Clofarabine-phytochemical combination exposures in CML cells inhibit DNA methylation machinery, upregulate tumor suppressor genes and promote caspase-dependent apoptosis

AGNIESZKA KAUFMAN-SZYMCZYK1, KATARZYNA MAJDA1, AGATA SZULAWSKA-MROCZEK1, KRYSZYNA FABIANOWSKA-MAJEWSKA2 and KATARZYNA LUBECKA1

1Department of Biomedical Chemistry, Faculty of Health Sciences, Medical University of Lodz, 92-215 Lodz; 2Faculty of Medicine, Lazarski University, 02-662 Warsaw, Poland

Received March 15, 2019; Accepted July 2, 2019

DOI: 10.3892/mmr.2019.10619

Abstract. Clofarabine (2-chloro-2'-fluoro-2'-deoxyarabinosyladenine, CIF), a second-generation 2'-deoxyadenosine analog, possesses a variety of anti-cancer activities, including the capacity to modulate DNA methylation marks. Bioactive nutrients, including resveratrol (RSV) and all-trans retinoic acid (ATRA) have been indicated to regulate epigenetic machinery in malignant cells. The purpose of the current study was to evaluate whether the tested phytochemicals, RSV or ATRA, can improve the therapeutic epigenetic effects of CIF in chronic myeloid leukemia (CML) cells. The present study investigates, to the best of our knowledge, for the first time, the influence of CIF in combination with RSV or ATRA on the expression of relevant modifiers of DNA methylation machinery, including DNA Methyltransferase 1 (DNMT1) and Cyclin dependent kinase inhibitor 1A (CDKN1A) in CML cells. Subsequently, the combinatorial effects on promoter methylation and transcript levels of methylation-silenced tumor suppressor genes (TSGs), including phosphatase and tensin homologue (PTEN) and retinoic acid receptor beta (RARB), were estimated using MSRA and qPCR, respectively. The tested TSGs were chosen according to bioinformatical analysis of publicly available clinical data of human DNA methylation and gene expression arrays in leukemia patients. The K562 cell line was used as an experimental CML in vitro model. Following a period of 72 h exposure of K562 cells, the tested combinations led to significant cell growth inhibition and induction of caspase-3-dependent apoptosis. These observations were accompanied by DNMT1 downregulation and CDKN1A upregulation, with a concomitant enhanced decrease in DNMT1 protein level, especially after ATRA treatment with CIF. Concurrent methylation-mediated RARB and PTEN reactivation was detected. The results of the current study demonstrated that CIF that was used in combination with the tested phytochemicals, RSV or ATRA, exhibited a greater ability to remodel DNA methylation marks and promote cell death in CML cells. These results may support the application of CIF combinations with natural bioactive agents in anti-leukemic epigenetic therapy.

Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative disorder characterized, in the vast majority of cases, by the presence of Philadelphia chromosome (Ph) formed by translocation of sections between chromosomes 9 and 22. Abnormally short chromosome 22 encodes the chimeric p210 BCR-ABL tyrosine kinase protein, a product of the oncogene BCR-ABL, constitutively active enzyme that drives uncontrolled cellular growth and differentiation of CML cells. The Ph chromosome with the BCR-ABL fusion gene is also present in 25-50% of adult patients with acute lymphoblastic leukemia (ALL) and rare cases of acute myeloid leukemia (AML) (1,2).

BCR-ABL is the target of tyrosine kinase inhibitors (TKIs) introduced, with great success, for the treatment of CML patients at the end of the last century. Despite the high therapeutic efficacy of TKIs, around 25% of CML patients develop resistance to 1st (Imatinib) and 2nd line (Desatinib, Nilotinib) of TKIs. This resistance may result from mutations within the kinase domain of BCL-ABL, although other mechanisms of primary or acquired resistance to TKIs have been investigated as well (2-5). Apart from these genetic abnormalities also epigenetic alterations may contribute to CML pathogenesis and drug resistance (6,7). TKIs effectively inhibit BCR-ABL kinase, although CML stem cell survival has been observed (5). Thus, it is reasonable to seek a novel epigenetic approach to improve CML treatment.

Epigenetic alterations regulate gene expression via DNA methylation, histone modifications and activity of non-coding RNAs (8,9). Interference between these epigenetic processes affects chromatin accessibility for transcription (8). Although,
it is still DNA methylation that is the most stable epigenetic reaction modulating gene expression. It consists of the attachment of methyl group to cytosine mainly in CpG islands within gene promoters. Dysregulated epigenetic code, including aberrant methylation patterns, is often observed and considered to be one of the causes, in addition to genetic changes, of the development and progression of neoplastic diseases (10,11). In cancer cells, a certain pool of genes (mainly tumor suppressor genes) is silenced by methylation of their promoter regions while other genes are activated (oncogenes and prometastatic genes) through the hypomethylation of their regulatory regions. Methyltransferase DNA methylation patterns within gene regulatory regions in cancer cells (13,14). All these molecular mechanisms of CIF anti-cancer action contributed to the FDA-approved therapeutic usage of this drug in ALL and some AML cases (15,16).

As we have shown in our previous studies deoxyadenosine analog-clofarabine (2-chloro-2'-fluoro-2'-deoxyarabinosyladenine, CIF), apart from its anticancer activity resulting from inhibition of ribonucleotide reductase and DNA polymerases, and apoptosis induction by altering mitochondrial activity, can also modulate gene expression via redesigning DNA methylation patterns within gene regulatory regions in cancer cells (13,14). All these molecular mechanisms of CIF anticancer action contributed to the FDA-approved therapeutic usage of this drug in ALL and some AML cases (15,16).

Natural phytochemicals have raised considerable interest not only as chemopreventive agents but also as chemotherapeutic adjuvants because of their anticancer properties demonstrated in a large number of studies (17). Resveratrol (3,4,5-trihydroxystilbene, RSV), the polyphenol from red grapes and peanuts, has been shown to modulate cell cycle, survival and apoptosis also through altering gene methylation patterns (18-22). Other possible molecular targets of RSV are AMPK and SIRT1, mTOR, NF-kB, PI3K/AKT, MAPK signaling pathways (23).

