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The effects of clofarabine in ALL inhibition through DNA methylation regulation*

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Clofarabine (2-chloro-2'-fluoro-2'-deoxyarabinosyladenine, ClF), a second-generation 2'-deoxyadenosine analog, possesses manifold anti-cancer activities. Our previous reports and some of others demonstrate the potential capacity of ClF to regulate the epigenetic machinery. The study presented here is the first to investigate the influence of ClF on modulators of the DNA methylation machinery, including DNMT1 and CDKN1A, in acute lymphoblastic leukemia (ALL) cells. ClF effects on promoter methylation and transcriptional activity of hypermethylated and silenced tumor suppressor genes (TSGs), including *APC*, *CDKN2A, PTEN*, and *RARB*, have been tested as well. Methylation level of the proximal promoter region of *APC*, *CDKN2A*, *PTEN*, and *RARB*, as well as expression of those TSGs, *DNMT1* and *CDKN1A*, were estimated by using a methylation-sensitive restriction analysis and qPCR, respectively. The Nalm-6 cell line was used as an experimental in vitro model of ALL cells. We observed ClF-mediated inhibition of cellular viability and apoptosis induction of Nalm-6 cells with an increased percentage of cells positive for active Caspase-3. Interestingly, exposure of Nalm-6 cells to ClF at 20 nM concentration for three days has led to a significant *DNMT1* downregulation, accompanied by robust *CDKN1A* upregulation. ClF caused hypomethylation of *APC*, *CDKN2A*, and *PTEN,* with a concomitant increase in their transcript levels. Taken together, our results demonstrate the ability of ClF to reactivate DNA methylation-silenced TSGs in ALL cells. This may implicate translational significance of our findings and support ClF application as a new epigenetic modulator in the anti-leukemia therapy.

Key words: clofarabine; DNA methylation; tumor suppressor genes; acute lymphoblastic leukemia; epigenetic therapy

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Abbreviations: ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; APC, APC regulator of WNT signaling pathway; CDKN1A, cyclin-dependent kinase inhibitor 1A; CDKN2A, cyclin dependent kinase inhibitor 2A; ClF, clofarabine; CML, chronic myeloid leukemia; dCK, deoxycytidine kinase; DNMT1, DNA methyltransferase 1; PTEN, phosphatase and tensin homolog; RARB, retinoic acid receptor beta; TSGs, tumor suppressor genes.

INTRODUCTION

Clofarabine (2-chloro-2'-fluoro-2'-deoxyarabinosyladenine, ClF), a second-generation analog of the natural purine nucleoside, has been shown to be effective in clinical treatment of many human malignancies, especially the acute lymphoblastic leukemia (ALL) and some acute myeloid leukemia (AML) cases (Ghanem *et al.*, 2010; Ghanem *et al.*, 2013). The U. S. Food and Drug Administration has approved ClF in December 2004 for the treatment of pediatric patients with relapsed or refractory ALL.

After intracellular phosphorylation by deoxycytidine kinase (dCK) to a dNTP derivative, ClF-dATP becomes an active cytotoxic agent. Key mechanisms responsible for ClF-dATP anti-cancer action involve inhibition of DNA synthesis, termination of DNA elongation, interference with DNA repair machinery, and apoptosis induction through DNA strand breaks and aberrant mitochondrial integrity, leading to release of proapoptotic proteins (Majda *et al.*, 2011; Xie *et al.*, 1995; Xie *et al.*, 1996).

ClF-dATP inhibits both, the DNA polymerases and the ribonucleotide reductase (RR), and has a high affinity to dCK. The chlorine and fluorine residues in the ClF structure render its resistance to deamination by the adenosine deaminase and to cleavage of the glycosidic linkage by bacterial purine nucleoside phosphorylase. These attributes stabilize this compound in an acidic environment and increase its oral bioavailability (Majda *et al.*, 2011; Xie *et al.*, 1995; Xie *et al.*, 1996).

ClF has demonstrated cytotoxicity to a variety of human hematologic and solid tumor cell lines and tumor xenograft models (the leukemia, colon, and breast tumor models) (Takahashi *et al.*, 2002; Majda *et al.*, 2010; Wang & Albertioni, 2010; Lubecka-Pietruszewska *et al.*, 2014; Yamauchi *et al.*, 2014; Rahmati-Yamchi *et al.*, 2015; Stumpel *et al.*, 2015). Although the mechanisms of ClF cytotoxic activity (the antiproliferative and proapoptotic effects) have been extensively studied in ALL *in vitro* and *in vivo* models, almost nothing is known about ClF possible role as a hypomethylating agent in ALL inhibition. To the best of our knowledge, there is only one study presenting ClF epigenetic effects in primary MLL-rearranged infant ALL. Interestingly, ClF (5–10 nM) induced promoter demethylation of *FHIT* (fragile histidine triad diadenosine triphosphatase) tumor suppressor gene that was accompanied by its subtle re-expression (Stumpel *et al.*, 2015). Moreover, our team has previously observed that ClF could modulate gene expression *via* redesigning DNA methylation patterns within gene regulatory regions in the chronic myeloid leukemia (CML, K-562 cell

