

Influence of Clofarabine on Transcriptional Activity of *PTEN*, *APC*, *RARB2*, *ZAP70* Genes in K562 Cells

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Abstract. *Background:* In this study, the effect of clofarabine, a new generation 2'-deoxyadenosine analogue, on promoter methylation and transcriptional activity of selected genes (*PTEN*, *APC*, *RARB2*, *ZAP70*) in K562 cells was assessed. *Materials and Methods:* Promoter methylation was estimated using methylation-sensitive restriction analysis. The mRNA level of the genes was measured with real-time PCR. *Results:* The inhibitory cytostatic index (IG_{50}) for clofarabine in K562 cells cultured for 72 (or 96) h was 8 nM. The drug (20 nM) caused: (i) potent diminution in methylation of *PTEN* promoter, moderate methylation reduction of *APC* and *RARB2* promoters, and complete methylation of *ZAP70* promoter; (ii) significant stimulation of *PTEN*, *APC*, *RARB*, and *p21* mRNA expression and (iii) decline in mRNA level of *ZAP70* and *DNMT1* genes. *Conclusion:* The results indicated that clofarabine is involved in epigenetic regulation of transcriptional activity of the tested tumour suppressor genes and genes encoding proteins involved in DNA methylation process.

Clofarabine (2-chloro-2'-fluoro-arabinosyladenine, Cl-F-ara-A manufactured as Clolar[™] or Evoltra[®]) is a second-generation purine nucleoside analogue, which combines the most favourable biochemical properties of its congeners: cladribine (2-chloro-2'-deoxyadenosine, 2CdA) and fludarabine (2-fluoro-arabinosyladenine, F-ara-A) (Figure 1). The nucleoside demonstrates antitumour activity in relatively low doses, significantly lower than its mentioned prototypes, in both *in vitro* and *in vivo* experiments with tumour models (1, 2).

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Clofarabine was synthesized in the late 1980s (3), and was approved by the U.S. Food and Drug Administration in 2004 and by the European Commission in 2006 for treatment of paediatric patients (1 to 21 years old) with refractory or relapsed acute lymphoblastic leukaemia. Currently, the drug is used in clinical trials against other types of haematological malignancies and solid tumours (4, 5). Additionally, the experimental clinical treatment with clofarabine is introduced in combination with ara-C or cyclophosphamide to usually unresponsive elderly patients with leukaemia (6-8).

Hitherto, existing results of studies concerning clofarabine cytotoxicity in cancer cells indicated that the molecular mechanism action of clofarabine is similar to that of cladribine. Clofarabine triphosphate nucleotide (Cl-F-ara-ATP) leads to cell apoptosis due to the inhibition of ribonucleotide reductase and DNA polymerase activities (9, 10) and to direct induction of apoptosis *via* mitochondria damage (11). Clofarabine triphosphate also inhibits RNA synthesis, but the effect is much smaller and is mainly directed towards poly(A)polymerase (12).

Recent data concerning epigenetic DNA modification (mainly DNA methylation) showed that clofarabine action results in hypomethylation of genomic DNA and hypermethylation of CT antigen genes that is associated with up-regulation of mRNA and protein expression of the antigens (13). This is consistent with previous studies devoted to cladribine and fludarabine in which it was noted that the drugs not only led to a reduction of genomic DNA methylation (mainly CpG islands) (2), but also to a strong decline in methylation of *PTEN* and *APC* promoters in K562 cells (14).

According to these findings for clofarabine and its prototypes, the present study aimed to estimate the effects clofarabine on methylation and expression (at the mRNA level) of *PTEN*, *APC*, *RARB2*, and *ZAP70* genes as well as expression of *DNMT1* and *p21* genes. These genes encode proteins important for regulation of intracellular signalling pathways (*PTEN*, *APC*, *ZAP70*) and for regulation of cell cycle (*RARB2*). The *PTEN*, *APC* and *RARB2* genes are often epigenetically silenced in cancer cell

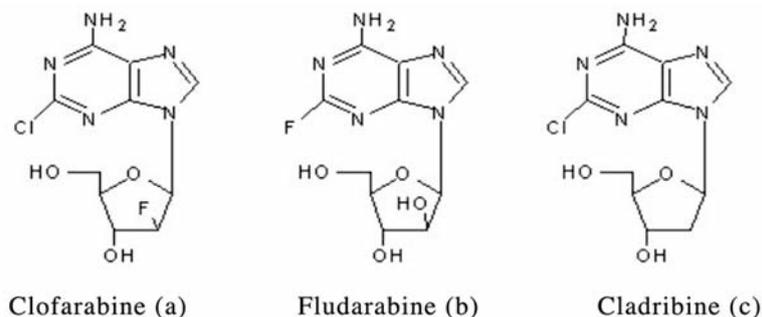


Figure 1. Structural formulae of antileukemic adenosine analogues: (a) clofarabine, (b) fludarabine and (c) cladribine.

