European Journal of Pharmacology 838 (2018) 32–40

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Full length article

Epigallocatechin-3-gallate and BIX-01294 have different impact on epigenetics and senescence modulation in acute and chronic myeloid leukemia cells

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ARTICLE INFO

Keywords:
Myeloid leukemia
EGCG
BIX-01294
Senescence
Epigenetic regulation

ABSTRACT

Myeloid leukemia treatment is quite successful nowadays; nevertheless the development of new therapies is still necessary. In the present study, we investigated the potential of epigenetic modulators EGCG (epigallocatechin-3-gallate) and BIX-01294 (N-(1-benzylpiperidin-4-yl)-6,7-dimethoxy-2-(4-methyl-1,4-diazepan-1-yl)quinazolin-4-amine) to alter epigenetic state and cause cellular senescence in acute and chronic myeloid leukemia NB4 and K562 cells. We have shown that after leukemia cell treatment with EGCG and BIX-01294 the proliferation and survival were inhibited of both cell lines; however, only NB4 cells underwent apoptosis. Both epigenetic modulators caused cell cycle arrest in G0/G1 phase as assessed by RT-qPCR (*p53*, *p21*, *Rb*) and flow cytometry analysis. Increased levels of *ATM*, *HMGA2*, phosphorylated ATM, and SA-β-galactosidase staining indicated that EGCG caused cellular senescence, whereas BIX-01294 did not. Immunoblot analysis of epigenetic players DNMT1, HP1α, H3K9me3, EZH2, and SUZ12 demonstrated beneficial epigenetic modulation by both agents with exception of mainly no epigenetic changes caused in K562 cells by EGCG. Therefore, we suggest EGCG as a promising epigenetic modulator for acute promyelocytic leukemia therapy and as a potential cellular senescence inducer in both acute and chronic myeloid leukemia treatment, whereas BIX-01294 could be beneficial as an epigenetic modifier for both myeloid leukemias treatment.

1. Introduction

Myeloid leukemia is a heterogenic disease classified into acute and chronic according to how quickly it progresses. Acute promyelocytic leukemia (APL) is a type of acute myeloid leukemia (AML) commonly characterized by chromosomal translocation t(15;17)(q22;q21), which generates the fusion protein PML-RARα. All-*trans*-retinoic acid (ATRA) and arsenic trioxide (ATO) were demonstrated to target PML-RARα and, therefore, are successfully used for APL treatment (Iland et al., 2014; Kakizuka et al., 1991; Zhang et al., 2010). However, there still are a small proportion of APL patients who relapse or show resistance to ATRA and/or ATO treatment and this is considered as a critical problem (Lou et al., 2015). Meanwhile, chronic myeloid leukemia (CML) is typically characterized by a reciprocal translocation t(9;22)(q34;q11) called Philadelphia chromosome, which results in a constitutively active BCR-ABL tyrosine kinase (Sawyers, 1999). Selective tyrosine kinase inhibitors (TKIs) such as imatinib, nilotinib, and dasatinib significantly reduced CML patient mortality rate and turned CML into highly manageable chronic disease. However, a number of patients demonstrate or develop resistance to TKIs or they undergo continued therapy for life (Baccarani et al., 2013).

Considering the mentioned pitfalls of APL and CML treatment, development of novel therapies is necessary. Since genetic abnormalities collaborate with epigenetic changes, scientists suggest that epigenetic therapy, often combined with other therapies, might be an important and powerful cancer treatment approach (Ahuja et al., 2016). Besides, when exposed to treatment, cancer (or normal) cells can choose their subsequent fate depending on type or level of cell damage: programmed cell death (apoptosis) or therapy-induced cellular senescence (TIS). TIS is described as a permanent arrest of cell proliferation. It has been suggested as a tumor suppression mechanism, thus as an option to enhance cancer therapy (Provinciali et al., 2013).

EGCG (epigallocatechin-3-gallate) is the most abundant and the most biologically active catechin in green tea (*Camellia sinensis*). Among many of its known activities (such as anti-oxidant, anti-bacterial, anti-inflammatory, etc.), EGCG has also been shown to be a potential anti-

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https://doi.org/10.1016/j.ejphar.2018.09.005
Received 12 June 2018; Received in revised form 3 September 2018; Accepted 4 September 2018
Available online 05 September 2018

cancer agent, which is involved in numerous biological mechanisms related with cancer development and progression (Granja et al., 2016). As an epigenetic modifier, this polyphenol features DNMT and HDAC inhibitory properties (Khan et al., 2015). BIX-01294 (1-benzylpiperidin-4-yl)-6,7-dimethoxy-2-(4-methyl-1,4-diazepan-1-yl)quinazolin-4-amine) is a synthetic inhibitor specific for EHMT2/G9a histone methyltransferase, which catalyzes the dimethylation of H3K9. Abnormally elevated levels of this repressive histone lysine methylation have been observed in many types of human cancers. EHMT2/G9a plays an important role in gene silencing, and therefore, it is an attractive target for cancer therapy (Huang et al., 2010; Kubicek et al., 2007). In this study, we used the NB4 cell line, which possesses the characteristic APL chromosomal translocation t(15;17) (Lanotte et al., 1991), and CML cell line K562, which has Philadelphia chromosome (Lozzio and Lozzio, 1975). We examined the potential of EGCG and BIX-01294 to cause epigenetic changes and cellular senescence in both APL and CML cells, with a view to clarify their presumptive therapeutic potential.

2. Materials and methods

2.1. Cell cultures, cell proliferation, apoptosis, and cell cycle assays

Authenticated NB4 cell line was purchased from DSMZ (Braunschweig, Germany). Authenticated K562 cell line was purchased from ATCC (Manassas, VA, USA). NB4 and K562 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco, Carlsbad, CA, USA) at 37 °C in a humidified 5% CO_2 atmosphere. Cells were seeded at density 0.5×10^6 cells/ml. Cell proliferation and survival were evaluated by trypan blue exclusion test using a hemocytometer. For detection of early apoptotic, necrotic and viable cells we used the staining assay with Annexin V-FITC and Propidium Iodide "ApoFlowEx® FITC Kit" (Exbio, Vestec, Czech Republic) according to manufacturer's instructions and analyzed cells on the BD FACS Canto II flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Cell cycle analysis was performed using standard propidium iodide staining procedure (Savickiene et al., 2014b).