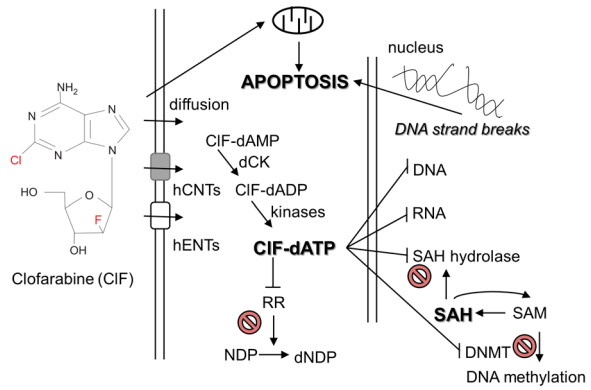

Figure 1. Potential mechanisms of anti-cancer action of clofarabine (ClF).
ClF requires the triple phosphorylation process to become an active drug derivate, ClF-dATP, that is incorporated into the DNA. ClF-dATP inhibits the ribonucleotide reductase (RR) activity, lessening the normal dNTPs formation and their competition for incorporation into the DNA. Apart from inhibition of DNA synthesis, ClF has been shown to affect DNA methylation by inhibition of S-adenosyl-L-homocysteine (SAH) hydrolase, resulting in subsequent SAH accumulation. It reduces formation of S-adenosyl-L-methionine (SAM), a methyl donor used by DNMT, and thereby inhibits the DNA methylation reaction. dCK, cytosolic deoxycytidine kinase; hCNTs, concentrative transporters. hENTs, equilibrative transporters.

line) and breast cancer cells (MCF7 and MDA-MB-231 cell lines) (Majda *et al*., 2010; Lubecka-Pietruszewska *et al*., 2014). We have also explored whether different natural bioactive compounds, such as all trans-retinoic acid (ATRA), resveratrol, sulforaphane, genistein and/or epigallocatechin-3-gallate (EGCG), can enhance the epigenetic mechanisms regulated by ClF (Lubecka-Pietruszewska *et al*., 2015; Lubecka *et al*., 2018a; Lubecka *et al*., 2018b; Kaufman-Szymczyk *et al*., 2019).

ClF is thought to inhibit DNA methylation either through inhibition of S-adenosyl-L-homocysteine (SAH) hydrolase activity, as demonstrated for cladribine (2-chloro-2'-deoxyadenosine, 2CdA), a ClF precursor (Wyczechowska *et al*., 2003), or through downregulation of the DNA methyltransferase 1 gene (*DNMT1*), encoding the main enzyme catalyzing the DNA methylation reaction (Majda *et al*., 2010; Lubecka-Pietruszewska *et al*., 2014; Stumpel *et al*., 2015).

Therefore, a query appears whether ClF may act through epigenetic mechanisms to inhibit ALL, represented by the Nalm-6 cell line? We hypothesize that ClF impacts DNA methylation patterns and thereby gene transcription *via* modulation of epigenetic enzymes' expression, such as the DNA methyltransferase 1 (DNMT1) in ALL cells. Changes in the levels of this enzyme may alter the occupancy of specific protein complexes in gene regulatory regions which determines the chromatin structure and as a result gene transcription. Through this mode of action, ClF reverses the cancer-specific patterns of DNA methylation. This leads to activation of the methylation-silenced tumor suppressor genes (Fig. 1).

Thus, the study presented here is the first to investigate the influence of ClF on modulators of the DNA methylation machinery, including *DNMT1* and cyclin-dependent kinase inhibitor 1A (*CDKN1A*) in ALL cells. ClF effects on promoter methylation and transcriptional activity of DNA methylation-silenced tumor suppressor genes (TSGs), including APC regulator of WNT signaling pathway (*APC*), cyclin dependent kinase inhibitor 2A (*CDKN2A*), phosphatase and tensin homolog (*PTEN*) and retinoic acid receptor beta (*RARB*), have been tested as well.

MATERIALS AND METHODS

Compounds and chemicals. Clofarabine (ClF) was purchased from MERCK. ClF was dissolved in sterile water (1 mM) and stored at –20°C. Subsequent dilutions were made in fresh growth medium.

Cell culture, growth and viability assays. The Nalm-6 cell line (human, B cell precursor leukemia, ATCC CRL-3273) was established from the peripheral blood of a 19-year-old man with acute lymphoblastic leukemia (ALL) in a relapse in 1976. The Nalm-6 cells were cultured in RPMI-1640 medium with HEPES (Lonza) supplemented with 2 mM L-glutamine, 10% foetal bovine serum (FBS), 1 U/ml penicillin and 1 μg/ml streptomycin (MERCK), at 37°C and a humidified atmosphere of 5% CO_2. In all experiments, the cells were seeded at the amount of 40×10^3 cells per ml and were cultured for 72 h with ClF at different concentrations (in the range from 5 nM to 50 nM). Cell growth and viability were determined using the trypan blue (MERCK) exclusion test to estimate the IG_{50} value. The number of viable cells in culture treated with ClF was expressed as a percentage of viable cells in the unexposed control culture (without ClF). The IG_{50} value represents the growth inhibitory concentration at which the compound causes a 50% decrease in the number of viable cells when compared to control (unexposed cells) after 72 h incubation. The number of dead cells that took up trypan blue was specified as the percentage of the total cell number.

