Progressive increase in death-rate caused by cancer diseases is a very important epidemiological problem in the world nowadays. Scientists make efforts to contribute to anticancer therapy progress through design and development of new anticancer drugs, among which nucleoside analogues constitute an important group. The synthetic nucleosides, which are antimetabolites in DNA and/or RNA syntheses, were successfully introduced to therapy of hematological malignancies and virus infections. The history of nucleoside analogues dates back to late 1950s when cytarabine (ara-C, cytosine arabinoside) was synthesized. The success of ara-C in treatment of leukemias brought about a development of other purine and pyrimidine nucleoside analogues such as: ara-A, fludarabine (F-ara-A, 2-fluoro-arabinosyladenine) and cladribine (2CdA, 2-chloro-2’-deoxyadenosine) (1–4). Although activities of these mentioned drugs indicated that they might be promising anticancer agents, there were some limitations to their widespread usage in anticancer therapy. For example, the main problems in further clinical investigations with cladribine and fludarabine were: their low oral bioavailability due to instabilities of these drugs in acidic solution (in gastrointestinal tract) and their susceptibility to bacterial cleavage (5). Moreover, the therapeutic action of these drugs was connected with moderate myelosuppression and both profound and prolonged immunosuppression. All those side effects were frequently associated with elevated risk of opportunistic infections and secondary malignancies. Additionally, high doses of cladribine and fludarabine resulted often in severe neurological toxicity (6).

The negative consequences of therapy with cladribine and fludarabine led to the design of a new 2’-deoxyadenosine analogue, clofarabine (Cl-F-ara-A, 2-chloro-2’-fluoro-2’-deoxyarabinosyladenine), which was synthesized in Research Institute in Birmingham (Alabama) in the late 1980s (7). The new analogue combines the most favorable pharmacokinetic properties of its prototypes. Clofarabine demonstrates antitumor activity in relatively low doses in both in vitro and in vivo studies with tumor models. This led to undertaking I and II phases of clinical trials with the drug (8, 9). After fifteen years of experimental studies with clofarabine, in 2004 the
FDA (U.S. Food and Drug Administration) approved the drug for treatment of pediatric patients (1–21 years old) with acute leukaemia. Then the drug was acquired by Genzyme Corporation and manufactured as Clolar™. In 2006, clofarabine was approved by The European Commission for treatment of relapsed or refractory ALL in pediatric patients. In Europe the drug under the name Evoltra® is produced by Bioenvision.

To evaluate more thoroughly properties of clofarabine, further studies focused on consequences of the drug action as well as on optimization of its oral and intravenous doses during the treatment of hematological and solid malignancies (9). Moreover, promising results of therapy in patients of other age groups, particularly in usually unresponsive elderly patients, were noted (10). To improve clofarabine therapeutic activity, the drug was recently combined with other chemotherapeutic agents in clinical applications (11).

Until now, studies with clofarabine on molecular level have indicated that the mechanism of the drug involves; inhibition, by Cl-F-ara-ATP, of ribonucleotide reductase activity and DNA synthesis, followed by DNA damage and direct induction of apoptosis. Additionally, there are some new hypotheses of other possible mechanisms of the drug’s action. One of them is influence of the drug on epigenetic regulation of genes expression. The latter effect still requires further investigations. Elucidation of the multidirectional molecular mechanism of clofarabine cytotoxicity in tumor cells seems to be crucial for understanding of the therapeutic and, in particular, antileukemic action of the drug.

**Clofarabine – structural formula and metabolism in cells**

Clofarabine was originally synthesized by Montgomery et al. as an analogue of two synthetic nucleosides: cladribine and fludarabine (7). Cl-F-ara-A, similarly to cladribine, has a chlorine atom at 2 position of adenine ring and 2'-deoxyarabinose moiety with fluorine atom at 2'position instead of arabinose moiety that fludarabine has (Fig. 1). The molecule of clofarabine assumes an *anti* conformation. Halogenation of adenine ring at 2 position, such as in clofarabine and its prototypes cladribine and fludarabine, causes that the amino group of adenosine is resistant to deamination by deoxyadenosine deaminase (ADA). Introduction of the fluorine atom to 2'position in 2'-deoxyarabinose causes higher stability of the drug at low pH than 2'-deoxyadeno-
sine and its halogenated congeners. Additionally, the arabinose moiety modified by fluorine is more resistant to enzymatic catabolism by bacterial (e.g., E. coli) purine nucleoside phosphorylase (PNP) which catalyzes cleavage of the glycosidic bond. All these properties make catabolism of the drug significantly limited in gastrointestinal tract (8, 9) (Scheme 1), what enhances oral bioavailability of clofarabine.