ATRA (all-trans retinoic acid) is a natural, physiologically active, predominant metabolite of vitamin A. ATRA acts as a hormone and impacts many physiological processes. ATRA through its binding to specific nuclear retinoic acid receptors RARs (RARA, RARB and RARG) that form heterodimers with retinoid X receptors RXRs can regulate transcription of some genes (24). Within promoters of these genes, the retinoic acid response elements (RAREs) have been found. According to present knowledge, the transcriptional activity of RAR/RXR complex results from the incorporation of ATRA to RAR receptors. This model of interaction is known as a classical or genomic pathway that regulates cell differentiation, cell cycle, and apoptosis (25). RARs and RXRs are able to create heterodimers with other receptors, such as vitamin D receptor (VDR), steroid receptors or peroxisome proliferator-activated receptor (PPAR). There is evidence that ATRA can also regulate the gene expression independently of the presence of RAREs. Furthermore, ATRA and its receptors may affect other critical signaling pathways, including NF-κB, IFN-G, TGFβ, VEGF, and MAPK pathways, as well as cause chromatin remodeling (24,26). Because of ATRA importance in cell physiology, the antitumor activity of retinoids has been broadly studied. Consequently, ATRA heretofore has gained FDA approval for treatment of APL (acute promyelocytic leukemia) and cutaneous T-cell lymphoma. There are some suggestions that the cause of the lack of ATRA anticancer activity in other types of leukemia and solid tumors might be associated with aberrant epigenetic marks, for example, frequent DNA methylation-mediated silencing of retinoic acid receptor beta (RARB) (26,27).

Interestingly, the growing body of literature demonstrates that some natural bioactive compounds, including ATRA and RSV, might be indirectly involved in the regulation of DNMT1 expression and/or DNMT1 activity. DNMT1 has been shown to be overexpressed in many types of cancer (28). The following mechanisms responsible for ATRA or RSV-mediated DNMT1 downregulation in cancer cells have been detected, i.e., cyclin-dependent kinase inhibitor 1A (CDKN1A) transcriptional reactivation (18,29) followed by decreased activity of E2F (elongation factor 2) transcription factor, as well as re-expression of DNA methylation-silenced tumor suppressor genes, phosphatase and tensin homologue (PTEN) and RARB, encoding proteins that may inhibit activity of AP-1 (activator protein-1) transcriptional complex (29,30). E2F and AP-1 transcription factors activate DNMT1 expression due to the presence of binding sites in DNMT1 regulatory region (31,32). Moreover, CDKN1A (p21) belongs to tumor suppressor genes and encodes a protein that competes with DNMT1 for the same binding site on proliferating cell nuclear antigen (PCNA), the homotrimeric ring surrounding DNA) during DNA replication. It disrupts the forming of DNMT1/PCNA complex and subsequently may lead to inhibition of DNA methylation reaction (33,34). PTEN was shown to be mutated or DNA methylation-silenced in a large number of malignancies. PTEN protein as a phosphatase negatively regulates intracellular levels of phosphatidylinositol-3,4,5-trisphosphate in cells which is crucial for its tumor suppressive activity. The dephosphorylated phosphoinositide through negative regulation of PI3K/AKT and MAPK/AP-1 signaling pathways modulates cell cycle progression and cell survival (35).

The promising results of combining nucleoside analogues, such as cladribine and fludarabine (CIF precursors), with ATRA or RSV in breast cancer cells, including methylation-mediated PTEN and RARB transcriptional reactivation (18,30), indicate that the combination of CIF with these phytochemicals (ATRA or RSV) may exhibit a new effective approach in anticancer epigenetic therapy.

As mentioned above, alterations in DNA methylation marks are common in cancer cells, including different leukemia cells. Thus, the present study aimed to evaluate anticancer potential of CIF combined with natural bioactive compounds, RSV or ATRA, in K562 cells representing an experimental in vitro model of CML cells. This is the first study to investigate the influence of CIF-phytochemical combination exposures on the regulation of DNA methylation machinery in CML cells. We focused on determining any changes in DNMT1 and CDKN1A expression, as well as in promoter methylation and expression of tumor suppressor genes PTEN and RARB.

Materials and methods

Compounds and chemicals. All tested compounds CIF, ATRA, and RSV were purchased from Sigma-Aldrich. CIF.

3598  KAUFMAN-SZYMCZYK et al: ANTI-CANCER EFFECTS OF CLOFARABINE-PHYSOCHEMICAL
was dissolved in sterile water (1 mM) and stored at -20°C. Solutions of ATRA (10 mM) and RSV (5 mM) were prepared in 96% ethanol and stored in the dark at -20°C. Subsequent dilutions were made in growth fresh medium with a final ethanol concentration of 0.1% (v/v), and this ethanol concentration was used as vehicle control in all experiments.

**Cell culture, growth and viability assay.** Human erythroleukemic cell line K562 (American Type Culture Collection, ATCC) was cultured in RPMI 1640 medium with HEPES (Lonza) supplemented with 2 mM L-glutamine and 10% foetal bovine serum (FBS), 1 U/ml penicillin and 1 µg/ml streptomycin (Sigma-Aldrich), at 37°C and a humified atmosphere of 5% CO2. K562 cell line was routinely verified by morphology, invasion and growth rate. The tested cell line was authenticated by DNA profiling using the short tandem repeat (ATCC), in 2018. In all experiments the cells were seeded at the amount of 4x10^4 cells per ml, and were cultured for 72 h with three different compounds, CIF, ATRA and RSV, used separately, at concentrations equal to GI_{50} concentrations (i.e., doses leading to 50% inhibition of cell growth), respectively: 8 nM (CIF), 30 µM (ATRA) and 11.5 µM (RSV). Additionally, the cells were treated for 72 h with the compounds administered in two combinations: CIF + ATRA (both at GI_{50} concentrations, i.e., 8 nM for CIF and 30 µM for ATRA) and CIF + RSV (both at GI_{50} concentrations, i.e., 8 nM for CIF and 11.5 µM for RSV).