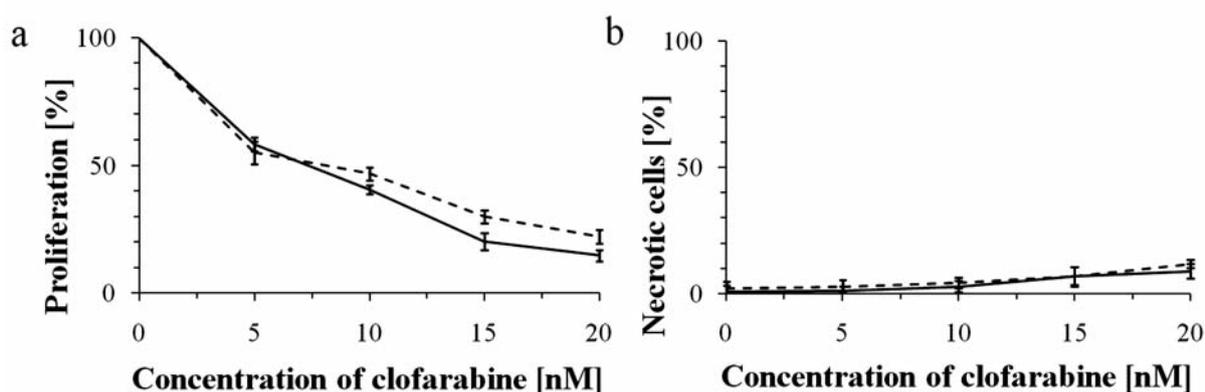


Figure 2. Cytostatic index (IG_{50}) and lethality of K562 cells for clofarabine. Full line, cells incubated for 96 hours; dashed line, cells incubated for 72 hours.

lines and cancer tissue (14-19). The *ZAP70* gene, encoding cytosolic zeta-chain associated tyrosine kinase, is normally expressed in T- and NK (natural killers) cells, but also in some chronic lymphocytic leukaemias (CLL) in B-cells. *ZAP70* expression in B-cells caused by hypomethylation of its promoter has been correlated with poor prognosis (20, 21). The *DNMT1* and *p21* genes encode proteins that are pivotal for the DNA methylation process and for this reason they are involved in regulation of gene transcriptional activity (22).

Materials and Methods

Compounds and chemicals. Clofarabine was obtained by Genzyme Co. (San Antonio, TX, USA). Basal reagents (for RNA and DNA isolation and purification) were obtained from Sigma-Aldrich Sp. z.o.o. (Poznan, Poland) and endonucleases (HpaII and Eco72I) from Fermentas UAB (Vilnius, Lithuania).

Cell culture, proliferation and viability assay. Human erythroleukemic K562 cell line (American Type Culture Collection, LGC Standards Sp. z.o.o., Lomianki, Poland) was cultured in RPMI medium containing: L-glutamine and HEPES (CytoGen Sp. z.o.o., Lodz, Poland),

supplemented with 10% foetal bovine serum (FBS), 1 U/ml penicillin, 1 μ g/ml streptomycin (Gibco - Europe, Paisley, Scotland, UK). Cells were grown at 37°C in a humidified atmosphere of 5% CO_2 . Tested cell lines were treated for 72 and 96 h with clofarabine at 5 to 20 nM concentrations. Cell proliferation and viability were determined using the Trypan blue (Sigma-Aldrich Sp. z.o.o., Poznan, Poland) exclusion test to estimate IG_{50} values, which represent the drug concentration resulting in a 50% inhibition of cell growth. The number of viable cells in culture treated with clofarabine was expressed as a percentage of viable cells in untreated control culture. In the viability assay, the number of necrotic cells that took up Trypan blue dye was expressed as a percentage of the total cell number. All other experiments were performed at the 20 nM concentration for which cell viability was higher than 90% after 96 h-culture.

RNA and DNA extraction. Total RNA from a human erythroleukemic cell line was isolated using Trizol Reagent (Invitrogen Life Technologies Sp. z.o.o., Warsaw, Poland) according to the manufacturer's protocol. Isolated RNA was diluted in water containing 1% DEPC (ribonuclease inhibitor) and stored at -70°C.