Similarly to other purine 2'-deoxynucleosides, Cl-F-ara-A crosses membranes into cells by facilitative and active nucleoside transporter mechanisms. Due to lipophilic properties of the drug it can enter cells by passive diffusion (12). The last mentioned mechanism takes place at higher concentration of Cl-F-ara-A or at longer exposure time (8, 13). Cl-F-ara-A is transported into cells by two types of active transport processes: equilibrative – by means of hENT1 and hENT2 transporters, and concentrative – mainly by means of hCNT3. hENTs are bi-directional sodium-independent nucleoside transporters driven by nucleoside concentration across cell membranes, whereas hCNT is sodium-dependent active transporter (against concentration gradient) for purine nucleosides and uridine and requires ATP as a cofactor. All mentioned halogenated 2'-deoxyadenosine analogues are transported into cells by the same mechanisms. King et al. showed in human leukemic CEM cells that among them Cl-F-ara-A had the highest affinity for hENT1, hENT2, and hCNT3, what can result in the highest cytotoxicity of clofarabine (13). It is necessary to emphasize that clofarabine nucleotides, 5’-mono-, di-, and triphosphate, are not substrates for nucleoside transporters and they must be enzymatically converted to nucleosides to be transported out of the cells (8, 13).

In cells, Cl-F-ara-A is phosphorylated to the nucleoside monophosphate (Cl-F-ara-AMP), like 2'-deoxyadenosine, cladribine and fludarabine, by cytosolic deoxycytidine kinase (dCK) and/or by mitochondrial deoxyguanosine kinase (dGK) which are present in lymphoblasts. Substrate specificity of dCK to clofarabine is as good as to the natural deoxycytidine and significantly greater than to cladribine and fludarabine (8, 14). Such higher substrate specificity of the kinase to clofarabine results probably from specific complex formed between enzyme, clofarabine and ADP. The crystal structure of the active complex was described by Zhang et al. (15). In this complex the nucleoside is very tightly surrounded by the enzyme molecule and several hydrogen bonds are formed between amino acids residues and 3’-, and 5’-hydroxyl groups as well as fluorine atom. In active site all nitrogen atoms of the adenine ring (i.e., N1, N3, N6, and N7) form hydrogen bonds with encircling amino acid residues (i.e., Gln, Tyr, Asp, and Arg, respectively). Moreover, 2-Cl atom makes van der Waals interactions with two hydrophobic amino acids residues Met and Leu. Additionally, at the active site stacking interaction between adenine ring and aromatic ring of Phe is formed. These hydrophobic interactions (i.e., van der Waals and stacking) are responsible for high catalytic activity of deoxycytidine kinase towards clofarabine.

It is necessary to underline that the deficiency of dCK activity in leukemic cell lines results in resistance of the cells to clofarabine. The deficiency of dCK activity is not due to epigenetic silencing by methylation of its gene promoter (16).

As it was mentioned above, clofarabine can be also phosphorylated by mitochondrial deoxyguanosine kinase which has specific activity to purine nucleosides. The good substrate specificity of dGK towards clofarabine may explain its role in mitochondrial damage (17). The dGK enzyme forms, similarly to dCK, enzyme-clofarabine-ATP complex, but in this case ATP (not ADP) is bound to the active site of enzyme (18). Studies of activities of both kinases in human leukemic cell lines (e.g., CEM, HL60 and Molt-4) indicated increased activities in proliferating cells (19).

The major metabolite of clofarabine is Cl-F-ara-AMP that is in contrast to other purine nucleosides analogues. The monophosphate derivative is successively converted in cells into di-, and triphosphate by proper kinases. In many authors’ opinion low efficiency of conversion of the nucleoside monophosphate to diphosphate derivative is rate-limiting reaction in formation of Cl-F-ara-ATP in cells (20). Succeeding phosphorylation reactions are dose-dependent what was documented in CEM cells. There was noted that at concentration of Cl-F-ara-A lower than 3 μM the major metabolite was Cl-F-ara-AMP, whereas at the nucleoside concentration greater than 3 μM, Cl-F-ara-ATP predominated. The studies concerning retention of clofarabine nucleotides inside cells (CEM cells) indicated that the half-life for Cl-F-ara-ATP was 29 h and the time was longer than for mono-, and diphosphate nucleotides of the drug. The prolonged retention of triphosphate nucleotide of clofarabine may be an important factor in clofarabine toxicity (20).

