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

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INTRODUCTION

Clofarabine (2-chloro-2'-fluoro-2'-deoxyarabinosyladenine, CIF), a second-generation 2'-deoxyadenosine analog, possesses manifold anti-cancer activities. Our previous reports and some of others demonstrate the potential capacity of CIF to regulate the epigenetic machinery. The study presented here is the first to investigate the influence of CIF on modulators of the DNA methylation machinery, including DNMT1 and CDKN1A, in acute lymphoblastic leukemia (ALL) cells. CIF 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 CIF-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 CIF at 20 nM concentration for three days has led to a significant DNMT1 downregulation, accompanied by robust CDKN1A upregulation. CIF caused hypomethylation of APC, CDKN2A, and PTEN, with a concomitant increase in their transcript levels. Taken together, our results demonstrate the ability of CIF to reactivate DNA methylation-silenced TSGs in ALL cells. This may implicate translational significance of our findings and support CIF 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; CIF, 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.
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% CO₂. In all experiments, the cells were seeded at the amount of 4×10⁴ 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 IG₅₀ 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 IG₅₀ 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-Piotruszewska 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 (Ann+/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
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). RARβ 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 RARβ, 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, RARβ, CDKN2A, ESR1 (estrogen receptor 1), BRCA1 (BRCA1 DNA repair associated), and/or CDH1 (cadherin 1). We found that only RARβ, 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, RARβ (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); RARβ (chr3:52549694; -139 bp from TSS; enhancer region; cg6720425 on Illumina 450K array), PTEN (chr10:89624078; +973 bp from TSS [5’UTR, 1°Exon]; 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 [1°Exon]; 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, RARβ, and CDKN2A – HpaII, C↓CGG, or – HpaII, C↓CGG, or APC – Eco72I, CACGTTG); control sample without the enzyme (undigested sample) andMsp1-digested sample were incubated under the same conditions, (ii) PCR amplification of undigested DNA and HpaII-, Eco72I-, Msp1-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-Piłucka et al., 2014; Lubecka et al., 2019).

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

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### Table 1. PCR primer sequences.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5’-3’)</th>
<th>Reverse primer (5’-3’)</th>
<th>Product (bp)</th>
<th>Annealing T (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTEN</td>
<td>cagccgttggaggtatttcc</td>
<td>ggcgttcctgtgagagttcc</td>
<td>214 bp [+848/+1062]; 61.1 °C</td>
<td></td>
</tr>
<tr>
<td>APC</td>
<td>ctgagcctgcgtggcgttgg</td>
<td>cggttaagcaaggtgagggag</td>
<td>319 bp [164/+155]; 61.1 °C</td>
<td></td>
</tr>
<tr>
<td>RARβ</td>
<td>ctgctgtgctgctggtcctgg</td>
<td>ggcgttcctgcatcgccgtc</td>
<td>295 bp; 58.4 °C</td>
<td></td>
</tr>
<tr>
<td>CDKN2A</td>
<td>ctgctgtgctgctggtcctgg</td>
<td>agaccccccttcacccacttg</td>
<td>253 bp [-88/+165]; 63.0 °C</td>
<td></td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Gene</th>
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<th>Reverse primer (5’-3’)</th>
<th>Product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNMT1</td>
<td>accgccccctgcaccacacccttg</td>
<td>agcacgctctctctctttatttagtgaggt</td>
<td>100 bp</td>
</tr>
<tr>
<td>CDKN1A</td>
<td>gcctgagggagccagctgaggg</td>
<td>cgcgttcgqaggtgtaagatctg</td>
<td>103 bp</td>
</tr>
<tr>
<td>PTEN</td>
<td>cggactgtgtgaatagtagatgtt</td>
<td>catgaacctgtgtctgccct</td>
<td>330 bp</td>
</tr>
<tr>
<td>APC</td>
<td>tgcgagaaggtggaggaatgtagagaaatcttg</td>
<td>tgacaaattctactaaggcactaatcgc</td>
<td>101 bp</td>
</tr>
<tr>
<td>RARβ</td>
<td>ttcaagcagcctcaaagtcttttcca</td>
<td>aggtaattacagcgtctgacatttag</td>
<td>292 bp</td>
</tr>
<tr>
<td>CDKN2A</td>
<td>cagcagctgctgccaggagt</td>
<td>cccaggttcttcagactc</td>
<td>268 bp</td>
</tr>
</tbody>
</table>
was synthesized using 2 μg of total RNA, 6 μl of random hexamers, 5 μl of oligo(dT)$_{19}$, 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 (β₂-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

CIF inhibits cell growth and induces apoptosis in ALL cells

CIF 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).

CIF 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 CIF were used, 10 and 20 nM. Additionally, cytotoxic effects of CIF 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 CIF at both examined concentrations. However, CIF 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 50% of caspase-3(+*) cells upon CIF 20 nM exposure (Fig. 3).

The cytotoxic mechanisms of active derivate of CIF, CIF-dATP, include a number of important pathways depicted in Fig. 1. The CIF-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 breakdown of DNA strands. These CIF-dATP-mediated actions may cause mitochondria dysfunction and induce cell apoptosis. Genini et al. reported that in primary chronic lymphocytic leukemia (B-CLL) cells, CIF-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 CIF-mediated apoptosis of human T-acute lymphocytic leukemia cells, CCRF-CEM. It has been shown that the proapoptotic effect upon CIF exposure was associated with downregulation of the Bcl-XL and/or Mcl-1 proteins of the Bcl-2 family. Moreover, CIF-induced apoptosis has been demonstrated in other types of cancer. Rahmati-Yamchi and others (Rahmati-Yamchi et al., 2015) reported that CIF has an apoptotic effect on the T47D breast cancer cells via regulation of P53/R2 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 CIF 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 CIF-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 CIF 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).

CIF 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 CIF, significant DNMT1 down-
Clofarabine effects in ALL inhibition via DNA methylation regulation

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, PTEF, and R-4RB that have been shown to be hypermethylated and silenced in various types of cancer (Majda et al., 2010; Lubecka-Pietruszewska et al., 2014).

**CIF impacts promoter methylation and transcriptional activity of PTEF, APC, RARB, and CDKN2A in ALL cells**

In Nalm-6 cells, DNA methylation of PTEF, 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.

PTEN (phosphatase and tensin homolog) and APC (APC regulator of WNT signaling pathway) tumor suppressor genes encode proteins involved in down-regulation 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, R-AR, 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 suble 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 might be indirectly related to transcriptional re-expression of these TSGs in Nalm-6 cells (Figs. 5 and 6). However, the robust R-ARB re-expression upon ClF exposure appears not to be related to promoter hypomethylation of this gene, but to other regulatory mechanisms. R-ARB transcriptional activity might be indirectly regulated by PTEN. Lefebvre’s studies revealed that through inhibition of PI3K/AKT signaling pathway, PTEN could influence the R-AR expression by blockage of NCOR2 (nuclear receptor corepressor 2) co-repressor binding within the R-AR promoter, resulting in histone acetylation and R-AR 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 CIF 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.

REFERENCES


