Commentary

Mechanisms of anti-cancer action and pharmacology of clofarabine☆

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ABSTRACT

Clofarabine, a next-generation deoxyadenosine analogue, was developed on the basis of experience with cladribine and fludarabine in order to achieve higher efficacy and avoid extramedullary toxicity. During the past decade this is the only drug granted approval for treatment of pediatric acute leukemia. Recent clinical studies have established the efficacy of clofarabine in treating malignancies with a poor prognosis, such as adult, elderly, and relapsed pediatric leukemia. The mechanisms of its anti-cancer activity involve a combination of direct inhibition of DNA synthesis and ribonucleotide reductase and induction of apoptosis. Due to this broad cytotoxicity, this drug is effective against various subtypes of leukemia and is currently being tested as an oral formulation and for combination therapy of both leukemias and solid tumors. In this review we summarize current knowledge pertaining to the molecular mechanisms of action and pharmacological properties of clofarabine, as well as clinical experiences with this drug with the purpose of facilitating the evaluation of its efficacy and the development of future therapies.

1. Introduction

Nucleoside analogues have had a substantial impact on the treatment of cancer, especially hematological malignancies. The most credible explanation is the dependency of hematopoietic cells on salvage synthesis of nucleosides [1] and their expression of several membrane transporters that take up nucleoside analogues [2]. Their structural similarity to physiological nucleosides allows these analogues to be taken up by cells, metabolized, and incorporated into DNA or RNA. Therefore, the main targets of these activated nucleoside analogues are the enzymes involved in de novo and salvage pathways of nucleoside synthesis.

The history of nucleoside analogues dates back to the late 1950s when cytosine arabinoside (cytarabine, AraC) was synthesized and subsequently became the backbone in the treatment of acute myeloid leukemia (AML) and one of the most widely used nucleoside analogues to date [3]. Single-dose treatment with AraC was not promising, but in combination with agents that interacted with topoisomerase, such as anthracyclines, a high rate of remission could be achieved in AML patients [4]. The success of AraC led to the development of other arabinosylpurine homologues (e.g. 9-β-D-arabinofuranosyladenine (araA)). Since the clinical activity of araA was found to be limited due to its rapid deamination by adenosine deaminase (ADA) [5], ADA-resistant halogenated arabinosyl derivatives such as 2-fluoro-9-β-arabino-furanosyladenine (fludarabine) were then developed [6]. Thereafter, because of the insolubility of fludarabine, its 5′-monophosphate derivative (fludarabine monophosphate, FaraA, Fludara®), was designed for conversion into fludarabine by endogenous phosphatases. However, treatment with this latter drug also results in the formation, by phosphorylase cleavage, of 2-fluoroadenine, a toxic compound with only minor anti-tumor activity [7]. In the early 1970s the realization that halogenated analogues exert an anti-leukemic effect led to the synthesis of 2-chlordeoxyadenosine (cladribine, CdA) [8], which, however, has reduced oral bioavailability due in part to its instability in the
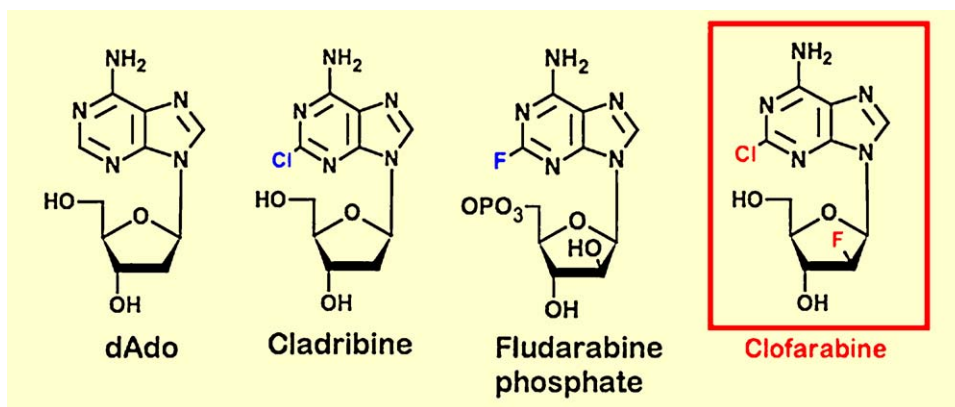
Fig. 1. Structural formulas of deoxyadenosine (dAdo) and its analogues: cladribine, fludarabine phosphate, and clofarabine.

acidic gastric environment as well as its susceptiblity to enzymatic cleavage [9–11].