Cl-F-ara-ATP is a good substrate for DNA polymerases α and ε that participate in DNA elongation and repair processes (21). The nucleotide incorporation increases in proportion up to 3 μM of the drug concentration. The nucleotide may be
Clofarabine molecular mechanism of antitumor activity

The study to explain molecular mechanism of clofarabine anticancer action started in the early 1990s. During the decades it was documented that clofarabine demonstrates, similarly to cladribine and fludarabine, cytotoxic activity against dividing and nondividing cells (9, 14). As it was mentioned previously, Cl-F-ara-ATP, the final active metabolite of clofarabine demonstrates, similarly to cladribine and fludarabine anticancer action started in the early 1990s. During the decades it was documented that clofarabine anticancer action started in the early 1990s. During the decades it was documented that clofarabine nucleotide with ANT protein affects the opening of the mitochondrial permeability transition pore, which allows the release of AIF (apoptosis inducing factor) and cytochrome c. The clofarabine nucleotides substitute in competitive manner for adenine nucleotide in the interaction with the mitochondrial proteins. These mitochondrial processes result in rapid reduction of mitochondrial transmembrane potential and lead to intensification of apoptosis process. The same pathway of mitochondrial damage provokes the activation of the caspase cascade as it was noted in CLL cells treated with nucleotides of 2'-deoxyadenosine or cladribine (17, 26).

Presented molecular mechanisms of Cl-F-ara-ATP anticancer action may be intensified by accumulation of non-metabolized 2'-deoxyadenosine. Clofarabine influence on ADA and S-adenosylhomocysteine hydrolase activities (important for 2'-deoxyadenosine metabolism) is hitherto unknown. The initial in vitro studies with K562 cells indicate that clofarabine inhibits activities of these two enzymes. Cl-F-ara-A used at very low concentration (5 nM) led to decrease in activities of ADA and SAH-hydrolase by 30% and 15%, respectively (unpublished data). The inhibition of ADA activity most probably causes accumulation of 2'-deoxyadenosine to toxic concentration for cells. Moreover, elevated 2'-deoxyadenosine concentration may lead to perturbation of DNA methylation due to SAH hydrolase inactivation followed by alteration of SAM pool. Such effect was demonstrated in studies with cladribine and K562 cells (27, 28). It is conceivable that clofarabine action during therapy in patients with chronic lymphocytic leukemia could lead to a decrease in activity of ADA and SAH hydrolase, similarly to observations during the therapy with cladribine (29). The suggestion is in accordance to Zhang’s studies. These data indicate that treatment of lymphoid tumor cells (delivered from leukemia patients) with low doses (1–10 nM) of clofarabine causes hypomethylation of CpG dinucleotide sequences as well as decreases in global DNA methylation. The observations were

inserted into DNA at internal or terminal sites of DNA chain depends on ratio of Cl-F-ara-ATP:dATP (20, 21). The incorporation site into DNA depends on ratio of Cl-F-ara-ATP:dATP (20, 21). The incorporation site into DNA depends on ratio of Cl-F-ara-ATP:dATP (20, 21). The incorporation site into DNA depends on ratio of Cl-F-ara-ATP:dATP (20, 21).
associated with elevated expressions of two cancer-testis antigens. Moreover, the DNA methylation decrease was connected with inhibition of tumor cell growth and induction of apoptosis (30). These observations are consistent with results of our studies devoted to clofarabine’s effect (used at 20 nM in K562 cells) on: (i) promoter methylation of selected tumor suppressor genes (PTEN, APC, and RARB2) involved in regulation of intracellular signalling pathways; (ii) expression of these genes on mRNA level as well as p21 and DNMT1 (DNA methyltransferase 1). Significant decrease in methylation status of PTEN, APC, and RARB2 promoters (by 57, 16, and 30%, respectively) was noted (data not shown). The decline levels in promoter methylation were associated with an increase in expression on mRNA level of the following tumor suppressor genes: p21 ~ 250%, PTEN ~ 76%, and RARB ~ 131%. Simultaneously, there was observed 30% reduction in mRNA of DNMT1 gene (data not shown).

At the moment, it is very difficult to define precise mechanism of clofarabine action in alterations of the promoter methylation and expression of tumor suppressor genes. It is possible to place two hypotheses. It might be an effect of disturbance of polystep “active methyl” cycle with perturbation of physiological balance between S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) by clofarabine and accumulated, non-metabolized 2’-deoxyadenosine. The other possibility is many-causal restoration of normal control function of intracellular signalling pathways resulting from inhibition of DNA synthesis, slowing down cell growth and intensification of cell apoptosis. The stronger induction of apoptosis is probably a consequence of the extra mitochondrial pathway (analogical to cladribine nucleotide effect on mitochondria), including interaction of clofarabine nucleotide with mitochondrial proteins and damage of mitochondrial integrity (17).

