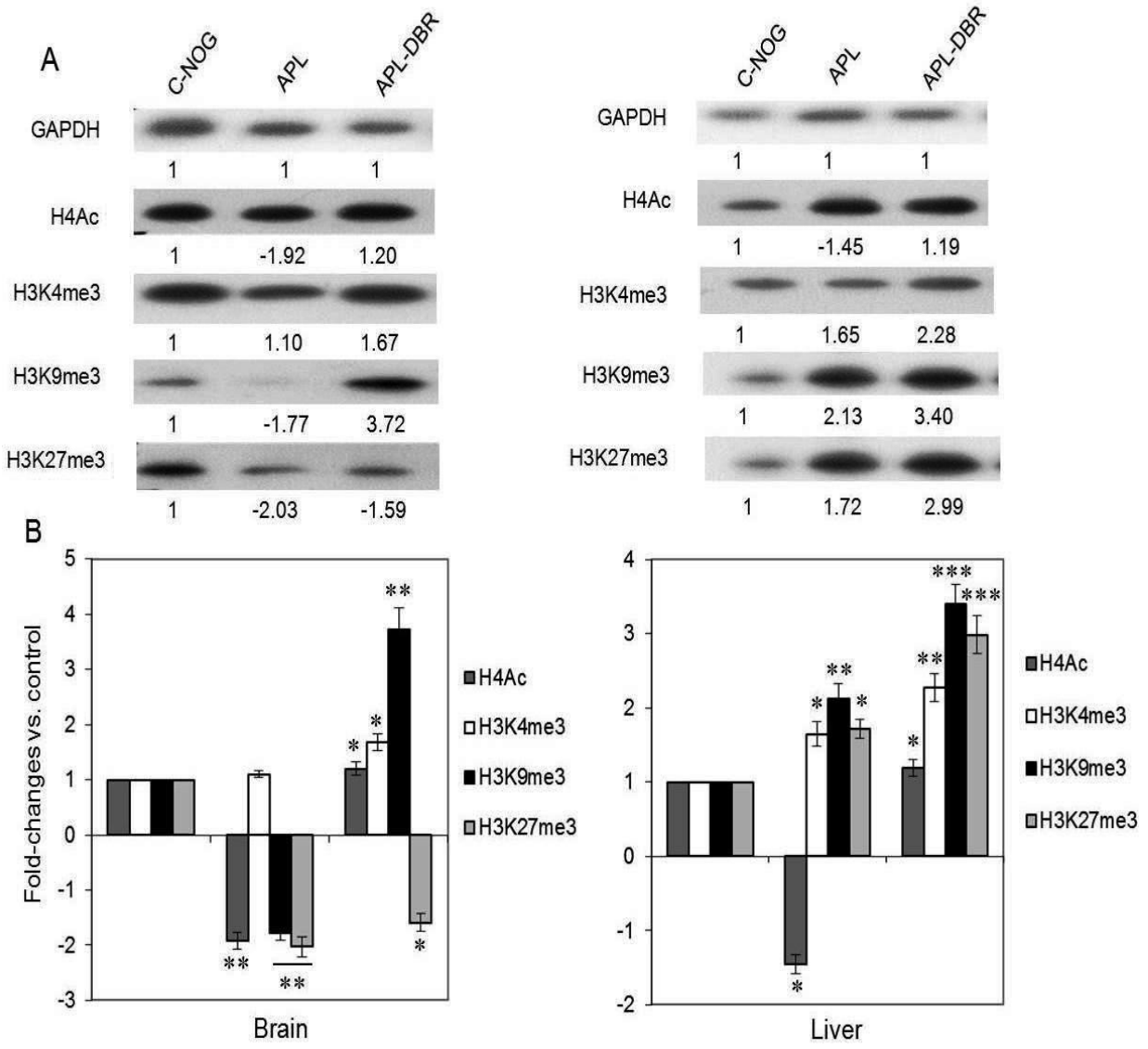

Fig. 3.4.3.1. Histone modifications in brain and liver of APL xenografs in response to epigenetic treatment. Results are mean ±SD of samples from three animals, $p \leq 0.05$ (*), $p \leq 0.001$ (**) and $p \leq 0.0001$ (***) (Valiulienė et al., 2016).