2.2. Reverse transcription quantitative PCR (RT-qPCR)

Total RNA was purified using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), cDNA was synthesized using Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Fisher Scientific, Waltham, MA, United States) and qPCR was performed using Maxima SYBR Green/ROX qPCR Master Mix (2×) (Thermo Fisher Scientific) on the Rotor-Gene 6000 system (Corbett Life Science, QIAGEN, Hilden, Germany). Primers sequences (Metabion international AG, Planegg/Steinkirchen, Germany) are outlined in Table 1. mRNA levels were normalized to GAPDH expression. Relative gene expression was calculated using ΔΔCt method (Schmittgen and Livak, 2008).

2.3. Methylation-specific PCR (MSP)

For genomic DNA purification, cells were washed twice with PBS, lysed in TNES buffer with 0.5 µg/µl Proteinase K (Thermo Fisher Scientific) overnight at 55 °C. After incubation, saturated NaCl solution was added up to 1.25 M and mixed vigorously. After centrifugation at 14,000 × *g* for 5 min, pellets were discarded. DNA was precipitated with 1 vol of ice-cold 95% ethanol and then dissolved in H_2O. Purified genomic DNA bisulfite conversion was performed using the EZ DNA Methylation-Direct™ (Zymo Research, Irvine, CA, USA). For positive controls Human Methylated & Non-methylated DNA Set (Zymo Research) were used. Methylation-specific PCR was performed using MeltDoctor HRM master Mix (Thermo Fisher Scientific) on the Rotor-Gene 6000 system (Corbett Life Science, QIAGEN). Primer sequences (Metabion international AG) are outlined in Table 1. PCR products were fractioned in agarose gel electrophoresis and detected on ChemiDoc™ XRS + System (BIO-RAD, Hercules, California).

Table 1
Primers used for RT-qPCR and MSP analysis.

Gene	Primers used for RT-qPCR analysis
ATM	F: CTCTGAGTGGCAGCTGGAAGA
	R: TTTAGGCTGGGATTGTTCGCT
CBFB	F: CAGGGAGAACAGCGACAAAC
	R: TCAGAATCATGGGAGCCTTC
CCNA2	F: AACTTCAGCTTGTGGGCACT
	R: AAACTCTGCTACTTCTGGGGG
GAPDH	F: AGTCCCTGCCACACTCAG
	R: TACTTTATTGATGGTACATGACAAGG
HMGA2	F: CCCAAAGGCAGCAAAAACAA
	R: GCCTCTTGGCCGTTTTTCTC
Pu.1	F: ACGGATCTATACCAACGCCA
	R: GGGGTGGAAGTCCCAGTAAT
p21	F: GGCAGACCAGCATGACAGATT
	R: GCGGATTAGGGCTTCCTCT
p53	F: TAACAGTTCCTGCATGGGCGGC
	R: AGGACAGGCACAAACACGCACC
Rb	F: GCAGTATGCTTCCACCAGGC;
	R: AAGGGCTTCGAGGAATGTGAG

Gene	Primers used for MSP analysis
ATM	Met F: GGAGTTCGAGTCGAAGGGC
	Met R: CTACCTACTCCCGCTTCCGA
	Unmet F: GTTTTGGAGTTTGAGTTGAAGGGT
	Unmet R: AACTACCTACTCCCACTTCCAA
p21	Met F: TACGCGAGGTTTCGGGATC
	Met R: CCCTAATATACAACCGCCCCG
	Unmet F: GGATTGGTTGGTTTGTTGGAATTT
	Unmet R: ACAACCCTAATATACAACCACCCCA
p53	Met F: ATTTACGGTATTAGGTCGGC
	Met R: ACACGCTCCCAACCCGAACG
	Unmet F: TTTAAAATGTTAGTATTTATGGTATTAGGTTGGT
	Unmet R: CATCATAAAAAACACACTCCCAACCCAAACA

2.4. Immunoblotting

Cell lysates were prepared as described previously (Savickiene et al., 2014b) and were fractionated in SDS-PAGE 7.5–15% gradient electrophoresis gel. After protein transfer on PVDF membrane, specific protein detection was performed using antibodies against DNMT1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (dilution ratio 1:500), EZH2 (Cell Signalling Technology, Danvers, MA, USA) (dilution ratio 1:1000), GAPDH (Abcam, Cambridge, UK) (dilution ratio 1:10000), Hp1α (Millipore, Billerica, MA, USA) (dilution ratio 1:1000), H2AX (Abcam) (dilution ratio 1:5000), H3K9me3 (Millipore) (dilution ratio 1:1000), H4K20me1 (Active Motif, Carlsbad, CA, USA) (dilution ratio 1:5000), H4K20me2 (Active Motif) (dilution ratio 1:1000), H4K20me3 (Active Motif) (dilution ratio 1:1000), LC3B (Abcam) (dilution ratio 1:3000), Phospho-ATM (Ser1981) (Abcam) (dilution ratio 1:15000), Phospho-H2AX (Ser139) (Millipore) (dilution ratio 1:5000), SUZ12 (Cell Signalling Technology) (dilution ratio 1:1000). GAPDH was used as a loading control. "SuperSignal West Pico Chemiluminescent Substrate" (Thermo Fisher Scientific) was used for chemiluminescent detection. Signal detection was carried out on ChemiDoc™ XRS + System (BIO-RAD, Hercules, California). Quantitative evaluation was performed using ImageJ software.

2.5. Senescence-associated β-galactosidase assay

Elevated senescence-associated β-galactosidase activity in senescent cells was assessed using "Senescence Cells Histochemical Staining Kit" (Sigma-Aldrich, St. Louis, MO, USA) according to manufacturer's protocol modified for suspension cells. Stained cells were incubated overnight and counted under light microscope. At least 500 cells were