Cell growth and viability were determined using the trypan blue (Sigma-Aldrich) exclusion test, to estimate GI_{50} values. The number of viable cells in culture treated with the tested compounds was expressed as a percentage of viable cells in control untreated culture (without the compounds, vehicle control). The following calculation has been used: (viable exposed/viable vehicle control) * 100%. 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 were determined after 72 h compound exposure by flow cytometry analysis using annexin V/providium iodide (PI) (FITC Annexin V Apoptosis Detection Kit II, BD Pharmingen) staining, according to the manufacturer's protocol (13). The following excitation/emission wavelengths have been used: FITC 488/519 nm and PI 488/617. Caspase-3 assay (PE Active Caspase-3 Apoptosis Kit, BD Pharmingen) was performed to estimate its activity as a marker of the early stage of the caspase-dependent apoptotic pathway. The excitation/emission wavelengths of 488/578 nm have been applied. The flow cytometry analysis was carried out using BD FACSuite™ version 1.2.1 software.

**Reverse transcription quantitative (RT-q) PCR.** Total RNA was isolated using TRIZOL® (Invitrogen, USA). cDNA 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 RT-qPCR reactions were carried out in a Rotor-Gene TG-3000 machine (Corbett Research, Australia) as we previously described (13,14). RPS17 (40S ribosomal protein S17), RPLP0 (60S acidic ribosomal protein P0), H3F3A (H3 histone family 3A), and BMG (β₂-microglobulin) were used as housekeeping control genes. The relative expression of each tested gene (DNMT1, CDKNIA, PTEN, and RARB) was normalized to the geometric mean of these four housekeeping genes, according to the method of Pfaffl et al (37). Primers sequences for RT-qPCR are shown in Table II.

**Measuring the amount of DNMT1 protein.** Protein nuclear extracts were isolated using the EpiQuik Nuclear Extraction Kit (Epigentek), according to manufacturer's protocol. The ELISA-like EpiQuik DNMT1 Assay Kit (Epigentek) was used for quantification of DNMT1 (DNA methyltransferase) in 10 µg of the total protein content. Each measurement was performed in triplicates according to the instructions in the manual. The absorbance at 450 nm was measured on a microplate reader (GloMax-Multi+ Microplate Multimode Reader, Promega) within 2-10 min.

**Statistical analysis.** Results from three independent experiments are presented as the mean ± standard deviation (SD). Statistical analysis of cell viability, apoptosis, MSRA, qPCR and ELISA-like EpiQuik DNMT1 assays was performed using two-way analysis of variance (ANOVA) followed by Bonferroni post hoc test. The results were considered statistically significant when P<0.05.

**Results and Discussion.**

**Effects of RSV and ATRA combined with CIF on inhibition of CML cell growth and apoptosis induction.** Following 72 h-exposure, all the tested compounds used alone, CIF, RSV, and ATRA, inhibited K562 cell growth in a dose-dependent manner with low cytotoxicity (Fig. 1A-C and F). The trypan blue exclusion test was carried out to determine concentrations leading to 50% inhibition of cell growth (GI_{50}) (Fig. 1A-C). The GI_{50} concentration for CIF was determined as equal to 8 nM in K562 cells (Fig. 1A), as we showed previously (13). The GI_{50} values for RSV and ATRA were determined as equal to 11.5 and 30 µM, respectively (Fig. 1B and C). The number of dead cells upon exposure to the tested compounds at GI_{50} concentrations did not exceed 10% (Fig. 1A-C), which support the use of all the compounds at GI_{50} concentrations in the combinatorial administrations, CIF and RSV, or CIF and ATRA (Fig. 1D-F).

Next, the cytotoxicity of all the compounds administered individually and in combinations was determined by flow cytometric assay (Fig. 2). The number of necrotic (Ann-/PI+) cells did not exceed 10% of all the cells upon any of the exposures, supporting low cytotoxicity of the tested concentrations (Fig. 2B, top and bottom panels). The use of CIF+RSV combination resulted in the most severe induction of apoptosis in K562 cells (Fig. 2C, upper panel). The
number of apoptotic cells increases from nearly 4% after CIF alone and 10% after RSV alone to 15% after combined administration CIF+RSV (Fig. 2C, upper panel). This enhanced pro-apoptotic effect of combinatorial CIF and RSV was associated with caspase-3 activation (Fig. 2D, upper panel). Upon 72 h-incubation with this combination over 9% of all K562 cells showed active caspase 3, whereas after CIF or RSV alone approximately 2% or 5.5% of all K562 bound antibodies against caspase 3, respectively (Fig. 2D, upper panel). The extent of the effects of ATRA alone and CIF+ATRA on cell viability and caspase-dependent apoptosis was not as robust as for RSV used alone or in combination with CIF (Fig. 2C, bottom panel). The number of apoptotic cells increases from 4-5% after CIF or ATRA used alone to slightly more than 6% after combined administration, CIF+ATRA (Fig. 2C, bottom panel). The percentage of K562 cells with active caspase-3 was similar after the individual (2-3%, CIF or ATRA) and combinatorial (3.5%, CIF+ATRA) exposures (Fig. 2D, bottom panel).

Hitherto, only Lee and colleagues demonstrated that RSV in combination with CIF induces relevant anti-proliferative effects in malignant mesothelioma MSTo‑211H and H‑2452 in combination with CIF. This enhanced pro-apoptotic effect of combinatorial CIF and RSV was associated with caspase-3 activation (Fig. 2D, upper panel). Upon 72 h-incubation with this combination over 9% of all K562 cells showed active caspase 3, whereas after CIF or RSV alone approximately 2% or 5.5% of all K562 bound antibodies against caspase 3, respectively (Fig. 2D, upper panel). The extent of the effects of ATRA alone and CIF+ATRA on cell viability and caspase-dependent apoptosis was not as robust as for RSV used alone or in combination with CIF (Fig. 2C, bottom panel). The number of apoptotic cells increases from 4-5% after CIF or ATRA used alone to slightly more than 6% after combined administration, CIF+ATRA (Fig. 2C, bottom panel). The percentage of K562 cells with active caspase-3 was similar after the individual (2-3%, CIF or ATRA) and combinatorial (3.5%, CIF+ATRA) exposures (Fig. 2D, bottom panel).