3.4.4. Discussion

In our model, NB4 cells *in vivo* have formed tumours in neck, bladder and abdominal areas of xenograftic mice. Previous studies by other research teams have showed that AML cells (HL-60, KG1, KG1a), injected into *BALB/c nude* new born mice, had formed disseminated tumours like myelosarcomas, whereas cancer cells in haematopoietic tissue had not been found (Machado et al., 1984). Worth noting is that inoculated leukaemia cells have retained an ability to proliferate. However, in AML engrafted BALB/c nude mice, the total and percent numbers of PB cells was found unchanged compared with normal mice (Machado et al., 1984). In our model, the

monitoring of PB counts after NB4 injection in immunodeficient NOG mice showed significant increase in human CD33+, CD45+ populations correlated with the increase of WBC (Table 3.4.1.1). These results are in accordance to the data (Testi et al., 2005), showing the incidence of hyperleucocytosis in some cases of APL. We demonstrated that APL xenograftic mice treatment with epigenetically active compounds (DBR) had decreased WBC, CD33+ and CD45+ cell count and increased CD15+ cell quantity in APL xenograftic mice peripheral blood. An upsurge of *CSF3* gene expression has also been detected (Table 3.4.1.1). Moreover, epigenetic therapy has prolonged APL xenograftic mice life expectancy and protected them from tumour formation.

It has been demonstrated that tumour suppressor *WT1* gene expression is being increased in AML suffering human blood and standard AML cell lines (Wang et al., 2015; Frairia et al., 2017). We have expanded our study to evaluate *WT1* gene and protein expression. We have tried to ascertain if WT1 expression could be used to evaluate therapeutic efficacy of APL. Results acquired have showed that WT1 protein expression is lower in tumours of APL xenograftic mice than in control NB4 cells (Fig. 3.4.2.1). *WT1* mRNA quantity in APL xenograftic mice peripheral blood has been increased, compared to control mice. Worth noticing is that treatment with epigenetically active compounds have decreased *WT1* gene expression 2.5-fold (Table 3.4.1.1). The engraftment of NB4 cells, bearing over-expressed WT1, in NOG mice caused the accumulation of WT1 in lungs, brain and liver (but not in kidneys and spleen) with some decrease upon epigenetic-based treatment (Fig. 3.4.2.2).

We have also evaluated *PML-RARA* (APL pathogenesis related marker) expression in APL xenograftic mice blood. *PML-RARA* mRNA has been found in APL xenograftic mice blood, but was lacking in control NOG mice and APL xenograftic mice, treated with epigenetically active compounds (Table 3.4.1.1). These results coincide with results, published by other research groups, showing that treatment with single RA can also significantly decrease *PML-RARA* gene expression in APL xenograftic mice (Patel et al., 2012).

We conclude that changes in histone modifications may represent the status of cells or tissues of malignant and healthy organism. As it is known (Bojang and Ramos, 2014), the promoters of tumour suppressor genes in normal cells are enriched with active transcription marks, H4ac and H3K4me3, while satellite regions are enriched with

repressive H3K9me3 and H3K27me3 marks. Tumour cells undergo epigenetic reorganisation with the loss of repressive marks at satellite regions, overall loss of acetylation and the occurrence of repressive marks, H3K9me3 and H3K27me3, on the promoters of tumour suppressor genes. From the earlier study (Grigoryev et al., 2006) it was apparent that the prevailing epigenetic modification in the wild type adult mice somatic tissues is H3K9me3. Also, histone deacetylation mediates gene repression and compaction into heterochromatin characteristic for somatic cells.