The number of viable, necrotic, early and late apoptotic cells after 72 h exposure was determined by flow cytometry analysis using the annexin V/propidium iodide (PI) (FITC Annexin V Apoptosis Detection Kit II, BD Pharmingen) staining, according to the manufacturer's protocol (Majda *et al*., 2010; Lubecka-Pietruszewska *et al*., 2014). The flow cytometry analysis has been performed by using the CellQuestPro software (BD Pharmingen). The following analysis allows to respectively distinguish: viable cells (Ann-/PI-; Q3, lower left part on the cytogram), early apoptotic cells (An+/PI-; Q4, lower right), late apoptotic cells (Ann+/PI+; Q2, upper right), and necrotic cells (Ann-/PI+; Q1, upper left). Caspase-3 assay (Caspase-3 Assay Kit, BD Pharmingen) was performed to estimate its activity as a marker of the early stage of the caspase-dependent apoptotic pathway.

Methylation-Sensitive Restriction Analysis (MSRA). Methylation level of the proximal promoter regions of *PTEN, APC, RARB*, and *CDKN2A (P16)* in Nalm-6 cells was estimated by using the methylation-sensitive restriction analysis (MSRA) according to the method of Iwase and others (Iwase *et al*., 1999).

The specific promoter fragments of the tested genes (*APC*, *CDKN2A, PTEN*, and *RARB*) were chosen for methylation analysis taking into consideration literature data and analysis of the promoter regions using CpG-plot software (Larsen *et al*., 1992). This analysis indicates the pivotal role of these fragments in regulation of transcriptional activity of these genes. Fragment of the *APC* promoter selected for methylation analysis contains one Eco72I site, which constitutes an E-box B element recognized by the upstream stimulatory factor 1 (USF1) and 2 (USF2), which are near the signaling sequence for transcription factor Sp1, SP1 (Jaiswal & Narayan, 2001). The tested fragment of the *CDKN2A* proximal

Table 1. PCR primer sequences.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)	Product (bp) Annealing T (°C)
PTEN	cagccgttcggaggattattc	gggcttcttctgcaggatgg	214 bp [+848/+1062]; 61.1 °C
APC	ctaggcaggctgtgcggttg	cggtttaagacagtgcgagg	319 bp [-164/+155]; 61.1°C
RARB	ctcgctgcctgcctctctgg	gcgttctcggcatcccagtc	295 bp; 58.4°C
CDKN2A	ctggctggtcaccagag	agaccctctacccacctg	253 bp [-88/+165]; 63.0°C

promoter region described in Hara's report (Hara *et al.,* 1996) includes a methylation-sensitive HpaII site and TSS (transcription start site). Fragment of the *PTEN* promoter encompasses one HpaII site near the binding sequence for methylation-sensitive transcription factor AP-4, TFAP4 (Salvesen *et al.,* 2001). *RARB* promoter fragment includes two retinoic acid response elements (RAREs) and three methylation-sensitive CpG dinucleotide sequences located close to the RAREs, including one HpaII site (Arapshian *et al.,* 2000).

Moreover, according to publicly available data from NCBI's Gene Expression Omnibus GEO (Illumina 450K Human Methylation Array) and Oncomine, the tested *RARB*, *PTEN*, *APC*, and *CDKN2A* genes have been shown to be transcriptionally silenced by promoter hypermethylation in many types of leukemia, including ALL (Kaufman-Szymczyk *et al.,* 2019). Additionally, in our previous *in vitro* studies on leukemia and breast cancer, with the HL-60 and K-562 cells, representing the acute (AML, data not published) and chronic myeloid leukemia (CML) respectively, and/or in breast cancer cells (MCF7 and MDA-MB-231 cell lines), we evaluated promoter DNA methylation level of several tumor suppressor genes, such as *APC, PTEN, RARB, CDKN2A, ESR1* (estrogen receptor 1)*, BRCA1* (BRCA1 DNA repair associated), and/or *CDH1* (cadherin 1). We found that only *RARB*, *PTEN, APC,* and *CDKN2A* (except for K-562 cells with *CDKN2A* homozygous deletion) promoters were differentially methylated in breast cancer cell lines (MCF7 and MDA-MB-231) with different invasive potential (Krawczyk *et al.,* 2007; Stefanska *et al.,* 2010, 2012), and/or between the AML and CML cells (not published). Additionally, nucleoside analogs, including clofarabine (ClF), that were investigated in our previous reports, affected the DNA methylation level only within *APC*, *PTEN, RARB (*Krawczyk *et al.,* 2007; Majda *et al.,* 2010; Stefanska *et al.,* 2010, 2012)*,* and *CDKN2A* (Lubecka *et al.*, 2018b) promoters in breast cancer and/or CML cells.