First of all, we analyzed the publicly available data from Oncomine for DNMT1 expression in different types of leukemia, as DNMT1 overexpression has been observed in many types of cancer. As depicted in Fig. 3A, in almost all types of leukemia DNMT1 expression is significantly higher compared to healthy individuals. Only in CML, the level of DNMT1 expression is lower than in normal blood cells, although the only available microarray data of CML, presented in Fig. 3A, are not statistically significant, so it is difficult to draw clear conclusions about the level of DNMT1 in CML cells. However, Mizuno et al. (40) reported relevant DNMT1 up-regulation in AML and CML cells as compared to normal blood cells.

In our study, in K562 cells treated with CIF at GI₅₀ concentration (8 nM) for 72 h, slight almost 10% reduction in DNMT1 gene expression, in comparison to control unexposed cells, was estimated using RT-qPCR and Pfaffl’s method (37) (Fig. 3D). The effects of exposure to RSV or ATRA administered alone, also at GI₅₀ concentrations, caused an even greater diminution in DNMT1 mRNA levels by 15 and 35%, respectively. However, the most robust, over 40% decrease in DNMT1 expression was noticed as the effect of combined exposure to CIF and ATRA (Fig. 3D, bottom panel). These changes in the expression of DNMT1 at the mRNA level correspond to changes in gene expression at the protein level, determined using ELISA-like commercial immunoassays (Fig. 3C). The combination CIF+ATRA caused almost 50% reduction in DNMT1 protein level as compared to control K562 cells (Fig. 3C, bottom panel). CIF and ATRA used alone led only to 11 and 29% decrease in DNMT1 protein levels, respectively (Fig. 3C). It has been shown that manifestation of the catalytic function of DNMT1 enzyme requires its binding to PCNA during DNA replication (33,34). Moreover, CDKN1A, as an antagonist of DNMT1, binds to the same DNA methylation pattern as a common feature of cancer cells. The purpose of the study was to investigate the interdependence between DNA methylation and expression of selected tumor suppressor genes and the expression of the main DNA methyltransferase, DNMT1, after treatment of model CML cells with a chemotherapeutic agent, CIF, combined with natural bioactive compounds, RSV and ATRA.

First of all, we analyzed the publicly available data from Oncomine for DNMT1 expression in different types of leukemia, as DNMT1 overexpression has been observed in many types of cancer. As depicted in Fig. 3A, in almost all types of leukemia DNMT1 expression is significantly higher compared to healthy individuals. Only in CML, the level of DNMT1 expression is lower than in normal blood cells, although the only available microarray data of CML, presented in Fig. 3A, are not statistically significant, so it is difficult to draw clear conclusions about the level of DNMT1 in CML cells. However, Mizuno et al. (40) reported relevant DNMT1 up-regulation in AML and CML cells as compared to normal blood cells.

In our study, in K562 cells treated with CIF at GI₅₀ concentration (8 nM) for 72 h, slight almost 10% reduction in DNMT1 gene expression, in comparison to control unexposed cells, was estimated using RT-qPCR and Pfaffl’s method (37) (Fig. 3D). The effects of exposure to RSV or ATRA administered alone, also at GI₅₀ concentrations, caused an even greater diminution in DNMT1 mRNA levels by 15 and 35%, respectively. However, the most robust, over 40% decrease in DNMT1 expression was noticed as the effect of combined exposure to CIF and ATRA (Fig. 3D, bottom panel). These changes in the expression of DNMT1 at the mRNA level correspond to changes in gene expression at the protein level, determined using ELISA-like commercial immunoassays (Fig. 3C). The combination CIF+ATRA caused almost 50% reduction in DNMT1 protein level as compared to control K562 cells (Fig. 3C, bottom panel). CIF and ATRA used alone led only to 11 and 29% decrease in DNMT1 protein levels, respectively (Fig. 3C). It has been shown that manifestation of the catalytic function of DNMT1 enzyme requires its binding to PCNA during DNA replication (33,34). Moreover, CDKN1A, as an antagonist of DNMT1, binds to the same
domain of PCNA. Thus, CDKN1A polypeptide may disturb the formation of PCNA-DNMT1 complex, and then leads to repression of DNA methylation processes (41). The estimation of CDKN1A expression on mRNA level (in connection and comparison with DNMT1 expression) allows defining the potential interrelations between DNA methylation processes and expression of DNMT1 and CDKN1A genes in cells exposed to natural bioactive compounds and CIF, also in combined therapy. So we found that changes in DNMT1 expression are associated with concomitant changes in CDKN1A mRNA level (Fig. 3D and E). Upon 72 h-incubation of K562 cells with ATRA at GI₅₀ concentration, CDKN1A transcript level increased almost three times, and almost two times in cells exposed to GI₅₀ concentration of RSV (Fig. 3E), in comparison to control unexposed cells. Since ATRA binds to nuclear RARs that heterodimerize with RXRs, it may further modulate transcription through cognate response elements in the promoters of the target genes including CDKN1A (42,43). Due to structural similarity of RSV to estradiol and its binding to estrogen receptors (ERs) it may elicit similar responses as upon endogenous estrogens and modulate the expression of estrogen-responsive genes, such as CDKN1A (44).

Combination of ATRA and CIF resulted in a 3.3-fold increase in CDKN1A mRNA level. CIF used alone did not influence the CDKN1A expression, and the combination of CIF+RSV did not increase the level of CDKN1A above that achieved with RSV used alone (Fig. 3E, upper panel). Our findings suggest that especially CIF+ATRA-mediated concomitant CDKN1A induction and DNMT1 downregulation in K562 cells may decrease DNA methylation efficiency of TSGs.

According to Oncomine publicly available data in all types of leukemia, CDKN1A expression is significantly decreased as compared to normal blood cells (Fig. 3B). Thus, reactivation of CDKN1A gene encoding protein capable of cell cycle arrest is one of the goals of anti-leukemic therapy (45). DNA methylation-mediated PTEN reactivation in K562 cells exposed to CIF combined with RSV or ATRA. PTEN is a multifunctional tumor suppressor gene, encoding a phosphatase with dual specificity for lipid and protein substrates, has been shown to be silenced in multiple cancers, including different types of leukemia (Fig. 4A). The PTEN downregulation in cancer cells may be related to genetic changes, but also it may result from hypermethylation of its promoter region, which partly implies epigenetic regulation of PTEN transcription (46-48). DNA methylation-mediated regulation of PTEN expression was observed for example in ALL (46), breast cancer (47) and colorectal cancer (48).