Cellular DNA, after incubation with proteinase K, was extracted using a phenol:chloroform: isoamyl alcohol (25:24:1) mixture. Then DNA was precipitated using ammonium acetate and ethanol. Pure DNA was dissolved in TE buffer and stored at -20°C.

Table I. Primers of tested gene promoter fragments used in PCR.

Genes	Fragment length (bp)	Sequence of primers (5' → 3') F-forward; R-reverse	Annealing temperature (°C)	Accession no. (amplified fragments)
<i>PTEN</i>	286	F:GCGGAAGCAGCCGTTCCGGAG R:GTCATGTCTGGGAGCCTGTG	60.9	GenBankno. AF143312 [-281 +5]
<i>APC</i>	317	F:CTAGGCAGGCTGTGCGGTTG R:CGGTTTAAGACAGTGCGAGG	61.1	GenBankno. U02509 [-88+230]
<i>RARB2</i>	295	F:CTCGCTGCCTGCCTCTCTGG R:GCGTTCTCGGCATCCCAGTC	58.4	GenBankno. X56849 [-23+187]
<i>ZAP70</i>	285	F:CTTCTGACGGTTCCTGTG R:CGGAAGGCCTGGTCTC	58.1	NCBI RefSeq No. NG_007727.1 [-175+58]

Table II. Primers of tested gene fragments used in real-time PCR.

Genes	Fragment length (bp)	Sequence of primers (5' → 3')	Annealing temperature (°C)
<i>PTEN</i>	330	F:CGAACTGGTGAATGATATGT R:CATGAACTTGTCTTCCCGT	50.0
<i>APC</i>	101	F:TGCGAGAAGTTGGAAGTGTGAAAGCATTG R:TGACAAATTCATAAGGCACTCAATACGC	60.0
<i>RARB2</i>	92	F:TTCAAGCAAGCCTCACATGTTTCCA R:AGGTAATTACACGCTCTGCACCTTTAG	56.0
<i>ZAP70</i>	2092	F:CGCTGCACAAGTTCCTGGT R:AGCTGCTGCACCAGGTGTC	53.0
<i>p21</i>	103	F:GCTCAGGGGAGCAGGCTGAAG R:CGGCGTTTGGAGTGGTAGAAATCTGT	60.0
<i>DNMT1</i>	100	F:ACCGCCCTGGCCAAAGCCATTG R:AGCAGCTTCTCTCTCTTTATTTTAGCTGAG	60.0

Methylation assay. The methylation status of promoter of selected genes (*i.e.* *PTEN*, *RARB2*, *APC* and *ZAP70*) was estimated using according to Iwase's method (23). The promoter methylation level was evaluated in DNA from cells incubated alone or in the presence of clofarabine used at 20 nM concentration. Methylation-sensitive restriction analysis includes the following experimental steps: (i) digestion of cellular DNA with methylation-sensitive restriction endonucleases (*Hpa*II, *Eco*72I recognizing non-methylated sequences C↓CGG and CAC↓GTG, respectively); (ii) PCR amplification of digested DNA; (iii) electrophoretic analysis of amplified promoter fragments in 6% polyacrylamide gel and (iv) densitometric analysis of gels using Quantity One computer program (BioRad Sp. z.o.o., Warsaw, Poland).

PCR was carried out at 95°C for 5 min, cycled for 1 min at 94°C, 1 min at annealing temperature (annealing temperatures and primer sequences used for selected fragments of tested gene promoters are presented in Table I), and 2 min at 72°C (30 cycles), followed by a 10 min extension at 72°C. PCR products were visualized in 6% polyacrylamide gel stained with ethidium bromide. Undigested DNA and DNA digested with appropriate endonucleases after amplification were used as controls. The methylation level in each sample was calculated on the basis of densitometric analysis and expressed as a percentage of undigested DNA after the comparison of band intensities for digested and undigested DNA. The percentage of

methylation inhibition was calculated by comparison of methylation level in cells treated with clofarabine and in control cells.

Quantitative analysis on mRNA level. In order to obtain cDNA, to 2 µg of total RNA there were added: 6 µl of random hexamers and 5 µl of oligo(dT)15 (Promega GmbH, Mannheim, Germany). The mixture was incubated at 70°C for 10 min and then cooled to 4°C. Reaction with ImProm-II reverse transcriptase (Promega GmbH, Mannheim, Germany) was carried out according to the manufacturer's protocol. The samples were incubated under the following conditions: 5 min at 25°C, 60 min at 42°C, and 15 min at 70°C. The solution with cDNA was stored at -20°C.