According to Human GRCh37/hg19 Assembly, the MSRA-tested CpG sites for the selected genes are located within the proximal promoter regions at the following locations: *APC* (chr5:112073538; -11 bp from transcription start site (TSS) [TSS200]; cg23938220 on Illumina 450K microarray platform); *RARB* (chr3:25469694; -139 bp from TSS; enhancer region; cg06720425 on Illumina 450K array), *PTEN* (chr10:89624078; +973 bp from TSS [5'UTR, 1stExon]; CpG island [chr10:89621773-89624128; 2356 bp; 171 CpG sites]; 24 bp from cg08859916 [+997 bp from TSS] on Illumina 450K array), *CDKN2A* (chr9: 21974761; +64 bp from TSS [1stExon]; CpG island [chr9:21974579-21975306; 728 bp; 63 CpG sites]; 58 bp from cg13601799 [+122 bp from TSS] on Illumina 450K array) (Kaufman-Szymczyk *et al.*, 2019; Lubecka *et al.,* 2018).

The methylation status of the tested CpG sites within gene promoters in Nalm-6 cells was estimated in unexposed control cells, as well as in cells exposed to ClF at 10 nM and/or 20 nM concentrations. The MSRA included four steps: (i) digestion of cellular DNA with endonuclease that recognizes only non-methylated sequences (*PTEN*, *RARB,* and *CDKN2A* – HpaII, C↓CGG, or *APC* – Eco72I, CAC↓GTG); control sample without the enzyme (undigested sample) and MspI-digested sample were incubated under the same conditions, (ii) PCR amplification of undigested DNA and HpaII-, Eco72I-, MspI-digested DNA with PCR primers shown in Table 1, (iii) electrophoretic analysis of amplified promoter fragments, and (iv) densitometric quantitative analysis of the band intensity. Densitometric analysis of band intensity was performed using the QuantityOne software (Bio-Rad Laboratories Ltd., UK). Methylation level in each sample was expressed as a percentage of undigested DNA after comparison of band intensities for digested and undigested DNA from the same sample, as shown below:

((Band intensity of DNA digested with HpaII or Eco72I/Band intensity of undigested DNA) ×100%) (Majda *et al.*, 2010; Lubecka-Pietruszewska *et al.*, 2014; Lubecka *et al.*, 2019).

Quantitative Real-Time PCR (qPCR). Total RNA was isolated using TRIZOL® (Invitrogen, USA). cDNA

Table 2. SYBR Green-based qRT-PCR primer sequences.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)	Product (bp)
DNMT1	accgcccctggccaaagccattg	agcagcttcctcctcctttattttagctgag	100
CDKN1A	gctcaggggagcaggctgaag	cggcgtttggagtggtagaaatctgt	103
PTEN	cgaactggtgtaatgatatgt	catgaacttgtcttcccgt	330
APC	tgcgagaagttggaagtgtgaaagcattg	tgacaaattccataaggcactcaatacgc	101
RARB	ttcaagcaagcctcacatgtttcca	aggtaattacacgctctgcacctttag	292
CDKN2A	catagatgccgcggaaggt	cccgaggtttctcagagcct	268

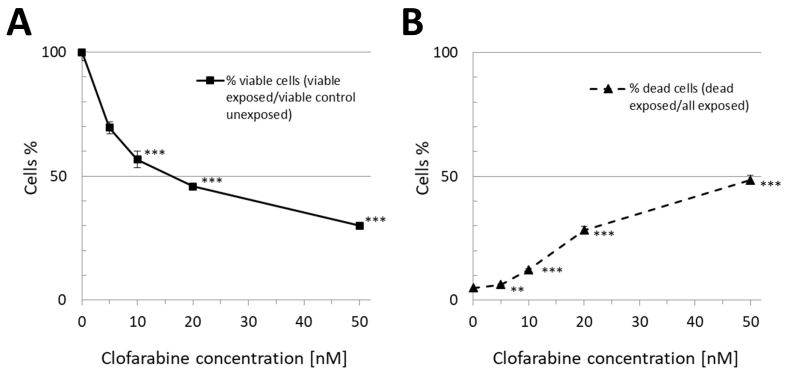

Figure 2. Effects of clofarabine (ClF) on the Nalm-6 cell growth (A) and viability (B), as measured by the trypan blue exclusion test, after 72 h incubation.
(**A**) The number of viable cells in culture treated with ClF was expressed as a percentage of viable cells in unexposed control culture (without ClF). The IG50 value represents the growth inhibitory concentration at which the compound causes a 50% decrease in the number of viable cells when compared with control (unexposed cells) after 72 h incubation. The IG50 value was estimated to be equal to 15 nM. (**B**) The number of dead cells that took up trypan blue was specified as the percentage of the total cell number. Data represents the mean ± S.D. of three independent experiments. Exposure *versus* control: ***P*<0.01, ****P*<0.001.