Oncomine data indicate that PTEN is transcriptionally silenced in three types of leukemia, including ALL, CLL and AML (Fig. 4A). According to the results of one available study with CML patients, no significant difference in PTEN expression has been noticed between cancer and normal blood cells (Fig. 4A). The proximal promoter region including CpG island of PTEN has been depicted in Fig. 4C. According to publicly available Illumina 450K data (GSE106600), currently the only available study for CML patients, any significant changes...
have not been observed in PTEN promoter methylation within CpGs covered on Illumina 450K microarray in CML cells as compared to normal blood cells (Fig. 4B). The detailed map in Fig. 4C shows the exact position of the tested CpG site, that is the CpG site within PTEN proximal promoter CpG island, i.e., 5’UTR and/or first exon (+973 bp from transcription start site, TSS), not covered on Illumina 450K array (marked in black), located between two CpGs from this microarray platform, i.e., cg03588460 (+337 bp from TSS, marked in gray) and cg08859916 (+997 bp from TSS, marked in gray) (Fig. 4C). In our previous studies, this CpG (chr10: 89624078, according to Human GRCh37/hg19 Assembly) has been shown to be differentially methylated between breast cancer cell lines with different level of invasiveness, suggesting its regulatory role in PTEN transcription (14,18,30). Putative transcription factor binding sites are demonstrated on the PTEN gene map (Fig. 4C), as predicted using TransFac. The multiple binding sites for DNA methylation-sensitive transcription factors within the tested PTEN promoter fragment support its potential regulatory role in PTEN transcription (Fig. 4C) (18,47,48).

Previously, we identified the role of CIF in the regulation of promoter methylation and expression of PTEN in K562 (CML) cells (13). In the present study, we checked if RSV and ATRA used alone can also affect the transcriptional activity of these genes through the remodeling of their promoter methylation. 72-hour exposure of K562 cells to RSV used alone at GI50=11.5 µM, and ATRA used alone at GI50=30 µM concentration led to significant decreases in PTEN promoter methylation by 51 and 24%, respectively (Fig. 4D), comparing to control unexposed cells (63%). CIF administrated alone mediated 7% diminution in PTEN promoter methylation level, although no significant changes in DNMT1 expression have been observed. Our initial unpublished studies in K562 cells indicate that CIF exposure leads to inhibition of the activity of two enzymes important for 2'-deoxyadenosine metabolism, deoxyadenosine deaminase (ADA) and S-adenosyl-L-homocysteine (SAH) hydrolase. CIF used at 5 nM concentration caused decreases in ADA and SAH-hydrolase activities by 30 and 15%, respectively. The CIF-mediated repression of ADA activity may lead to 2'-deoxyadenosine accumulation up to the level of toxic concentration in exposed cells. The raised levels of 2'-deoxyadenosine in cells can indirectly disrupt DNA methylation reaction via SAH-hydrolase inhibition leading to SAM pool depletion. A similar effect was shown by Wyczekowska and Fabianowska-Majewska (49) in K562 cells exposed to cladribine (49).

Upon exposure of K562 cells to CIF combined with ATRA, we observed almost complete demethylation of PTEN promoter compared to control K562 cells (Fig. 4D, bottom panel), whereas the extent of PTEN hypomethylation followed by CIF+RSV administration was similar to that caused by RSV alone (by approximately 50%) (Fig. 4D, upper panel). These alterations in the PTEN methylation pattern in K562 cells were accompanied by enforced expression of this gene (Fig. 4E). The robust PTEN upregulation was detected after both combinatorial administrations, CIF+RSV or CIF+ATRA, that caused increases in PTEN transcript level by 59 and 44%, when compared to control K562 cells, respectively (Fig. 4E). Surprisingly, although CIF and RSV used alone did not lead to any significant changes in PTEN expression in K562 cells upon 72 h of exposure, those compounds together exerted significant 59% PTEN upregulation (Fig. 4E, bottom panel).

Possibly, different concentration of CIF and RSV used alone as well as other exposure time could benefit in stronger PTEN
re-expression (13). It may also suggest that these compounds may cooperate in other unknown mechanisms driving changes in PTEN expression.

Our findings suggest partial involvement of DNA methylation in the regulation of PTEN transcriptional activity, although other mechanisms can play an additional role as well (46-48).
RARB transcriptional reactivation followed by combinatorial exposures in K562 cells partly related to its promoter hypomethylation. Expression of some tumor suppressor genes might be indirectly regulated by PTEN, one of them is RARB. Lefebvre et al (50) reported that PTEN via negative regulation of PI3K/AKT signaling pathway could affect RARB expression by blocking of SMRT co-repressor recruitment to RARB promoter region, which enhances histone acetylation.
and promotes *RARB* transcription (50). Moreover, according to publically available data (Oncomine), tumor suppressor gene *RARB* is downregulated in all types of leukemia (Fig. 5A). In Fig. 5B, the methylation status of CpG sites at TSS200 promoter region of *RARB* enhancer in CML and healthy individuals has been depicted (analyzed by Illumina 450K Human Methylation Array, publicly available datasets from NCBI's Gene Expression Omnibus GEO no. GSE106600). Among the 5 CpG sites within the demonstrated fragment of the *RARB* promoter, the CpG site located -139 bp from transcription start site (TSS), which methylation state was tested by MSRA, is indicated by a black oval shape. The CpG sites located nearby, covered on Illumina 450K microarray platform, are depicted by gray ovals. Putative transcription factor binding sites are marked as predicted using TransFac. The effects of CIF, RSV and ATRA used alone, as well as CIF in combination with RSV or ATRA on (D) methylation of *RARB* promoter, and (E) expression on mRNA level of *RARB* gene in K562 cells. All compounds used at GI<sub>50</sub> concentrations in all experiments. Data represent the mean ± SD of three independent experiments. *P<0.05 and ***P<0.001 vs. vehicle control; ###P<0.001 vs. CIF alone; ^P<0.05 and ^^^P<0.001 vs. RSV or ATRA alone. RARB, retinoic acid receptor beta; SD, standard deviation; CIF, clofarabine; RSV, resveratrol; ATRA, all-trans retinoic acid.
In K562 cells exposed to CIF, RSV or ATRA, used alone, as well as to CIF+RSV and CIF+ATRA, statistically significant demethylation of RARB gene promoter was observed. CIF reduced the RARB promoter methylation level by approximately 10% in comparison to control cells, RSV by 41% and ATRA by 26% (Fig. 5D). Combinational treatment with CIF+ATRA caused almost total demethylation of RARB promoter with concomitant over 3-fold increase in gene expression (Fig. 5D and E, bottom panels). Interestingly, the exposure to RSV and CIF+RSV, despite robust alteration in promoter methylation led to less pronounced RARB up-regulation, by 60-70% (Fig 5D and E, upper panels).