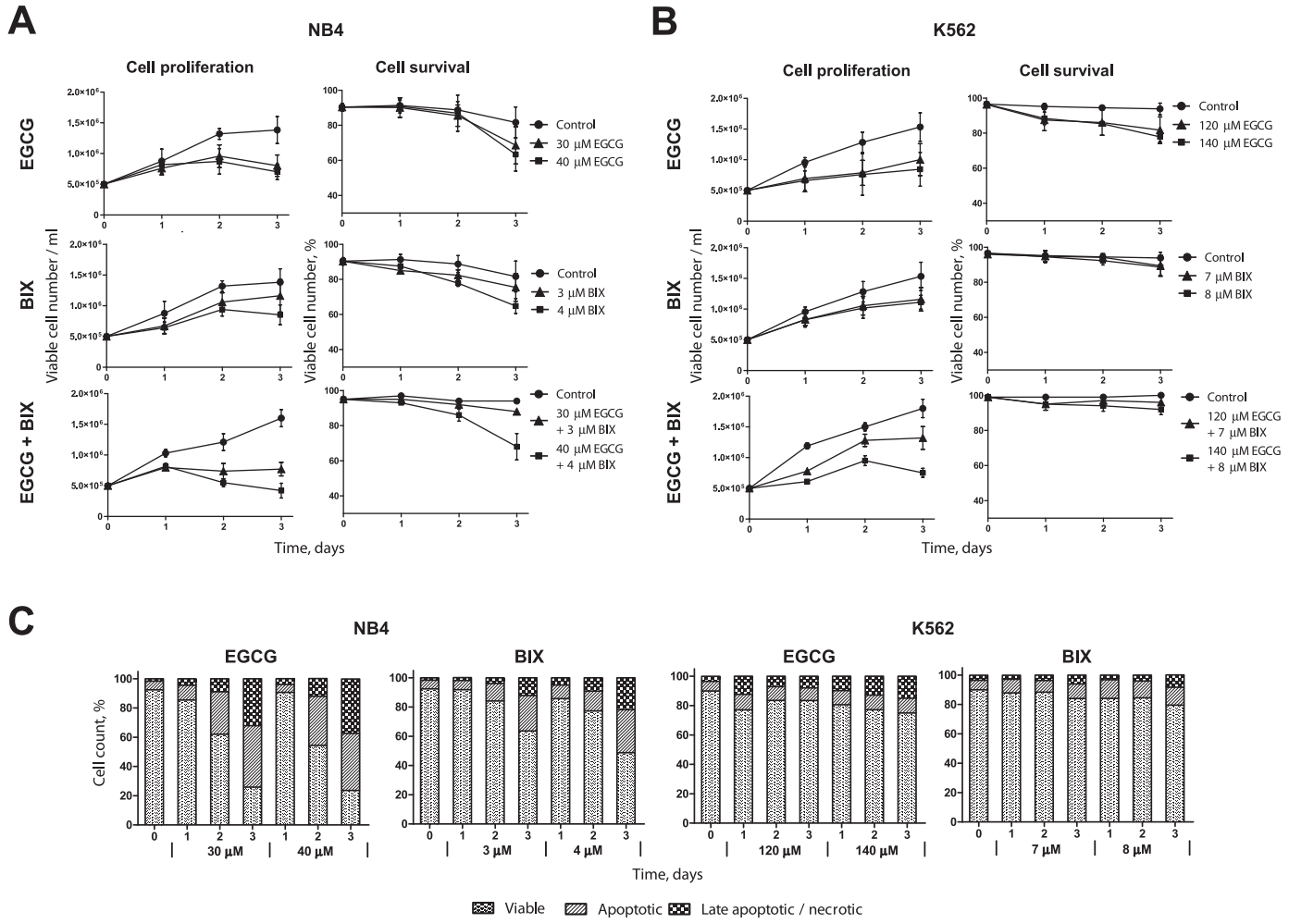

Fig. 1. EGCG and BIX-01294 effect on myeloid leukemia cell proliferation and death. NB4 and K562 cells were treated with different concentrations of EGCG and BIX-01294 for 3 days. Cell proliferation, survival, and apoptosis were analyzed. (**A**), (**B**) NB4 and K562 cell proliferation and survival were evaluated by trypan blue exclusion test. Results are mean ± S.D. (n = 3). (**C**) NB4 and K562 cell apoptosis analyzed after treatment with EGCG and BIX-01294 by staining with Annexin V and Propidium Iodide.

counted.

2.6. Statistical analysis

Data are expressed as mean ± standard deviation (S.D.). Two-tailed Student's *t*-test was used to determine the significance of difference between groups of treated and untreated samples; significance was set at $P \leq 0.05$ (*).

3. Results

3.1. EGCG and BIX-01294 effect on cell proliferation and death

NB4 and K562 cells were treated with EGCG and BIX-01294. Cell proliferation and survival were evaluated by trypan blue exclusion test every day for 3 days. After NB4 cell treatment with 30 μM and 40 μM EGCG or 3 μM and 4 μM BIX-01294, dose and time dependent inhibition of cell proliferation and survival was observed (Fig. 1A). Same doses of EGCG and BIX-01294 did not show any effect on K562 cell proliferation and survival (data not shown), thus, higher doses of these chemical agents were tested. 120 μM and 140 μM EGCG or 7 μM and 8 μM BIX-01294 had similar effect on K562 cell proliferation as lower doses of these agents had on NB4 cells (Fig. 1B). Therefore, proliferation is impaired by both EGCG and BIX-01294 in both cell lines, however, NB4 cells showed higher sensitivity. K562 cell treatment with BIX-01294 had only slight effect on cell survival. NB4 and K562 cell treatment with the combination of EGCG and BIX-01294 was not too toxic and showed slightly higher effect than treatment with agents alone (Fig. 1A, B).

Furthermore, EGCG and BIX-01294 ability to induce apoptosis was assessed. Cells were treated with EGCG and BIX-01294 for 3 days and stained with Annexin V and Propidium Iodide every 24 h. Flow cytometry results revealed increasing numbers of apoptotic NB4 cells over time (Fig. 1C). Meanwhile, K562 cells demonstrated resistance to apoptosis induction (Fig. 1C). Since K562 cells demonstrated resistance to induction of apoptosis, we further examined whether tested leukemia cell lines undergo autophagy after treatment with EGCG or BIX-01294. We examined expression changes in autophagy-related genes *BCNA1* and *ATG5* by RT-qPCR and LC3 II protein level by immunoblot, however obtained results were not consistent and did not show clear autophagy induction neither by EGCG nor by BIX-01294 (data not shown). To sum up, EGCG and BIX-01294 inhibit cell proliferation in both myeloid leukemia cell lines with a higher effect on NB4 cells. Apoptotic death occurred in NB4 cells after treatment with tested chemical agents, while K562 demonstrated apoptosis resistance. Thus, inhibited K562 cell survival might be caused by necrosis, while inhibited proliferation with low effect on survival might be caused by cell cycle arrest.