was synthesized using 2 µg of total RNA, 6 µl of random hexamers, 5 µl of oligo(dT)$_{15}$, and ImProm-II reverse transcriptase (Promega, USA). All quantitative real-time PCR reactions were carried out in a Rotor-Gene TG-3000 machine (Corbett Research, Australia), as we previously described (Majda *et al.*, 2010; Lubecka-Pietruszewska *et al.*, 2014; Lubecka *et al.*, 2019). *RPS17* (40S ribosomal protein S17), *RPLP0* (60S acidic ribosomal protein P0), *H3F3A* (H3 histone family 3A), and *BMG* (β_2-microglobulin) were used as housekeeping reference genes. The relative expression of each tested gene (*DNMT1*, *CDKN1A*, *PTEN, APC, RARB*, and *CDKN2A*) was normalized to the geometric mean of these four housekeeping genes, according to the method of Pfaffl and others (Pfaffl *et al.*, 2002). Primer sequences for real-time PCR are shown in Table 2.

Statistical analysis. Results from three independent experiments are presented as the mean ± standard deviation (S.D.). Statistical analysis of cell viability, apoptosis, MSRA, and qPCR assays was performed using a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. The results were considered statistically significant when $P<0.05$.

RESULTS AND DISCUSSION

ClF inhibits cell growth and induces apoptosis in ALL cells

ClF 72 h-exposure inhibits Nalm-6 cell viability in a dose-dependent manner. The trypan blue exclusion test was used to estimate the viability of the cells (Fig. 2A) and the percentage of necrotic cells in the cultures (Fig. 2B).

ClF concentration leading to a 50% decrease in the number of viable cells (IG$_{50}$), was determined as equal to 15 nM. In all further experiments, two different concentrations of ClF were used, 10 and 20 nM. Additionally, cytotoxic effects of ClF used at 10 and 20 nM concentrations were determined by employing a flow cytometric assay (Fig. 3).

We observed a statistically significant increase in the number of apoptotic Nalm-6 cells (in comparison to the number of apoptotic cells in control) after 72 h exposure of ClF at both examined concentrations. However, ClF used at the higher 20 nM concentration caused a severe apoptosis induction. Almost a 40% increase in the number of apoptotic cells was associated with a significant caspase-3 activation, reaching 30% of caspase-3(+) cells upon ClF 20 nM exposure (Fig. 3).

The cytotoxic mechanisms of active derivate of ClF, ClF-dATP, include a number of important pathways depicted in Fig. 1. The ClF-dATP anti-cancer activity is attributed to inhibition of DNA synthesis *via* RR and DNA polymerases inhibition, as well as repression of DNA elongation and breakage of DNA strands. These ClF-dATP-mediated actions may cause mitochondria dysfunction and induce cell apoptosis. Genini et al. reported that in primary chronic lymphocytic leukemia (B-CLL) cells, ClF-dATP leads to damage of mitochondrial DNA, aberrant mitochondrial metabolic function, and impairment of mitochondrial integrity. This may result in the release of proapoptotic factors, cytochrome c and AIF, and stimulation of the apoptosis pathway *via* the caspase cascade (Genini *et al.,* 2000).

Similarly to our results, Takahashi and others (Takahashi *et al.*, 2002) observed ClF-mediated apoptosis of human T-acute lymphocytic leukemia cells, CCRF-CEM. It has been shown that the proapoptotic effect upon ClF exposure was associated with downregulation of the Bcl-XL and/or Mcl-1 proteins of the Bcl-2 family. Moreover, ClF-induced apoptosis has been demonstrated in other types of cancer. Rahmati-Yamchi and others (Rahmati-Yamchi *et al.,* 2015) reported that ClF has an apoptotic effect on the T47D breast cancer cells *via* regulation of *P53R2* gene expression in a time- and dose-dependent manner. In the Wang and Albertioni's studies, exposure of human HCT116 epithelial colon cancer cells to ClF has caused a rapid reduction in thymidine incorporation into DNA during DNA synthesis and a 3-fold increase in apoptosis induction (Wang & Albertioni, 2010).

Moreover, our team observed a ClF-stimulated apoptosis in the MCF7 and MDA-MB-231 breast cancer cells with different invasiveness (Lubecka-Pietruszewska *et al.*, 2014), and the K-562 cells, representing the CML cells (Majda *et al.*, 2010). Yamauchi's findings revealed that ClF exposure has led to apoptosis in the human HL-60 and HL/ara-C20 cells, representing AML and AML-resistant to cytarabine cells (Yamauchi *et al.*, 2014).