It is worth pointing out, that the higher extent of changes mediated by CIF+ATRA combination in exposed K562 cells, i.e., CDKN1A transcriptional reactivation that may result in decreased E2F activity, as well as re-expression of PTEN and RARB encoding proteins that inhibit AP-1 activity, strongly support enhanced DNM1 promoter methylation level by approximately 10% in comparison to control cells, RSV by 41% and ATRA by 26% (Fig. 5D). Combinational treatment with CIF+ATRA caused almost total demethylation of RARB promoter with concomitant over 3-fold increase in gene expression (Fig. 5D and E, bottom panels). Interestingly, the exposure to RSV and CIF+RSV, despite robust alteration in promoter methylation led to less pronounced RARB up-regulation, by 60-70% (Fig 5D and E, upper panels).

As some authors suggest, CML is the ‘poster child’ for targeted cancer therapy. The identified target, a product of abnormal gene BCR-ABL became the aim of drug development and as mentioned above the TKIs inhibitors were introduced to CML therapy. Nowadays the first- or second-generation TKIs are the first-line treatment of patients with newly diagnosed CML. Although initial responses are high, in more than 25% of patients the therapy fails and/or they develop resistance to the treatment. For several years, intensive work has been underway to explain treatment failure and to identify different mechanisms of the drug resistance. The resistance to TKIs based on clinical outcomes can be explained by genomic mechanisms (mutations in the BCR-ABL domain), but also by BCR-ABL-independent mechanisms (poor compliance, drug influx and efflux, activation of alternative signaling pathways, plasma TKI concentration, insensitivity of quiescent stem cells) (51). However, epigenetic dysregulation of the expression of the CML-associated genes has been reported as well (7). Thus, in order to improve the effectiveness of CML therapies, there is a strong need to develop new treatment strategies.

Nishioka et al reported that hypermethylation of PTEN promoter is associated with this gene downregulation and activation of pro-survival signaling mediated by AKT in leukemia cells. According to the other authors’ findings, the PTEN silencing induced by DNA methylation requires EZH2 and DNA methylation enzymes. Moreover, the authors claim that the epigenetic silencing of PTEN is one of the mechanisms that cause drug resistance in individuals with leukemia after exposure to Imatinib (52,53). In this context demethylation and re-expression of PTEN seem to be a promising way to achieve long term therapeutic response. Our initial results show that natural bioactive compounds, mainly ATRA but also RSV, especially in combination with CIF, might positively modulate PTEN expression. Additionally, a combination of RSV with CIF indicates high pro-apoptotic activity in K562 CML cells. In work of Can et al (54) RSV (used alone in high dose) has also effectively induced apoptosis of K562/IMA-3 cells (resistant CML cells). These results may suggest the potential use of ATRA and/or RSV in CML therapy not only in patients with primary but also with acquired resistance to TKIs.

In summary, our study is the first to demonstrate the epigenetic anticancer capacity of the combinatorial exposures of CIF and ATRA or RSV in CML cells. Upon 72 h-treatment, the tested combinations led to significant cell growth inhibition and greater induction of caspase-3-dependent apoptosis. These observations may be related to accompanied relevant DNM1I downregulation and robust CDKN1A upregulation, with a concomitant, enhanced decrease in DNM1I protein expression due to the presence of binding sites in DNM1 regulatory region. A competition of CDKN1A (p21) with DNMT1 for the same binding site in proliferating cell nuclear antigen. Shc, SH2-containing collagen-related proteins; PI2P, phosphatidylcholineinolinositol (4,5)-bisphosphate; PI3P, phosphatidylcholineinolinositol (3,4,5)-trisphosphate; SMRT, thyroid-, retinoic-acid-receptor-associated corepressor. CIF, clofarabine; DNM1, DNA methyltransferase 1; RSV, resveratrol; ATRA, all-trans retinoic acid; PTEN, phosphatase and tensin homologue; CDKN1A, Cyclin dependent kinase inhibitor 1A.
level, especially after CIF with ATRA. Concurrent methylation-mediated RARB and PTEN reactivation have been detected. The proteins encoded by these genes are crucial for the regulation of important intracellular oncogenic signaling pathways, including PI3K/AKT and MAPK/AP-1 pathways. Taken together, our results reveal that CIF used in combination with the tested phytochemicals, RSV or ATRA, has the higher ability to remodel DNA methylation marks and promote cell death in CML cells.

Future studies will focus on assessing the efficacy of clofarabine-phytochemical combination exposures in other CML in vitro and in vivo models. We believe that further extensive studies of this new combinatorial strategy, the CIF combinations with ATRA or RSV, may support its translational application as a therapeutic epigenetic approach against CML.

Acknowledgements

The authors would like to thank Dr Justyna Jakubowska from The Department of Pediatrics, Oncology and Hematology, as well as Professor Piotr Smolewski and Dr Barbara Cebula-Obrzut from Department of Experimental Hematology, Medical University of Lodz, Poland, for their support in performing and analyzing flow cytometry experiments.

Funding

This study was supported by Medical University of Lodz in Poland (grant nos. 503-6-099-01/503-61-001, 502-03-6-099-01/502-64-007, 502-03-6-099-01/502-64-089 and 502-03-6-099-01/502-64-133) granted to The Department of Biomedical Chemistry, Faculty of Health Sciences at the Medical University of Lodz (Lodz, Poland).