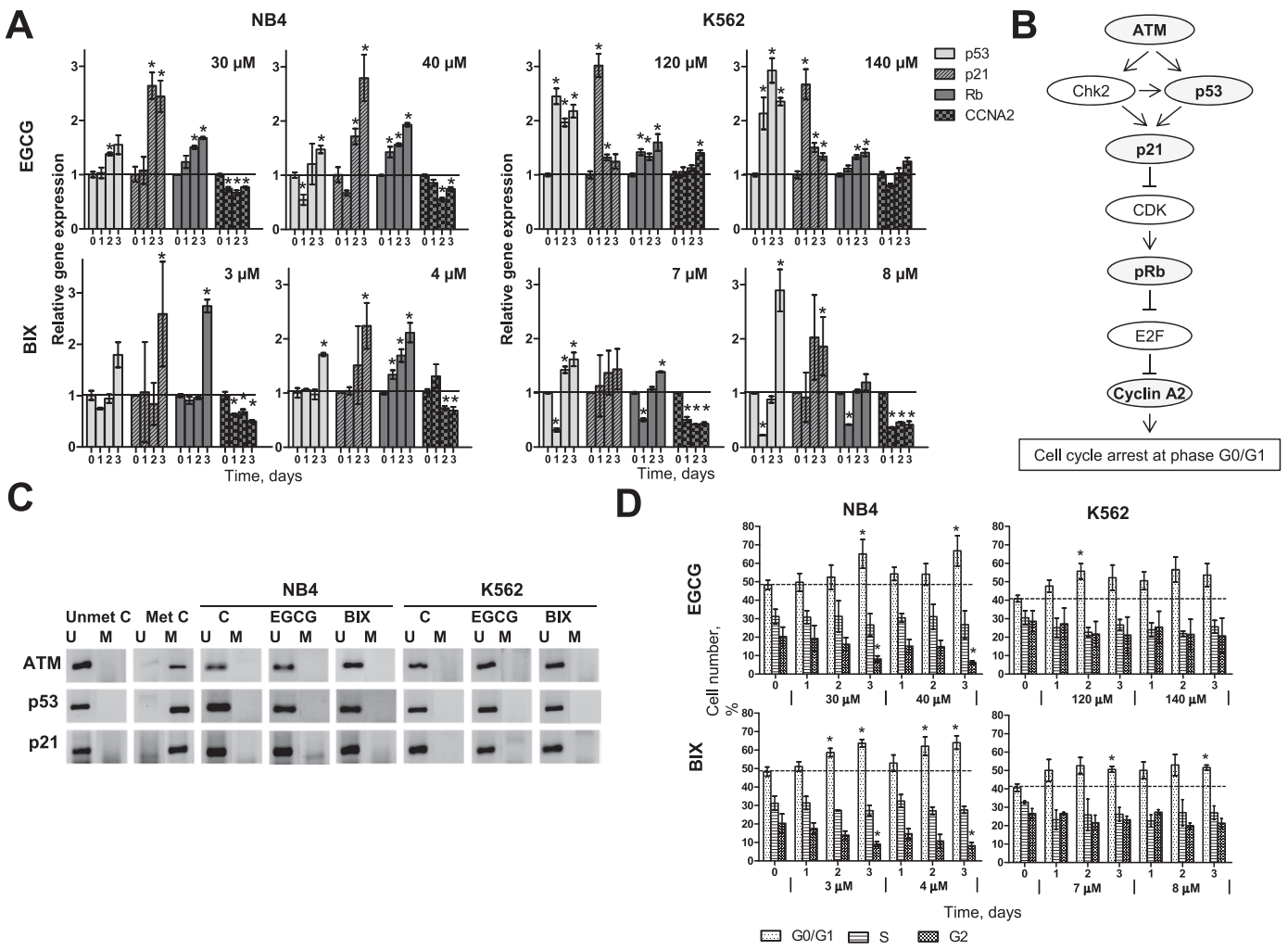

Fig. 2. Myeloid cell treatment with EGCG and BIX-01294 influenced cell cycle progression. NB4 and K562 cells were treated with different concentrations of EGCG and BIX-01294 for 3 days. Gene expression of cell cycle regulators and cell cycle analysis were performed. (**A**) Gene expression changes of cell cycle inhibitors *p53*, *p21*, *Rb* and cell cycle activator *CCNA2* were evaluated using RT-qPCR ΔΔCt method; GAPDH was used for mRNA level normalization; results are presented as changes in comparison to untreated cells; results are mean ± S.D. (n = 3); *P ≤ 0.05. (**B**) Suggested cell cycle arrest mechanism: elevated ATM level cause tumor suppressor p53 activation and CHK2 kinase activation, which turns on p21; the latter one is CDK inhibitor therefore cyclin/CDK complexes are incapable of phosphorylating (inactivating) pRb; hypo-phosphorylated (active) pRb binds and so inactivates transcription factor E2F; inactivated E2F is unable to activate transcription of cyclins (e.g. cyclin A2 encoded by gene *CCNA2*) thus cell cycle is arrested at phase G1 (Aliouat-Denis et al., 2005; Campisi and d'Adda di Fagagna, 2007). (**C**) *ATM*, *p53* and *p21* promoter methylation status was analyzed using MSP method. NB4 cells were treated with 40 μM EGCG and 4 μM BIX-01294 and K562 cells were treated with 140 μM EGCG and 8 μM BIX-01294 for 3 days. Unmet C – unmethylated control DNA; Met C – methylated control DNA; C – untreated cells; lane U – amplified product with primers recognizing unmethylated promoter sequence; lane M – amplified product with primers recognizing methylated promoter sequence. (**D**) Cell cycle analysis was performed using standard propidium iodide method. Results are mean ± S.D. (n = 3); *P ≤ 0.05.

3.2. EGCG and BIX-01294 influence cell cycle progression

Altered expression of cell cycle inhibitors and activators together with cell cycle arrest in the G0/G1 phase, although not exclusive, is the main hallmark of senescent cells (Campisi and d'Adda di Fagagna, 2007). We examined gene expression changes of cell cycle inhibitors *p53*, *p21*, *Rb* and activator *CCNA2* after treatment with EGCG or BIX-01294 by RT-qPCR (Fig. 2A, B). Treated cell samples were taken for analysis every 24 h for 3 days. Treatment with EGCG and BIX-01294 caused significantly elevated levels of cell cycle inhibitors *p53*, *p21*, *Rb* in both NB4 and K562 cell lines. K562 cell line possesses *p53* inactivating frameshift mutation that results in truncated protein (Law et al., 1993), however, its downstream targets may also be activated by p53-independent pathway (Aliouat-Denis et al., 2005) (Fig. 2B). Cell cycle activator *CCNA2* level was reduced with the exception of K562 cells after treatment with EGCG. In addition, we tested whether an increase of *p53* and *p21* expression might be associated with promoter demethylation in these genes. MSP analysis demonstrated that *p53* and *p21* promoter regions in NB4 and K562 cells were unmethylated even before the treatment (Fig. 2C). 3-day treatment with EGCG or BIX-01294 did not cause *p53* and *p21* promoter methylation changes. Thereby, p53 and p21 expression changes were not generated by promoter demethylation but apparently by the other mechanisms. For example, we have demonstrated decreased global levels of gene silencers such as EZH2, SUZ12, Hp1α, and H3K9me3 after treatment with EGCG and BIX-01294 that might be responsible for tested gene expression changes (Fig. 4A). Our observations that EGCG and BIX-01294 altered gene expression of cell cycle inhibitors (*p53*, *p21*, *Rb*) and activator (*CCNA2*) suggest that these chemical agents might arrest cell cycle progression. Thus, cell cycle analysis using standard propidium iodide method was performed. It supports gene expression analysis results – both chemical agents caused cell cycle arrest in G0/G1 phase with a lesser effect of EGCG on K562 cells (Fig. 2D).