In this study, we analysed histone modifications in APL xenograft tumours and tissues in response to epigenetic agents-based treatment. The comparative data demonstrated higher levels of H3K4me3 and H3K9me3 and lower of H4Ac and H3K27me3 in APL xenograft tumours than in leukaemic NB4 cells. As compared to control NOG mice, the level both active and repressive histone marks decreased in lungs, spleen (except H3K4me3) and kidneys (except H3K9me3) and continued to decrease after APL xenograft treatment by DBR combination. The most apparent changes were found in lungs with increased tumourigenic WT1 mark. APL xenograft brain exhibited lower levels of all modifications compared with control. However, after epigenetic APL xenograft treatment both active marks and especially repressive H3K9me3 mark were elevated. Interestingly, the highest increase in the level of all epigenetic marks (except H4Ac) was detected in liver of APL xenograft. Noticeably, APL xenograft liver retained high levels of WT1 with some decrease upon DBR treatment in parallel with the increase of all histone modifications tested. This may be associated with the functional liver activity in response to APL cell infiltration and drug treatment.

Findings presented here demonstrate, for the first time that APL xenograft mice tumours and tissues display complex changes of histone modifications showing the modulation of the chromatin epigenetic environment in adult tissues of malignant organism.

4. CONCLUSIONS

1. Treatment with HMT inhibitors BIX-01294 and 3-deazaneplanocine A as well as HDAC inhibitor belinostat suppresses the growth and viability of APL cells, whereas combined application of BIX-01294, belinostat and 3-deazaneplanocine A enhances RA-induced granulocytic differentiation.

2. Both the application of single epigenetic modifier inhibitors (BIX-01294, 3-deazaneplanocine A and belinostat) and their combined treatments with RA reduce the expression of APL cell genes and their coded proteins, related to chromatin rearrangement (*SUZ12*, *EED*, *EZH2*, *HDAC1*, *HDAC2*), while facilitating the expression of genes and their coded proteins, related to cell cycle regulation (*CDKN1B*) and differentiation (*CEBPE*, *PPARG*).

3. After treatment with single compounds (BIX-01294, 3-deazaneplanocine A and belinostat) the number of transcription activating histone modifications (histone H4Ac) increases while suppressive modifications (H3K9me2, H3K9me3 and H3K27me3) decreases. These effects are enlarged by applying combined treatments.

4. In untreated APL cells hyperacetylated histone H4 is found in protein complexes with proteins involved in gene transcription and translation processes, whereas after treatment with belinostat hyperacetylated histone H4 is found in association with proteins, necessary for apoptotic response, defence against oxidative stress and retinoic acid signalling pathways.

5. Epigenetic therapy protects APL xenograftic NOG mice from tumour formation and prolongs their lifespan. Treatment with epigenetically active compounds has an influence on epigenetic changes of APL xenograftic mice tissues.

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1. Clinical trials data base: www.clinicaltrials.gov. Last time used 20th of September, 2017.

Websites:

1. National Cancer Institute. *www.cancer.gov/types/leukemia*. Last revised 2017.

6. PUBLICATIONS

Related to the topic of doctoral dissertation

1. **Valiulienė G**, Stirblytė I, Jasnauskaitė M, Borutinskaitė V, Navakauskienė R. Anti-leukemic effects of HDACi Belinostat and HMTi 3-Deazaneplanocin A on human acute promyelocytic leukemia cells. Eur J Pharmacol. 2017; 799:143-53.

2. **Valiulienė G**, Treigytė G, Savickienė J, Matuzevičius D, Alksnė M, Jarašienė-Burinskaja R, Bukelskienė V, Navakauskas D, Navakauskienė R. Histone modifications patterns in tissues and tumours from acute promyelocytic leukemia xenograft model in response to combined epigenetic therapy. Biomed Pharmacother. 2016; 79:62-70.

3. **Valiuliene G**, Stirblyte I, Cicenaite D, Kaupinis A, Valius M, Navakauskiene R. Belinostat, a potent HDACi, exerts antileukaemic effect in human acute promyelocytic leukaemia cells via chromatin remodelling. J Cell Mol Med. 2015; 19(7):1742-55.

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