Cl-F-ara-ATP is also incorporated to RNA, but according to Xie’s and Plunkett’s study, the inhibition of RNA and proteins syntheses requires higher concentration of clofarabine than the concentration needed for inhibition of DNA synthesis (22). The authors indicate that the incorporation rate of clofarabine nucleotide into RNA is only 1% in comparison to the rate of insertion of clofarabine monophosphate into DNA (22).

In year 2008, Chen et al. documented in in vitro studies that clofarabine triphosphate is a substrate for yeast poly(A) polymerase, and the nucleotide causes termination of the chain elongation in both absence and presence of ATP. Incorporation of Cl-F-ara-ATP blocks the ability of the yeast poly(A) polymerase to extend RNA chain, during polyadenylation of RNA chain (31). The inhibitory effect of Cl-F-ara-ATP on RNA polyA-tail synthesis is concentration-dependent, and at 50 µM concentration the nucleotide reduces polyadenylation to 50% of control.

In summary, the mechanism of clofarabine action includes a number of important pathways...
which are responsible for cytotoxicity of the drug (Scheme 2). The drug action is directed to: (i) inhibition of DNA synthesis due to inhibition of ribonucleotide reductase and DNA polymerases activities. Blockage of DNA elongation and DNA strand breakage induce apoptosis, in consequence; (ii) dysfunction of mitochondria by the following effects: damage of mitochondrial DNA, destruction of mitochondrial metabolic function due to interaction of mitochondrial proteins with clofarabine nucleotide, damage of mitochondrial integrity resulting in release of cytochrome c and AIF proteins and stimulation apoptosis pathway via caspase cascade (17, 26); (iii) perturbation of DNA methylation leading to: hypomethylation of CpG islands, decrease in methylation of global DNA and in methylation of promoters of some tumor suppressor genes (PTEN, APC, RARβ2). The decrease in methylation of the gene promoters is associated with an increase in mRNA level of these genes and p21 mRNA level, and with diminution of DNMT1 mRNA level; (iv) inhibition of ADA and SAH hydrolase activities by clofarabine, which was only demonstrated in initial assays in K562 cells; (v) reduction of polyadenylated tail of RNA.

The cytotoxic mechanism of clofarabine imitates cladribine action in high degree. However, clofarabine action is not only more rapid but often its efficacy is higher than cladribine because of: more effective transmembrane transport, rapid accumulation of Cl-F-ara-ATP, greater disruption of DNA elongation (due to clofarabine triphosphate higher substrate specificity for DNA polymerase α), and inhibition of tumor cell growth at over 10-fold lower concentration of the drug than cladribine. The full elucidation of all multidirectional actions already documented and others, which are here presented as hypotheses, require further studies. It is especially necessary to explain the role of clofarabine in epigenetic regulation of expression of many tumor suppressor genes and protooncogenes.

Pharmacokinetic and clinical studies of clofarabine

Clofarabine is currently under investigation in clinical trials for treatment in pediatric and adult patients with acute myeloid leukemias (AML), acute lymphoblastic leukemias (ALL) and myelodysplastic syndromes (MDS) (9, 32). Up till now, these trials are conducted at following research centers: MD Anderson Cancer Center in Houston (USA), Division of Hematology at “Sapienza” University Rome (Italy), Comprehensive Cancer Center at Johns Hopkins in Baltimore (USA), Cardiff University School of Medicine (UK) and Collegium Medicum at Nicolaus Copernicus University in Bydgoszcz (Poland) (33). Treatment with Cl-F-ara-A in patients with other disorders as chronic lymphoproliferative disorders (LPD) and solid tumors indicates substantial activity of the drug, but such trials are still very limited. Recently, combination of clofarabine with cytarabine (ara-C) or cyclophosphamide for therapy of older patients was introduced (39).

Clofarabine is strong growth inhibitor of leukemic cell lines. For example, inhibitory growth indexes (IG50) of clofarabine for the following cells, L1210 (mouse leukemic cell line) (32), K562 (human erythroleukemic cell line) and MDA-MB-231 (breast cancer cell line) are equal to 5 µM, 8 nM, and 70 nM, respectively (unpublished data).