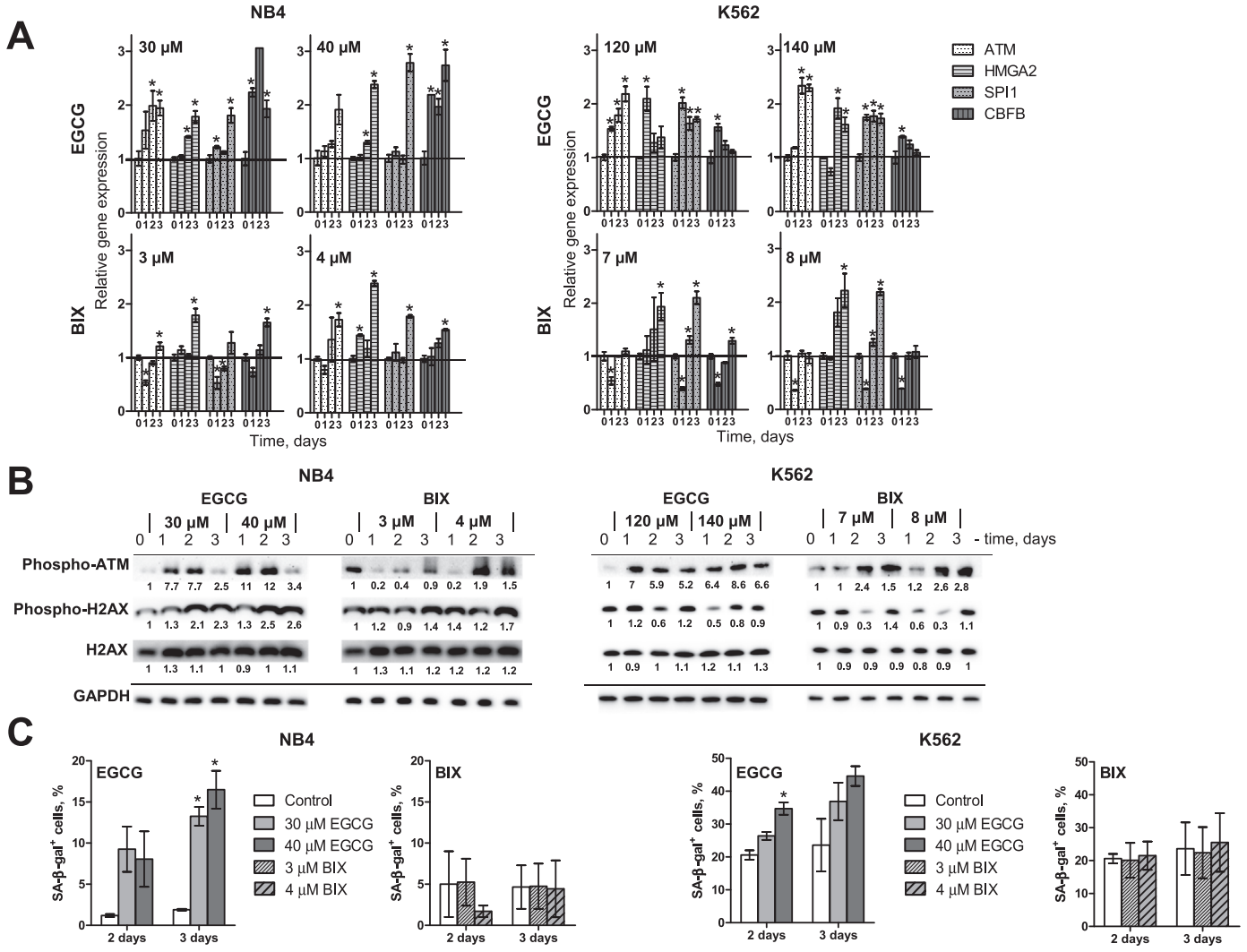

Fig. 3. EGCG and BIX-01294 impact on myeloid leukemia cell senescence and differentiation. NB4 and K562 cells were treated with different concentrations of EGCG and BIX-01294 for 3 days. (**A**) Gene expression changes of senescence associated genes *ATM*, *HMGA2* and differentiation associated genes *SPI1*, *CBFB* were evaluated using RT-qPCR ΔΔCt method; GAPDH was used for mRNA level normalization; results are presented as changes in comparison to untreated cells; results are mean ± S.D. (n = 3); *P ≤ 0.05. (**B**) ATM and H2AX phosphorylation changes were assessed using immunoblot; GAPDH was used as a loading control. For easier result interpretation, intensity of protein bands was measured using ImageJ software; every sample amount was corrected according to GAPDH from the same immunoblot membrane; results are presented as changes in comparison to untreated cells. The experiment was repeated at least two times; representative results are shown. (**C**) Senescence-associated-β-galactosidase (SA-β-gal) activity assay was performed; results are mean ± S.D. (n = 3); *P ≤ 0.05.

3.3. Cellular senescence assessment in NB4 and K562 cells after treatment with EGCG and BIX-01294

As mentioned before, demonstration of cell cycle arrest is not sufficient to determine cellular senescence. Therefore, we evaluated EGCG and BIX-01294 influence on DNA damage response (DDR) effectors ATM and H2AX that might trigger cellular senescence. Also, we analyzed gene expression changes of *HMGA*, which is required for senescence-associated heterochromatin foci (SAHF) formation. Finally, senescence-associated-β-galactosidase (SA-β-gal) activity was assessed. Altogether these elements indicate a senescent phenotype in many different types of cells (Campisi and d'Adda di Fagagna, 2007).