ClF regulates DNA methylation machinery in ALL cells

First, we analyzed the Oncomine publicly available data for *DNMT1* expression in ALL patients, as *DNMT1* upregulation has been observed in various types of cancer (Zhang & Xu, 2017; Mizuno *et al.*, 2001). As depicted in Fig. 4A (left panel), in ALL patients, *DNMT1* expression is significantly higher (P=1.9E-8) when compared to healthy individuals. As we hypothesized, in Nalm-6 cells (ALL cells) exposed to ClF, significant *DNMT1* down-

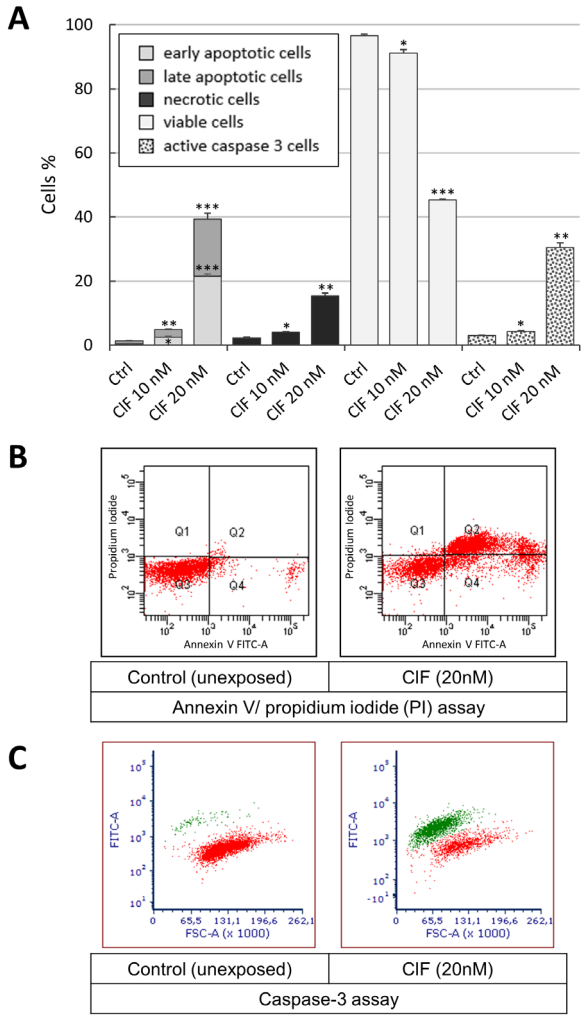

Figure 3. Effects of clofarabine (ClF) used at the indicated concentrations (10 and 20 nM) on the number of viable, necrotic, apoptotic, and Caspase-3(+) cells in the Nalm-6 cell cultures (A). Example cytograms of Annexin V/ propidium iodide (PI) (B) and Caspase-3 (C) assays for experiments in Nalm-6 cells upon ClF (20 nM) exposure.
Values are means of at least three independent experiments, with ±S.D. represented by vertical bars. Exposure *versus* control: **P*<0.05, ***P*<0.01, ****P*<0.001. Q1, necrotic cells (Ann-/PI+); Q2, late apoptotic cells (Ann+/PI+); Q3, viable cells (Ann-/PI-); Q4, early apoptotic cells (Ann+/PI-).

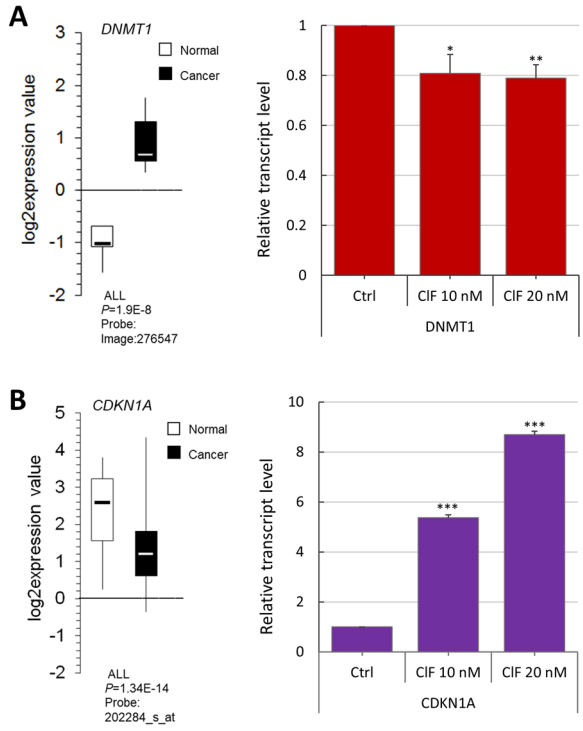

Figure 4. Expression of *DNMT1* and *CDKN1A* genes in ALL patients and Nalm-6 cells.
Gene expression microarray data for *DNMT1* (**left panel part A**) and *CDKN1A* (**left panel part B**) in ALL patients compared to healthy controls. The gene expression data (normal versus cancer) were obtained from Oncomine and are presented as log2-transformed median centered per array, and SD-normalized to 1 per array. Effects of ClF on mRNA level of *DNMT1* (**right panel part A**) and *CDKN1A* (**right panel part B**) in Nalm-6 cells. Data represent the mean ±S.D. of three independent experiments. Exposure *versus* control: **P*<0.05, ** *P*<0.01, ****P*<0.001.

regulation (by 20%) has been detected (Fig. 4A, right panel).