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

AKS and KM conducted the experiments. AKS, KM, ASM, KFM and KL performed the analysis and contributed to writing and editing the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Chen Y, Peng C, Li D and Li S: Molecular and cellular bases of chronic myeloid leukemia. Protein Cell 1: 124-132, 2010.
2. Soverini S, de Benedittis C, Mancini M and Martinelli G: Mutations in the BCR-ABL1 kinase domain and elsewhere in chronic myeloid leukemia. Clin Lymphoma Myeloma Leuk 15 (Suppl): S120-S128, 2015.
3. Druker BJ, Talpaz M, Resta DJ, Peng B, Buchdunger E, Ford JM, Lydon NB, Kantarjian H, Capdeville R, Ohno-Jones S and Sawyers CL: Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. N Engl J Med 344: 1031-1037, 2001.
4. Bhatia R, Holtz M, Niu N, Gray R, Snyder DS, Sawyers CL, Arber DA, Slovak ML, and Forman SJ: Persistence of malignant hematopoietic progenitors in chronic myelogenous leukemia patients in complete cytogenetic remission following imatinib mesylate treatment. Blood 101: 4701-4707, 2003.
5. Chomel JC, Bonnet ML, Sorel N, Bertrand A, Meunier MC, Fiechelson S, Melkus M, Bennaceur-Griscelli A, Guihot F and Turhan AG: Leukemic stem cell persistence in chronic myeloid leukemia patients with sustained undetectable molecular residual disease. Blood 118: 3657-3660, 2011.
6. Leo E and Martinelli G: DNA methylation in chronic myeloid leukemia. J Mol Genet Med 8: 118, 2014.
7. Koschmieder S and Vetrie D: Epigenetic dysregulation in chronic myeloid leukaemia: A myriad of mechanisms and therapeutic options. Semin Cancer Biol 51: 180-197, 2018.
8. Robertson KD: Epigenetic mechanisms of gene regulation. In: DNA Methylation and Cancer Therapy, Medical Intelligence Unit, Springer, Boston, MA, pp13-30, 2005.
9. Jaensch R and Bird A: Epigenetic regulation of gene expression: How the genome integrates intrinsic and environmental signals. Nat Genet 33 (Suppl): S245-S254, 2003.
10. Jones PA and Baylin SB: The epigenomics of cancer. Cell 128: 683-692, 2007.
11. Chik F, Szfy M and Rabbani SA: Role of epigenetics in cancer initiation and progression. Adv Exp Med Biol 720: 91-104, 2011.
12. Chen T and Li E: Structure and function of eukaryotic DNA methyltransferases, Curr Top Dev Biol 60: 55-89, 2004.
13. Majda K, Kaufman-Szymczyk A, Lubecka-Pietruszewska K, Bednarak A and Fabianowska-Majewska K: Influence of clofarabine on transcriptional activity of PTEN, APC, RARB2, ZAP70 genes in K562 cells. Anticancer Res 30: 4601-4606, 2010.
14. Lubecka-Pietruszewska K, Kaufman-Szymczyk A, Stefanska B, Cebula-Obrzut B, Smolewski P and Fabianowska-Majewska K: Clofarabine, a novel adenine analogue, reactivates DNA methylation-silenced tumour suppressor genes and inhibits cell growth in breast cancer cells. Eur J Pharmacol 723: 276-287, 2014.
15. Ghanem H Jabbour E, Faderl S, Ghandi V, Plunkett W and Kantarjian H: Clofarabine in leukemia. Expert Rev Hematol 3: 15-22, 2010.
16. Ghanem H, Kantarjian H, Ohanian M and Jabbour E: The role of clofarabine in acute myeloid leukemia. Leuk Lymphoma 54: 688-698, 2013.
17. Stefanska B, Karlic H, Varga F, Fabianowska-Majewska K and Haslberger A: Epigenetic mechanisms in anti-cancer actions of bioactive food components-the implications in cancer prevention. Br J Pharmacol 167: 279-297, 2012.
18. Stefanska B, Salamé P, Bednarak A and Fabianowska-Majewska K: Comparative effects of retinoic acid, vitamin D and resveratrol alone and in combination with adenosine analogues on methylation and expression of phosphatase and tensin homologue tumour suppressor gene in breast cancer cells. Br J Nut 107: 781-790, 2012.
19. Lubecka K, Kurzawa L, Flower K, Buvula H, Zhang H, Teegarden D, Camarillo I, Suderman M, Kuang S, Andrisiani O, et al: Stibbensid remodel the DNA methylation patterns in breast cancer cells and inhibit oncogenic NOTCH signaling through epigenetic regulation of MAML2 transcriptional activity. Carcinogenesis 37: 656-668, 2016.
20. Lee YJ, Lee YJ, Im JH, Won SY, Kim YB, Cho MK, Nam HS, Choi YJ and Lee SH: Synergistic anti-cancer effects of resveratrol and chemotherapeutic agent clofarabine against human malignant mesothelioma MSTO-211H cells. Food Chem Toxicol 52: 61-68, 2013.
22. Sui T, Ma L, Bai X, Li Q and Xu X: Resveratrol inhibits the phosphatidylinositol 3-kinase/protein kinase B/mammalian target of rapamycin signaling pathway in chronic myelogenous leukemia cells by McI-1 down-regulation and caspase-3 activation. BMB Rep 48: 166-171, 2015.

23. Kulkarni SS and Cantó C: The molecular targets of resveratrol. Biochim Biophys Acta 1852: 1114-1123, 2015.

24. Theodosiou M, Lauter V and Schubert M: From carrot to clinic: An overview of the retinoic acid signaling pathway. Cell Mol Life Sci 67: 1443-1455, 2010.

25. Tang XH and Gudas LJ: Retinoins, retinoic acid receptors, and cancer. Annu Rev Pathol 6: 345-364, 2011.

26. Connolly R, Nguyen NK and Sukumar S: Molecular pathways: Retinoids, retinoic acid receptors, and cancer. Clin Cancer Res 19: 1651-1659, 2013.

27. Schenk T, Stengel S and Zelent A: Unlocking the potential of natural compounds in breast cancer cells. Eur J Pharmacol 638: 47-53, 2010.

28. Zhang W and Xu J: DNA methyltransferases and their roles in tumor metastasis and drug resistance. Cancer Res 69: 5624-5632, 2009.