Protein kinase *ATM* gene expression analysis by RT-qPCR revealed that after cell treatment with EGCG for 3 days, *ATM* expression level increased about two times, whereas treatment with BIX-01294 for 3 days caused only a slight *ATM* level increase in NB4 cells and no changes in the K562 cell line (Fig. 3A). MSP analysis of the *ATM* promoter region showed that *ATM* promoters are unmethylated in untreated NB4 and K562 cells (Fig. 2C). No changes were observed after 3-day treatment with EGCG or BIX-01294. Thus, *ATM* expression changes are not conditioned by methylation changes. It is known that DNA damage causes protein kinase ATM phosphorylation. Histone variant H2AX is one of the substrates phosphorylated by ATM. After H2AX phosphorylation by ATM, modified chromatin recruits multiple downstream DDR proteins (Campisi and d'Adda di Fagagna, 2007). ATM and H2AX protein phosphorylation changes were examined after treatment with EGCG and BIX-01294 by immunoblot (Fig. 3B). We showed that ATM phosphorylation increased in both cell lines after both treatments; nevertheless phosho-H2AX level only increased in NB4 cells after EGCG and BIX-01294 treatments. Total histone variant H2AX amount remained unchanged in NB4 and K562 cells after treatment with EGCG and BIX-01294. This means that in both cell lines, EGCG and BIX-01294 triggered DNA damage that is sensed by ATM phosphorylation but DDR occurs only in NB4 cells. It is known that ATM phosphorylates other substrates such as p53; therefore we suggested that in K562 cells such a mechanism could cause cell cycle arrest, senescence or apoptosis.

After treatment with EGCG and BIX-01294, *HMGA2* gene expression up-regulation was detected by RT-qPCR (Fig. 3A). Since HMGA2 is required for SAHF formation, these results support the idea that EGCG and BIX-01294 might cause cellular senescence. SA-β-gal assay

demonstrated that after treatment with EGCG, an increase in the SA-β-gal$^+$ cell number was observed in a time dependent manner (Fig. 3C). K562 background staining was more intensive, thus, the increase induced by EGCG on SA-β-gal activity in both cells lines is very similar. Meanwhile, treatment with BIX-01294 did not cause any changes in SA-β-gal activity (Fig. 3C).

Taken together, increased *ATM* and *HMGA2* gene levels, elevated ATM phosphorylation, SA-β-gal$^+$ cell staining and increased expression of cell cycle inhibitors *p53*, *p21*, and *Rb* after NB4 and K562 cell treatment with EGCG support the idea that EGCG causes cellular senescence in these cell lines. Meanwhile, although BIX-01294 causes cell cycle arrest, increase ATM phosphorylation and *HMGA2* gene expression, it does not affect SA-β-gal activity. Therefore, we assume that BIX-01294 does not cause cellular senescence in NB4 and K562 cell lines.

3.4. EGCG and BIX-01294 upregulate certain genes important in hematopoiesis

PU.1 is a transcription factor (coded by gene *SPI1*) crucial to myeloid development. It regulates the activity of numerous myeloid- and lymphoid-specific promoters and enhancers (Vangala et al., 2003). CBFβ protein (coded by gene *CBFB*) is a component of the core binding factor (CBF) complex, which is a transcription regulator required for myeloid, lymphoid, and megakaryocytic maturation in adult hematopoiesis (Link et al., 2010). RT-qPCR analysis showed that treatment with EGCG and BIX-01294 increased *SPI1* and *CBFB* expression in NB4 and K562 cells (Fig. 3A). Thus, both modifiers have positive effect on hematopoietic cell differentiation-associated genes *SPI1* and *CBFB*.

3.5. EGCG and BIX-01294 cause epigenetic changes in NB4 and K562 cells

Proteins HP1α, DNMT1 and histone modification H3K9me3 are associated with gene silencing, which might lead to tumor suppressor gene repression when their levels are deregulated (Estève et al., 2006). In this study, we examined their changes after treatment with EGCG and BIX-01294 (Fig. 4A). In NB4 cells, the levels of HP1α and DNMT1 protein expression and H3K9me3 modification were shown to be reduced after treatment with EGCG and BIX-12094 independently. However, K562 cells did not show these changes – BIX-01294 caused only a decrease of HP1α and DNMT1 protein levels, while no changes were observed in H3K9me3 modification. Moreover, K562 cell treatment with EGCG caused variable changes of these proteins: only DNMT1 level decreased while H3K9me3 modification intensity did not change and HP1α level even increased. Polycomb repressive complex 2 (PRC2) is also responsible for heterochromatin formation. Immunoblot analysis demonstrated that PRC2 components EZH2 and SUZ12 levels decreased in NB4 cells after both treatments while in K562 cells only BIX-01294 reduced levels of tested PRC2 components.

H4K20 methylation level was evaluated after cell treatment with EGCG and BIX-01294 (Fig. 4A). H4K20me1 level declines during G1 phase thus supporting cell cycle progression and then increases after S phase, while H4K20me2 and H4K20me3 levels increase in quiescence. H4K20me1 and H4K20me2 are involved in DNA damage repair and DNA replication, whereas H4K20me3 is linked with silenced heterochromatin (Jørgensen et al., 2013) and is important for transposable element silencing. Its loss has been identified in many cancers (Fraga et al., 2005). We demonstrated that cell treatment with EGCG did not have significant effect on H4K20 methylation levels (only the H4K20me3 level declined in NB4 cells). BIX-01294 caused an increase of all three histone modifications in NB4 cells, whereas in K562 cells, only H4K20me1 level decreased after treatment with BIX-01294. Therefore, the results comply with previously described ones – acute promyelocytic leukemia NB4 cells show higher sensitivity to epigenetic modulation by EGCG and BIX-0124 compared to chronic myeloid leukemia K562 cells.

4. Discussion

In this study, we investigated two new approaches for myeloid leukemia treatment. Green tea polyphenol EGCG has been widely studied because of its anti-cancerous activity (Granja et al., 2016). EGCG inhibited proliferation and induced apoptosis in various cancer cells (Gan et al., 2016). Epigenetic modifier BIX-01294 (EHMT2/G9a histone methyltransferase inhibitor) has been studied less extensively in comparison to EGCG. However, it has been revealed that higher *G9A* expression is observed in various cancers and enhances the proliferation and tumorigenicity of cancer cells (Ding et al., 2013). BIX-01294 was shown to reduce proliferation and cause apoptosis in neuroblastoma cells (Ding et al., 2013; Lu et al., 2013) and it also suppressed proliferation of bladder, bone, brain, breast, cervix, colon, liver, and lung cancer cell lines (Ding et al., 2013).