Real-time PCR was performed in a Rotor-Gene TG-3000 machine (Corbet Research, Mortlake, New South Wales, Australia). The reaction mixture (20 µl) was prepared according to the manufacturer's protocol. Amplification consisted of 50 cycles performed under the following conditions: denaturation at 94°C for 30 s, annealing for 15 s at temperatures characteristic for every pair of primers (Table II), and elongation at 72°C for 30 s. Four housekeeping genes, *RPS17*, *RPLP0*, *H3F3A*, and *BMG*, were used as reference genes. Analysis of the results was performed according to Pfaffl's method (24), in which so-called relative level of expression (relative to geometric mean of expression level of reference genes) was calculated.

Table III. Clofarabine effects on methylation of gene promoters (A) and mRNA levels (B) in K562 cells. The methylation level in control cells was estimated densitometrically as a percentage of band intensities of digested relatively to undigested DNA. Inhibition or stimulation of the gene promoter methylation by clofarabine is expressed as a percentage of the gene methylation level in cells cultured with clofarabine and in control cells (cultured without the drug). Data represent the mean±standard deviation of four independent experiments.

Gene	(A) Methylation status			(B) Gene expression		
	Control cells (%)	Cells with clofarabine (%)	Change of methylation (%)	Control	Relative mRNA level in cells with clofarabine	Change of gene expression (%)
<i>PTEN</i>	61.1±3.2	26.0±4.0	↓ 57.4	1	1.76±0.16	↑ 76.03
<i>APC</i>	57.0±1.9	47.9±2.5	↓ 15.9	1	1.33±0.14	↑ 32.74
<i>RARB2</i>	45.3±1.5	32.1±3.6	↓ 29.1	1	2.51±0.30	↑ 150.66
<i>ZAP70</i>	86.9±1.5	100.0±3.4	↑ 13.1	1	0.74±0.11	↓ 26.16
<i>p21</i>	-	-	-	1	4.31±0.28	↑ 331.50
<i>DNMT1</i>	-	-	-	1	0.72±0.09	↓ 28.14

Statistical analysis. Statistical calculation was performed with usage of Microsoft Office Excel 2007 spreadsheet. Each value is given as the mean ± standard deviation of four independent experiments. The Student's *t*-test was used to determine the significance of results, *p*<0.05 was regarded as statistically significant.

Results

Cell proliferation. IG₅₀ (the inhibitory cytostatic index) value for clofarabine and K562 cells cultured for both 72 and 96 hours in the presence of the drug is approximately eight nM. Trypan blue exclusion properties allowed the estimation of cell viability (Figure 2a) and percentage of necrotic cells in cultures (Figure 2b). Incubation of K562 cells with clofarabine for 96 hours at concentration equal to 20 nM did not lead to cell death greater than 10%.

Methylation level. The influence of clofarabine on the methylation status of the tested gene promoters is shown in Table III, (A). In control K562 cells (cultured without clofarabine) *PTEN*, *APC*, *RARB2* and *ZAP70* promoters were methylated in the following percentages: 61, 57, 45 and 87, respectively. The exposure of the cells to clofarabine (20 nM concentration) led to a strong decrease in *PTEN* promoter methylation (approximately by 57%), and a lesser reduction of *APC* and *RARB2* promoter methylation (16% and 29%, respectively). A tested fragment of *ZAP70* promoter after incubation with the drug was completely methylated.

mRNA level estimation. Changes of mRNA level of tested genes in K562 cells exposed to clofarabine used at 20 nM concentration are shown in Table III, (B). The increase in expression of *PTEN*, *APC* and *RARB2* genes was 76%, 33% and 150%, respectively, whereas full methylation of tested *ZAP70* promoter fragment led to decline in mRNA level by

over 25%. The effect of clofarabine on expression of two other genes, *p21* and *DNMT1*, encoding proteins pivotal for DNA methylation was also estimated. A greater than 3.3-fold increase in mRNA level of *p21* gene and almost 30% reduction of *DNMT1* gene expression, compared to expression of the genes in control K562 cells was noted.

It should be noted that there was a reverse correlation between demethylation of *PTEN* and *APC* promoters and mRNA level elevation of the genes in the K562 cells exposed to clofarabine. In the case of *RARB2*, demethylation of the gene promoter was associated with an inadequately high increase in mRNA level of the gene, whereas the increase in *ZAP70* promoter methylation was associated with decline in the gene mRNA level.

Discussion

This study presents experimental evidence that clofarabine demonstrates antitumour activity in relatively low doses. Clofarabine, a potent cytostatic agent, inhibits K562 cell proliferation with a low IG₅₀ value (approximately 8 nM), which is lower than for its congeners, cladribine and fludarabine (15-fold and over 400-fold lower, respectively) (2).