Furthermore, reactivation of the *CDKN1A* (*P21*) gene, encoding a protein capable of cell cycle arrest, is one of the goals of the anti-leukemic therapy (Parveen *et al.,* 2016). According to the Oncomine publicly available data, the *CDKN1A* expression is significantly decreased (*P*=1.34E-14) in ALL, as compared to normal blood cells (Fig. 4B, left panel). Therefore, we tested the mRNA level of a tumor suppressor gene *CDKN1A* (*P21*), encoding a protein that competes with DNMT1 for the same binding site on the proliferating cell nuclear antigen (PCNA, the homotrimeric ring surrounding DNA) during DNA replication (Chuang *et al.*, 1997; Iida *et al.*, 2002). Upon ClF exposure, we observed a robust *CDKN1A* upregulation in Nalm-6 cells. Over 5- and 8-fold increases in *CDKN1A* transcript level have been demonstrated at 10 and 20 nM ClF, respectively (Fig. 4B, right panel). The Chuang's and Iida's studies revealed that CDKN1A (P21) disrupts formation of the DNMT1/PCNA complex during DNA replication and subsequently may cause inhibition of the DNA methylation reaction (Chuang *et al.*, 1997; Iida *et al.*, 2002). Since our present findings indicate such a strong *CDKN1A* re-expression, this may support involvement of this mechanism in suppression of the DNA methylation processes (Fig. 4B).

Moreover, the ClF-mediated *DNMT1* downregulation may be related to alterations in the binding of specific protein complexes within the gene regulatory regions. This can change the chromatin structure and determine the gene transcriptional activity. It has been shown that ClF activates methylation-silenced tumor suppressor genes in CML and breast cancer cells *in vitro* (Majda *et al.*, 2010; Lubecka-Pietruszewska *et al.*, 2014). Through this epigenetic mode of ClF action, this drug may reverse cancer-specific patterns of DNA methylation (Fig. 1).

Therefore, the next step of our studies was to assess the levels of promoter methylation and gene expression of the selected TSGs, *APC*, *CDKN2A*, *PTEN,* and *RARB* that have been shown to be hypermethylated and silenced in various types of cancer (Majda *et al.*, 2010; Lubecka-Pietruszewska *et al.*, 2014).

ClF impacts promoter methylation and transcriptional activity of *PTEN, APC, RARB,* and *CDKN2A* in ALL cells

In Nalm-6 cells, DNA methylation of *PTEN*, *APC*, and *CDKN2A* promoter fragments was decreased, in

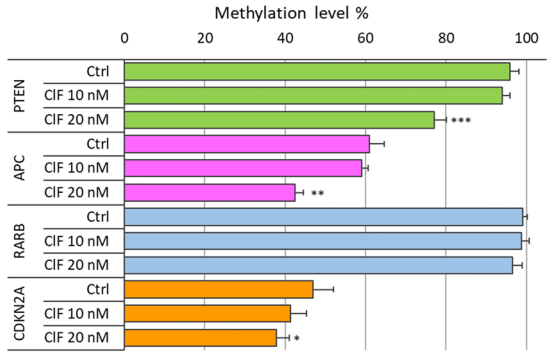

Figure 5. Effects of ClF on the promoter methylation status of *PTEN*, *APC*, *RARB*, and *CDKN2A* tumor suppressor genes in Nalm-6 cells upon 72 h exposure.
Data represent the mean ± S.D. of three independent experiments. Exposure *versus* control: **P*<0.05, ***P*<0.01, ****P*<0.001.

comparison to control cells, upon ClF exposure (Fig. 5). The higher concentration of ClF, equal to 20 nM, led to a more pronounced decrease in methylation of all gene promoters. For the *PTEN* gene, reduction in promoter methylation level was the most severe, from 96% in control cells to 77% in cells exposed to ClF at 20 nM ($P<0.001$). We observed a similar effect for the *APC* gene, where the methylation status of the tested region of gene promoter dropped from 61% in unexposed control to 42% ($P<0.01$) upon 20 nM ClF. In case of the *CDKN2A* gene, ClF also led to a decrease in promoter methylation, in a dose-dependent manner. ClF used at 10 nM concentration caused a decrease in methylation in the tested promoter fragment (from 47% in control) to 41%, while after ClF application at 20 nM concentration, it diminished to 38%. Meanwhile, in the *RARB* gene, the drug-mediated methylation changes were minor and not significant (Fig. 5).

Interestingly, we observed concomitant ClF-mediated increases in expression of the tested TSGs in Nalm-6 cells at the mRNA level, as summarized in Fig. 6. The most robust alteration in the transcript level, an almost 44-fold increase (in comparison to unexposed control cells), was detected for the *RARB* gene upon ClF 20 nM exposure (Fig. 6). 10 nM concentration of ClF has caused around a 4-fold increase in the *RARB* mRNA level. The *PTEN* expression was also significantly elevated. ClF used at 10 and 20 nM led to over 1.5- and 3-fold increases in the *PTEN* transcript level, respectively (Fig. 6). In the case of *APC* and *CDKN2A* genes, upregulation by 18-52% was observed upon ClF exposure at both tested concentrations (Fig. 6).