In this study, we demonstrated that EGCG and BIX-01294 inhibited proliferation of acute promyelocytic leukemia NB4 and chronic myeloid leukemia K562 cells. We also revealed that acute promyelocytic leukemia cells underwent apoptosis. Although we used higher doses of EGCG and BIX-01294 on K562 cells compared to NB4 cells (120–140 μM and 7–8 μM instead of 30–40 μM and 3–4 μM, respectively), chronic myeloid leukemia cells showed apoptosis resistance. Since treatment with BIX-01294 reduced K562 cell proliferation but only slightly reduced their survival, BIX-01294 might have caused only cell cycle arrest, whereas EGCG-treated cells might have died by necrosis. Chronic myeloid leukemia cells have been shown before to be highly resistant to various chemotherapeutic drugs due to Bcr-Abl expression, which blocks apoptosis (Amarante-Mendes et al., 1998). However, it has been shown that K562 cells showed apoptosis resistance to interferon and imatinib treatment, but after co-treatment with BIX-01294, K562 proliferation was strongly inhibited and apoptosis was induced (Loh et al., 2014).

Previous research showed that EGCG induces cell cycle arrest in G0/G1 phase in various cancer cells (Gan et al., 2016). Cell cycle arrest was confirmed by expression changes of various cell cycle activators and inhibitors: EGCG reduced gene expression of cell cycle activators *CCNA2*, *CCNB1*, *CCND1* and *E2F1* and enhanced *p21* gene expression in biliary tract cancer cells (Mayr et al., 2015), down-regulated protein expression of cyclin D1, CDK4/6 and phosphorylated Rb protein in bladder tumor cell (Chen et al., 2004), down-regulated cyclin D1 and up-regulated p21 expression in colorectal cancer cells (Zhang et al., 2012), etc. Previously we demonstrated that BIX-01294 induced cell cycle arrest in G0/G1 phase in acute promyelocytic leukemia NB4 and HL-60 cells (Savickiene et al., 2014a). Therefore, our observations that acute and chronic myeloid leukemia cells accumulated in G0/G1 cell cycle phase after treatment with EGCG or BIX-01294 conform to previous findings. Further, we showed that both chemical agents up-regulated cell cycle inhibitors *p53*, *p21*, *Rb* in both NB4 and K562 cell lines and down-regulated cell cycle activator *CCNA2* in both cell lines with an exception of lower effect of EGCG on K562 cells.

p53 gene promoter tends to be methylated in some cancer cases, for example it has been shown to be methylated in approximately half of all ovarian cancer cases (Chmelarova et al., 2013), and significant hypermethylation was detected among cervical cancer patients (Jha et al., 2012). It has also been demonstrated that *p53* methylation correlates with decreased gene expression in a subset of acute lymphoblastic leukemia patients (Agirre et al., 2003). However, in our study, *p53* promoter was shown to be unmethylated in NB4 and K562 cells. Thus, elevated p53 expression after treatment with EGCG and BIX-01294 could not be explained by gene promoter demethylation. The same conclusion could be made for *p21* gene as its promoter was found to be unmethylated as well. This correlates with previous findings that *p21* promoter is unmethylated in most types of human cancer including various myeloid leukemias (Scott et al., 2006).

Depending on DNA damage extent and origin of cell cycle arrest, cells might be induced to undergo apoptosis or enter permanent cell