Furthermore, this study demonstrated that clofarabine action leads to altered expression of genes (*i.e.* *PTEN*, *APC*, and *ZAP70*) that participate in key intracellular signalling pathways, such as: Wnt/β-catenin/TCF (negatively regulated by *APC*), phosphoinositide 3-kinase (PI3K)/Akt, and Shc/Ras/Raf/MAPK (modulated by *PTEN*, a dual specificity phosphatase for lipid and protein substrates). In K562 cells, the drug action (similarly to cladribine and fludarabine in MCF-7 cells) (16) provokes promoter demethylation of *PTEN* and *APC* genes to a lesser extent. This epigenetic effect is associated with a significant increase in mRNA level

of the genes (data not shown). The reverse correlation between changes in methylation status and increased gene expression suggests that the epigenetic modification (*i.e.* methylation) of the promoters is probably one of the major mechanisms responsible for transcriptional activity of *PTEN* as well as *APC*. The present results are in accordance with data of other studies on many cancer tissue and cancer cell lines and indicating that a decrease in the biological activity of *PTEN* may result from hypermethylation of the promoter region of the gene (17-19).

Additionally, it was noted that effect of clofarabine, leading to demethylation of promoters of the tested genes, was associated with a decline in *DNMT1* mRNA level. It can be suggested that reduction of DNA methylation by clofarabine (analogical to cladribine and fludarabine) probably results from indirect inhibition of *DNMT1* through irreversible inhibition of *S*-adenosylhomo-cysteine hydrolase activity and perturbation of the 'active methyl' cycle as well as from inhibition of DNA synthesis. It is possible that clofarabine (like its congeners) 'switches' cellular signals regulating expression of *DNMT1* as a consequence of a moderate increase in *PTEN* expression, resulting in impairment of Ras/MAPK oncogenic pathway, and strong stimulation of *p21* expression which was revealed in the current study (Table III). The p21 protein competes with *DNMT1* for the same binding site on proliferating cell nuclear antigen (PCNA) which contributes to an-agonising *DNMT1* activity by blocking the DNMT1-PCNA interaction (25). It should be noted that APC, a regulatory protein of the Wnt1/beta-catenin/TCF signalling pathways, is indirectly dependent on PTEN phosphatase activity. Negative regulation Akt kinase by *PTEN* activates GSK3beta serine-threonine kinase that phosphorylates two cooperating proteins, beta-catenin and *APC*. The binding of the two phosphorylated proteins diminishes the effect of beta-catenin, inducing expression of oncogenes (26).

Mechanism of clofarabine action also include the elevation of *ZAP70* promoter methylation and suppression of gene expression. *ZAP70* is a member of the SYK tyrosine kinase family, mainly involved in T-cell receptor signalling initiation, but in chronic lymphocytic leukaemia the kinase may be involved in B-cell receptor signalling (27). In transformed T-cells, activity of *ZAP70* gene may be modulated by methylation in the presence of *DNMT1* after its activation by STAT3 and kinase NPM-ALK (28). Functional *ZAP70*, through activation of intermediates (*i.e.* B- or T-cell linker proteins), stimulates downstream signalling pathways which include the MAPK and PI3 kinase (27). This is the evidence that two proteins, *ZAP70* and *PTEN*, play opposing roles in regulation of two intracellular signalling pathways.

In K562 cells exposed to clofarabine, demethylation of *RARB2* gene promoter was associated with a high mRNA level of the gene. A similar increased expression has been

observed in the case of cladribine in MCF-7 cells (29). According to Lefebvre *et al.*, *PTEN* phosphatase, through negative regulation of PI3 kinase/Akt signalling pathways, may affect *RARB2* expression *via* SMRT co-repressor recruitment to the promoter (30). Loss of Akt activity, due to protein dephosphorylation by *PTEN*, blocks the tethering of SMRT to the *RARB2* promoter, which promotes an increase in histone acetylation and stimulates gene expression (30). These facts support the hypothesis that *RARB2* is mainly regulated by histone deacetylation.

In summary, this study clearly demonstrated that clofarabine affects promoter methylation and expression (at the mRNA level) of the genes that encode the proteins which participate in regulation of intracellular signalling pathways (*e.g.* *PTEN*, *APC*, and *ZAP70*). This is significant for therapy of cancer associated with gene silencing due to hypermethylation of the gene regulatory regions. This effect of clofarabine action on promoter methylation and expression of selected genes participating in important intracellular signalling pathways is a new, unreported element of the clofarabine cytotoxic mechanism and is very important for a full understanding of the anti-leukemia action of the drug.

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