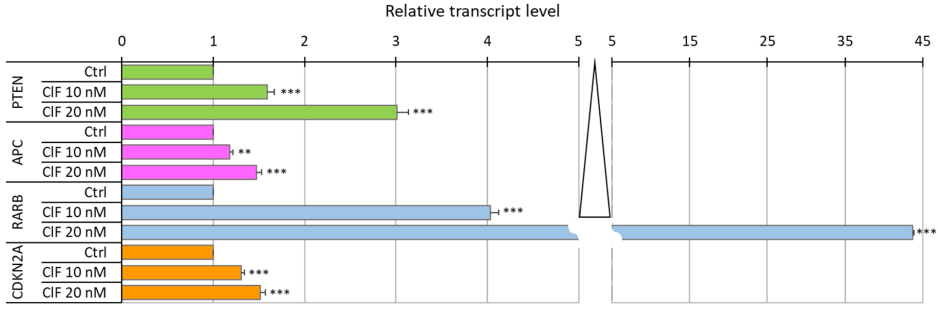

Figure 6. Effects of ClF on expression of the selected tumor suppressor genes *PTEN*, *APC*, *RARB*, and *CDKN2A* at the mRNA level, in Nalm-6 cells (72 h exposure).
Data represent the mean ± S.D. of three independent experiments. Exposure *versus* control: ***P*<0.01, ****P*<0.001.

PTEN (phosphatase and tensin homolog) and *APC* (APC regulator of WNT signaling pathway) tumor suppressor genes encode proteins involved in downregulation of intracellular oncogenic signaling pathways: the mitogen-activated protein kinase (MAPK)/activator protein 1 (AP-1) and phosphoinositide 3-kinase (PI3K)/AKT regulated by PTEN (Cantley *et al.*, 1999; Gu *et al.*, 1998), and Wnt-1/beta-catenin/T-cell factor (TCF) regulated by APC (Goss & Groden, 2000; Polakis, 2000). *CDKN2A* (*P16*; cyclin dependent kinase inhibitor 2A) tumor suppressor encodes a protein involved in downregulation of the Rb/E2F intracellular oncogenic signaling pathway (Kimura *et al.*, 2003). RARB (retinoic acid receptor beta) is a tumor suppressor protein involved in regulation of cell proliferation and differentiation, cell cycle progression, and apoptosis (Alvarez *et al.*, 2007). RARB can act as a potent repressor of transcriptional activity of the AP-1 protein complex (Lin *et al.*, 2000; Yang *et al.*, 1997). Thus, the proteins encoded by *PTEN*, *RARB, APC*, and *CDKN2A* that are negative regulators of AP-1, TCF, and E2F might be indirectly involved in the *DNMT1* downregulation (Bigey *et al.*, 2000; Qin *et al.,* 2011).

As we mentioned before, there is only one study demonstrating ClF-mediated epigenetic effects in primary MLL-rearranged infant ALL. Stumpel et al. observed that ClF led to *FHIT* demethylation and this gene subtle re-expression. Moreover, these changes in the *FHIT* methylation and expression were associated with DNMT1 downregulation (Stumpel *et al.,* 2015).

These new findings of ClF anti-cancer epigenetic effects in ALL cells are similar to those observed by our team in CML (Majda *et al.*, 2010) and breast cancer cells *in vitro* (Lubecka-Pietruszewska *et al.*, 2014). In the case of *APC*, *PTEN,* and *CDKN2A*, the ClF-mediated promoter methylation changes seem to correspond to the expression alterations of these TSGs in Nalm-6 cells (Figs. 5 and 6). However, the robust *RARB* re-expression upon ClF exposure appears not to be related to promoter hypomethylation of this gene, but to other regulatory mechanisms. *RARB* transcriptional activity might be indirectly regulated by PTEN. Lefebvre's studies revealed that through inhibition of PI3K/AKT signaling pathway, PTEN could influence the *RARB* expression by blockage of NCOR2 (nuclear receptor co-repressor 2) co-repressor binding within the *RARB* promoter, resulting in histone acetylation and *RARB* reactivation (Lefebvre *et al.,* 2006).

In the study presented here, we provide evidence that ClF is involved in epigenetic regulation of transcriptional activity of TSGs in ALL cells. This potent antimetabolite leads to hypomethylation of TSG promoters, which is accompanied by alterations in *DNMT1* and *CDKN1A* mRNA levels. Importantly, promoter demethylation is associated with TSGs reactivation, inhibition of cell growth, and caspase-dependent apoptosis in ALL cells. We believe that this may implicate translational significance of our findings

and support ClF application as a new epigenetic modulator in anti-leukemic therapy.

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Authors' Contributions

Conceptualization: Kaufman-Szymczyk A, Lubecka K; Methodology: Kaufman-Szymczyk A, Lubecka K; Formal analysis: Kaufman-Szymczyk A, Lubecka K; Investigation: Kaufman-Szymczyk A; Writing – original draft preparation: Kaufman-Szymczyk A, Lubecka K; Writing – review and editing: Kaufman-Szymczyk A, Lubecka K. Approval of final manuscript: all authors.

Disclosure

The authors declare no conflict of interest.

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