Results of pharmacologic study indicate that clofarabine efficacy was dose-dependent. At the dose of 40 mg/m2, which is equal to maximum tolerated dose (MTD) for acute leukemia, the median indexes (IG50) of clofarabine for the following cells, L1210 (mouse leukemic cell line) (32), K562 (human erythroleukemic cell line) and MDA-MB-231 (breast cancer cell line) are equal to 5 µM, 8 nM, and 70 nM, respectively (34). In leukemic cells in patients with acute and chronic leukemias, Cl-F-ara-ATP intracellular concentration showed prolonged retention (24 h) which was associated with a decrease in DNA synthesis which also lasted to 24 h (35, 36).

The clinical experiences with clofarabine were preceded by animal model study. In mice, the dose of 25–100 mg/kg (75–300 mg/m2) daily for 7 days, given intraperitoneally, were safe. In dogs, regiments of 7.5 mg/kg (150 mg/m2) per day for 5 days, used intravenously, resulted in severe myelosuppression and gastrointestinal toxicities and the deaths of the dogs on days 5–8 of therapy, whereas for 10-fold lower doses of clofarabine no toxicities occurred. The results of animal models allowed to calculate safe daily dose of 15 mg/m2 for 5 days for human phase I clinical trials (32).

The phase I study of clofarabine in adult patients with refractory-relapsed solid and hematologic malignancies let to determine MTD and dose-limiting toxicities (DLT). In patients with solid tumors, the MTD was 2 mg/m2 daily for 5 days, whereas in the case of patients with acute leukemias the MTD was determined to be 40 mg/m2 daily for 5 days. The last-mentioned MTD value was recommended for phase II dose schedule for adult acute leukemia. It is interesting that the MTD for acute leukemia was 20-fold higher than the value in patients with solid tumor. In the case of adult patients, an overall response rate was 16% (31). The
DLT value for clofarabine (like for cladribine and fludarabine) was myelosuppressive and hepatotoxic (34). In the cases of advanced leukemias for pediatric patients clofarabine was given at 52 mg/m$^2$ daily for 5 days. The dose is higher than for adult patients. An overall response rate was 32% (37). In phase II study (at the MD Anderson Cancer Center in Houston) comprising 62 patients with relapsed and refractory AML, MDS, CML in blastic phase, and ALL, clofarabine was administered at 40 mg/m$^2$ intravenously for over 1 h daily for 5 days, every 3 to 6 weeks; the overall response rate was of 48% (30/62 patients) (38).

Recently, combination of clofarabine (40 mg/m$^2$/1 h for 5 days) with cytarabine (1 g/m$^2$/2 h for 5 days given 4 h after cladribine) have been introduced to I-II phases of clinical trials for treatment of adult patients with relapsed AML, ALL, high-risk MDS, and CML (39–41). An overall response rate was 38%, but no responses occurred in patients with ALL and CML (41). Clofarabine with low-dose cytarabine has a higher response rate than clofarabine alone, but with comparable toxicity (40). The better clinical efficacy of the combined therapeutic strategy may be a consequence of synergistic effect of two used arabinono-nucleosides (i.e., clofarabine and cytarabine) (41).

The clinical application (for older patients with relapsed/refractory acute leukemias) of clofarabine followed by cyclophosphamide at dose level equal to 20 mg/m$^2$ of (clofarabine + cyclophosphamide) resulted in 50% of overall response rate. The observed clinical side effects of combined application of clofarabine with cyclophosphamide (i.e., prolonged marrow aplasia, fungal pneumonia, or multorgan failure) suggested some limitation in intensity of such therapy (42, 43).

It was previously mentioned that the drug stability in acidic solution enhance its oral bioavailability. The oral drug application was used for older patients with high-risk myelodysplastic syndrome. There were three doses of clofarabine used: 40 mg/m$^2$, 30 mg/m$^2$, and 20 mg/m$^2$ daily for 5 days. Courses were repeated every 4 to 8 weeks. An overall response rate was 43%. Although the toxicity profile was better with lower doses of clofarabine (44) the optimal dose, schedule and the appropriate patient profile for the oral therapy require further investigation.

In conclusion, hitherto reported data concerning clofarabine have been encouraging. The high anticancer activity of clofarabine in low doses, its stability in acidic environment, resistance to degradation by enzymatic and non-enzymatic pathways, multidirectional mechanism of the drug action, (e.g., action, including inhibition of DNA synthesis, induction of apoptosis and participation in epigenetic modulation of gene expression), the optimistic results of clinical trials where clofarabine was applied alone and in combination with ara-C or cyclophosphamide together with high percentage of overall response rate, suggest that the drug may be very useful in therapy of advanced leukemias in pediatric and older patients. Nevertheless, further investigation with clofarabine against solid tumors, optimization of treatment dose of clofarabine combined with other drugs, and studies on clofarabine oral application in hematological malignancies should be continued in the future.

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REFERENCES


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