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

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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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INTRODUCTION

Clofarabine (2-chloro-2′-fluoro-2′-deoxyarabinosyladenine, CIF), a second-generation analog of the natural pyrimidine, 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 CIF 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, CIF-dATP becomes an active cytotoxic agent. Key mechanisms responsible for CIF-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).

CIF-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 CIF 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).

CIF 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 CIF cytotoxic activity (the antiproliferative and proapoptotic effects) have been extensively studied in ALL in vitro and in vivo models, almost nothing is known about CIF possible role as a hypomethylating agent in ALL inhibition. To the best of our knowledge, there is only one study presenting CIF epigenetic effects in primary MLL-rearranged infant ALL. Interestingly, CIF (5–10 nM) induced promoter demethylation of FHIT (fragile histidine triad diaphanosine triphosphatase) tumor suppressor gene that was accompanied by its subtle re-expression (Stumpel et al., 2015). Moreover, our team has previously observed that CIF could modulate gene expression via redesigning DNA methylation patterns within gene regulatory regions in the chronic myeloid leukemia (CML, K-562 cell

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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.

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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 of the tested fragments of the tested genes (TSGs), including APC regulator of WNT signal dependent kinase inhibitor 1A (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% CO2. In all experiments, the cells were seeded at the amount of 4×10⁵ 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-Piuterzewska 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 (Jaishwal & 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). 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 (Arasphian 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-Szmaczyk 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 (CIF), that were investigated in our previous reports, affected the DNA methylation level 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 (chr3:52469694; -139 bp from TSS; enhancer region; cg6720425 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-Szmaczyk 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 CIF at 10 nM and/or 20 nM concentrations. The MSRA included four steps: (i) digestion of cellular DNA withendonuclease that recognizes only non-methylated sequences (PTEN, RARB, and CDKN2A – HpaII, CCGG), or APC – Eco72I, CACGTG); 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-, 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:

\[
\text{Methylation level} = \left( \frac{\text{Band intensity of DNA digested with HpaII or Eco72I}}{\text{Band intensity of undigested DNA}} \right) \times 100\% \\
\]

(Majda et al., 2010; Lubecka-Piutrowska et al., 2014; Lubecka et al., 2019).

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

| Table 1. PCR primer sequences. |
|------------------------------|
| **Gene** | **Forward primer (5’-3’)** | **Reverse primer (5’-3’)** | **Product (bp)** |
| PTEN | cagcggctcgagggaccttc | gggctttctcttcctttatgttttagctag | 214 bp [+84/+1062]; 61.1°C |
| APC | cagcggctcgagggacctttcc | gggctttctcttcctttatgttttagctag | 245 bp [+84/+1062]; 61.1°C |
| RARB | cagcggctcgagggacctttcc | gggctttctcttcctttatgttttagctag | 295 bp; 58.4°C |

| Table 2. SYBR Green-based qRT-PCR primer sequences. |
|-----------------------------|
| **Gene** | **Forward primer (5’-3’)** | **Reverse primer (5’-3’)** | **Product (bp)** |
| DNMT1 | accgcgctcgtcggacacgattcc | agaccggtcctcgtcctttatgttttagctag | 253 bp [-88/+165]; 63.0°C |
| CDKN1A | gcctcagcggctcgaggtgctag | cggcgtttgacgttgtgaataaatc | 295 bp; 58.4°C |
| PTEN | cagcggctcgagggacctttcc | gggctttctcttcctttatgttttagctag | 319 bp [+164/+155]; 61.1°C |
| APC | cagcggctcgagggacctttcc | gggctttctcttcctttatgttttagctag | 292 bp; 60.0°C |
| RARB | cagcggctcgagggacctttcc | gggctttctcttcctttatgttttagctag | 295 bp; 58.4°C |
| CDKN2A | cagcggctcgagggacctttcc | gggctttctcttcctttatgttttagctag | 292 bp; 60.0°C |
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 breakage 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-Yamechi and others (Rahmati-Yamechi et al., 2015) reported that CIF 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 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-Pietruszewsk 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).

**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 (IG50), 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).

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-Pietruszewsk et al., 2014; Lubecka et al., 2019). RPPH1 (40S ribosomal protein S17), RPLP0 (60S acidic ribosomal protein P0), H3F3A (H3 histone family 3A), and BMG (β2-microglobulin) were used as housekeeping references. 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.

**CIF and DNA Methylation 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-

**Figure 2. Effects of clofarabine (CIF) 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 CIF was expressed as a percentage of viable cells in unexposed control culture (without CIF). 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.
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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 R-IRB 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 Pten, APC, Rarb, and Cdkn2a in ALL cells**

In Nalm-6 cells, DNA methylation of Pten, APC, and CDKN2A promoter fragments was decreased, in
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 \textit{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 \textit{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 \textit{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 \textit{R-ARBP} 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 \textit{R-ARBP} gene upon ClF 20 nM exposure (Fig. 6). 10 nM concentration of ClF has caused around a 4-fold increase in the \textit{R-ARBP} mRNA level. The \textit{PTEN} expression was also significantly elevated. ClF used at 10 and 20 nM led to over 1.5- and 3-fold increases in the \textit{PTEN} transcript level, respectively (Fig. 6). In the case of \textit{APC} and \textit{CDKN2A} genes, up-regulation by 18-52% was observed upon ClF exposure at both tested concentrations (Fig. 6).

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

As we mentioned before, there is only one study demonstrating ClF-mediated epigenetic effects in primary MLL-rearranged infant ALL. Stumpel \textit{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 \textit{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 \textit{et al.}, 2010) and breast cancer cells \textit{in vitro} (Lubecka-Pietruszewska \textit{et al.}, 2014). In the case of \textit{APC}, \textit{PTEN}, and \textit{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 \textit{R-ARBP} re-expression upon ClF exposure appears not to be related to promoter hypomethylation of this gene, but to other regulatory mechanisms. \textit{R-ARBP} transcriptional activity might be indirectly regulated by PTEN. Lefebvre’s studies revealed that through inhibition of PI3K/AKT signaling pathway, PTEN could influence the \textit{R-ARBP} expression by blockage of NCOR2 (nuclear receptor co-repressor 2) co-repressor binding within the \textit{R-ARBP} promoter, resulting in histone acetylation and \textit{R-ARBP} reactivation (Lefebvre \textit{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 \textit{DNMT1} and \textit{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 modula-
tor in anti-angiogenesis therapy.

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Authors’ Contributions

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Disclosure

The authors declare no conflict of interest.

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