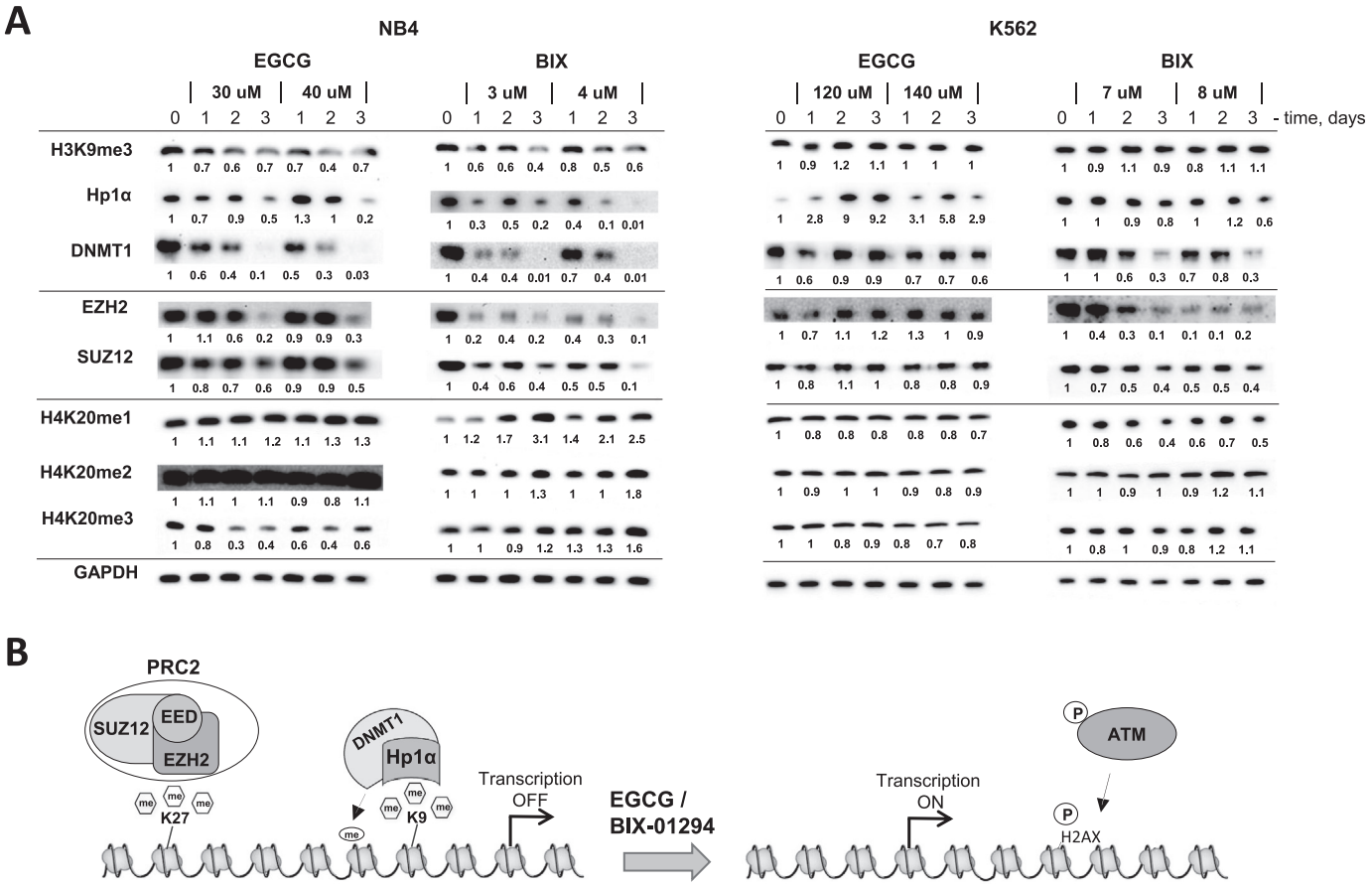

Fig. 4. EGCG and BIX-01294 caused epigenetic changes in myeloid leukemia cells. NB4 and K562 cells were treated with different concentrations of EGCG and BIX-01294 for 3 days. (**A**) Protein level changes were assessed using immunoblot; GAPDH was used as a loading control. For easier result interpretation, intensity of protein bands was measured using ImageJ software; every sample amount was corrected according to GAPDH from the same immunoblot membrane; results are presented as changes in comparison to untreated cells. The experiment was repeated at least two times; representative results are shown. (**B**) EGCG and BIX-01294 modulate acute promyelocytic leukemia cell chromatin: reduce levels of PRC2 complex proteins, of histone modification H3K9me3 and HP1α, DNMT1 proteins thus causing heterochromatin decondensation and tumor suppressor gene transcription activation. Also, both agents cause DNA damage response: induce ATM phosphorylation leading to histone H2AX phosphorylation.

cycle arrest called cellular senescence. Thus, altered expression of cell cycle inhibitors and activators together with cell accumulation in G0/G1 cell cycle phase is one of main features of senescent cells (Campisi and d'Adda di Fagagna, 2007). EGCG was shown to inhibit telomerase activity. Telomerase inhibition can cause replicative cellular senescence due to telomere shortening in time. Cellular senescence was detected after prolonged passage of cancer cells with EGCG (Naasani et al., 1998). Therefore, we evaluated whether tested agents can induce cellular senescence. We demonstrated that EGCG caused cellular senescence in both investigated myeloid leukemia cell lines even after 3 days of treatment (induced cell cycle arrest, up-regulation of *ATM* and *HMGA2*, phosphorylation of ATM and elevated number of SA-β-gal$^+$ positive cells) whereas BIX-01294 did not (although induced cell cycle arrest and up-regulation of *HMGA2* was observed, but neither up-regulation, nor phosphorylation of ATM was extensive and there was no effect on SA-β-gal$^+$ cell staining). Induction of either apoptosis or senescence depends on dose and type of used chemical agent – senescence-like phenotype is relatively more prominent at less cytotoxic drug doses (Chang et al., 1999); however, some agents do not cause senescence at any tested doses (Schwarze et al., 2005). Although apoptosis is thought to be more successful cancer treatment approach than induction of senescence, nevertheless the ability of chemical agents to induce cellular senescence is an advantage. It means that lower doses of chemical agent are less toxic but still have a positive effect in cancer therapy (Nardella et al., 2011). On the other hand, senescent cells can promote cancer by their characteristic secretory phenotype, and thus, accumulating high amounts of senescent cells might be harmful for the organism (Maria and Ingrid, 2017). Therefore, senescence inducing agents could be more beneficial if used in combination with senolytic therapy, which depletes senescent cells.

It is known that after induction of differentiation, cells start to accumulate in G0/G1 phase (Savickiene et al., 2014b). Previously it was shown that EGCG treatment up-regulated expression of some differentiation-associated proteins or genes (CD11b, CD15, *C/EBPε*, *CSF3R*) in acute promyelocytic leukemia NB4 and HL-60 cells and significantly enhanced cell differentiation when cells were co-treated with ATRA (Britschgi et al., 2010). Meanwhile, BIX-01294 alone did not induce acute promyelocytic leukemia NB4 and HL-60 cell differentiation to granulocytes but co-treatment with ATRA enhanced such differentiation as assessed by NBT test, increased CD11b surface protein level and up-regulated *C/EBPε* gene expression (Savickiene et al., 2014a). Here we investigated *SPI1* and *CBFB* (other myeloid hematopoiesis-associated genes) expression changes after treatment with EGCG or BIX-01294 alone. Correlating with previous results, a positive effect on these hematopoietic cell differentiation-associated genes was observed in both tested cell lines. Thus, our findings support the idea that both chemical agents have the ability to enhance myeloid cell differentiation when used in combined treatment with cell differentiation inducers.

Since epigenome aberrations are a prominent cancer characteristic we analyzed EGCG and BIX-01294 effect on myeloid leukemia epigenetic modulation. Chromatin enriched with histone modification H3K9me3 serves as a platform for HP1α binding, which recruits

DNMT1 for repressor complex formation, thus elevated levels of these proteins are associated with tumor suppressor gene silencing (Estève et al., 2006). PRC2 complex protein overexpression has been identified in various cancers as well (Sparmann and van Lohuizen, 2006). EGCG was shown to down-regulate PRC2 component EHZ2 (Balasubramanian et al., 2010) and DNMT1 (Singh et al., 2013). In this study, we analyzed H3K9me3, HP1α, DNMT1, PRC2 complex proteins (EZH2 and SUZ12) expression and H4K20 methylation changes after treatment with EGCG and BIX-01294. Acute promyelocytic leukemia cells showed sensitivity to epigenetic modulation by both EGCG and BIX-01294 (Fig. 4B). However, K562 cells showed resistance to EGCG – only DNMT1 level was down-regulated. Also, BIX-01294 demonstrated lower effect on K562 cells in comparison to NB4 cells. Hence, EGCG as epigenetic modifier could be useful for acute promyelocytic leukemia treatment, while BIX-01294 is effective in both myeloid leukemia cell lines.

5. Conclusions

EGCG and BIX-01294 impair myeloid leukemia cell proliferation and survival: APL cells show higher sensitivity than CML cells. Moreover, acute promyelocytic leukemia NB4 cells undergo apoptosis whereas chronic myeloid leukemia K562 cells remain apoptosis resistant. We suggest that epigenetic modifier EGCG as cellular senescence inducing agent could be important for myeloid leukemia therapy with an advantageous epigenetic modulation on acute promyelocytic leukemia cells. BIX-01294 although not inducing cellular senescence, could cause anti-cancerous epigenetic changes in both acute and chronic myeloid leukemias. Indeed, both EGCG and BIX-01294 might be beneficial for development of new myeloid leukemia treatment strategies.

Funding

This work was supported by the Research Council of Lithuania (Grant no. SEN-12/2015).

Declarations of interest

None.

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