29. Wu Q, Chen ZM and Su WJ: Anticancer activity of retinoic acid via AP-1 activity repression is mediated by retinoic acid receptor alpha and beta in gastric cancer cells. Int J Biochem Cell Biol 34: 1102-1114, 2002.

30. Stefanska B, Rudnicka K, Bednarek A and Fabianowska-Majewska K: Hypomethylation and induction of retinoic acid receptor beta 2 by concurrent action of adenosine analogues and natural compounds in breast cancer cells. Eur J Pharmacol 638: 1-9, 2010.

31. McCabe MT, Davis JN and Day ML: Regulation of DNA methyltransferase 1 by the pRb/E2F1 pathway. Cancer Res 65: 3624-3632, 2005.

32. Kulkarni SS and Cantó C: The molecular targets of resveratrol. Biochim Biophys Acta 1852: 1114-1123, 2015.

33. Chuang LS, Ian HI, Koh TW, Ng HH, Xu G and Li BF: Human DNA-(cytosine-5) methyltransferase-PCNA complex as a target for p21WAF1/CIP1. Oncogene 18: 5381-5392, 1999.

34. Iwase H, Omoto Y, Iwata H, Toyama T, Hara Y, Ando Y, Ito Y, Fujii Y and Kobayashi S: DNA methylation analysis at distal and proximal promoter regions of the oestrogen receptor gene in breast cancers. Br J Cancer 80: 1982-1986, 1999.

35. Paffi MW, Horgan GW and Dempfle L: Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res 30: e36, 2002.

36. Sui T, Ma L, Bui X, Li Q and Xu X: Resveratrol inhibits the phosphatidylinositol 3-kinase/protein kinase B/mammalian target of rapamycin signaling pathway in the human chronic myeloid leukemia K562 cell line. Oncol Lett 7: 2093-2098, 2014.

37. Wang B, Liu J and Gong Z: Resveratrol induces apoptosis in K562 cells via the regulation of mitochondrial signaling pathways. Int J Clin Exp Med 8: 16926-16933, 2015.

38. Mizuno S, Chijwiwa T, Okamura T, Akashi K, Fukumaki Y, Niho Y and Sasaki H: Expression of DNA methyltransferases DNMT1, 3A, and 3B in normal hematopoiesis and in acute and chronic myelogenous leukemia. Blood 97: 1172-1179, 2001.

39. Tan HH and Porter AG: p21(WAF1) negatively regulates DNMT1 expression in mammalian cells. Biochem Biophys Res Commun 382: 171-176, 2009.

40. Liu M, Iavarone A and Freedman LP: Transcriptional activation of the human p21(WAF1/CIP1) gene by retinoic acid receptor. Correlation with retinoid induction of U937 cell differentiation. J Biol Chem 271: 31723-31728, 1996.

41. Yu Z, Li W, Lu Q, Wang L, Zhang X, Han P, Chen P and Pei Y: Palbociclib is required for abiraterone-mediated inhibition of MEPM cells, which involves RAR. J Cell Biochem 104: 2185-2192, 2008.

42. Bowers JL, Tyulmenkov VV, Jernigan SC and Klinge CM: Resveratrol acts as a mixed agonist/antagonist for estrogen receptors alpha and beta. Endocrinology 141: 3657-3667, 2000.

43. Parvez A, Akash MS, Rehman K and Kyunn WW: Dual role of p21 in the progression of cancer and its treatment. Crit Rev Eukaryot Gene Expr 26: 49-62, 2016.

44. Montiel-Duarte C, Cordeu L, Agirre X, Román-Gómez J, Jiménez-Velasco A, Jose-Eneriz EÁ, García L, Andreu EJ, Calasanz MJ, Heliniger A, et al: Retinoids: A review of their role in cancer. Mol Cancer 13: 111, 2014.

45. Parvez A, Akash MS, Rehman K and Kyunn WW: Dual role of p21 in the progression of cancer and its treatment. Crit Rev Eukaryot Gene Expr 26: 49-62, 2016.

46. Montiel-Duarte C, Cordeu L, Agirre X, Román-Gómez J, Jiménez-Velasco A, Jose-Eneriz EÁ, García L, Andreu EJ, Calasanz MJ, Heliniger A, et al: Retinoids: A review of their role in cancer. Mol Cancer 13: 111, 2014.

47. García JM, Silva J, Peña C, García V, Rodríguez R, Cruz MA, Cantos B, Provencio M, España P and Bonilla F: Promoter methylation of the PTEN gene is a common molecular change associated with PTEN down-regulation due to promoter hypermethylation. Leuk Res 32: 709-716, 2008.

48. García JM, Silva J, Peña C, García V, Rodríguez R, Cruz MA, Cantos B, Provencio M, España P and Bonilla F: Promoter methylation of the PTEN gene is a common molecular change in breast cancer. Genes Chromosomes Cancer 41: 117-124, 2004.

49. Goel A, Arnold CN, Niedzwiecki D, Carethers JM, Dowell JM, Yu Z, Li W, Lu Q, Wang L, Zhang X, Han P, Chen P and Pei Y: Palbociclib is required for abiraterone-mediated inhibition of MEPM cells, which involves RAR. J Cell Biochem 104: 2185-2192, 2008.

50. Lefebvre B, Brand C, Flajollet S and Lefebvre P: Down-regulation of the tumour suppressor gene retinoic acid receptor beta2 through the phosphoinositide 3-kinase/Akt signaling pathway. Mol Endocrinol 20: 2109-2121, 2006.

51. Lussana F, Intermesoli T, Stefanoni P and Rambaldi A: Apoptotic effects of resveratrol, a grape polyphenol, on imatinib-sensitive and resistant K562 chronic myeloid leukemia cells. Biochem Pharmacol 65: 219-225, 2003.

52. Lefebvre B, Brand C, Flajollet S and Lefebvre P: Down-regulation of the tumour suppressor gene retinoic acid receptor beta2 through the phosphoinositide 3-kinase/Akt signaling pathway. Mol Endocrinol 20: 2109-2121, 2006.

53. Lussana F, Intermesoli T, Stefanoni P and Rambaldi A: Apoptotic effects of resveratrol, a grape polyphenol, on imatinib-sensitive and resistant K562 chronic myeloid leukemia cells. Biochem Pharmacol 65: 219-225, 2003.