In the 1990s, in order to tackle all the above-mentioned limitations of early nucleoside analogues, a next-generation deoxyadenosine analogue, clofarabine (2-chloro-2′-arabino-fluoro-2′-deoxyadenosine, CAFdA, Cl-F-ara-A), a 2′-arabino-fluoro derivative of cladribine, was synthesized [12]. The rationale behind its design was to combine the structural features of cladribine and fludarabine (Fig. 1). Like cladribine and fludarabine, clofarabine is toxic to both non-proliferating human lymphocytes and rapidly proliferating cells, while being resistant to phosphorylitic cleavage and deamination-stable in acidic environments.

Preclinical studies indicated a high degree of efficacy of clofarabine and subsequently led to human trials. In 2004 the FDA approved clofarabine (Clolar™, Genzyme) for the treatment of pediatric leukemic patients – a decision followed by European Commision approval in 2006 (Evoltra®, Bioenvision). Today the anti-cancer activity of clofarabine toward other types of tumors, as well as in other age groups, is being actively investigated.

2. Molecular mechanisms of action

2.1. Structure and physicochemical properties

Clofarabine was designed as an adenosine analogue with improved stability both in the plasma and the acidic gastric environment. And, indeed, the introduction of a fluorine atom at the 2′-arabino position of cladribine increases its stability at pH 1.0 [13]. As predicted, the pK_a value of clofarabine (1.75) is higher than that of cladribine (1.28). Furthermore, it exhibits the highest lipophilicity among related purine analogues [13] and is resistant to degradation by *E. coli* nucleoside phosphorylase [14].

2.2. Transport and metabolism

Clofarabine metabolites are retained by cells to a greater extent than metabolites of cladribine, which is believed to be one factor in the more pronounced anti-tumoral effect and higher hematological toxicity of the former [15]. Other factors contributing to its higher anti-cancer activity are thought to be clofarabine's high affinity for nucleoside transporters and deoxycytidine kinase (dCK), as well as for key enzymes involved in DNA synthesis, e.g. ribonucleotide reductase (RR) and DNA polymerase-α and -ε.

Most cells have two types of active nucleoside transporters: human equilibrative nucleoside transporter (hENT) and human concentrative nucleoside transporter (hCNT). Transport via hENTs is a sodium-independent mechanism involving primarily bi-directional facilitative diffusion driven by a gradient in the nucleoside concentration across the cell membrane. In contrast, the hCNTs are sodium-dependent active transporters that depend on ATP for their ability to transport purine nucleosides into the cell against a concentration gradient.

Clofarabine is believed to enter cells by both facilitated and active nucleoside transport mechanisms as well as, at higher concentrations and upon longer exposure, by passive diffusion across lipid membranes [16]. The importance of membrane nucleoside transporters in clofarabine metabolism is indicated by the fact that the rate of clofarabine uptake is ∼10-fold higher by transport-competent than transport-deficient human leukemia cells [16]. In particular, cells expressing hCNT3 exhibit the highest rate of clofarabine uptake compared to the cells expressing hCNT2, hENT1, and hENT2, whereas transport-deficient and hCNT1-expressing cells demonstrate no uptake, consistent with the fact that hCNT1 is specific for pyrimidines. In addition, clofarabine was more cytotoxic to cells expressing hENT1 than fludarabine or cladribine.

Upon entry into cells, clofarabine is phosphorylated to its monophosphate derivatives, primarily by dCK, a constitutively expressed key cytosolic enzyme involved in the salvage pathway of DNA synthesis, but also by the mitochondrial enzyme deoxyguanosine kinase (dGK) [17]. Further intracellular phosphorylation results in the final metabolite, clofarabine triphosphate. The cytotoxicity of nucleoside analogues in quiescent cells (i.e. lymphocytes) depends mainly upon selective and progressive accumulation of the triphosphate metabolites because of the high ratio of dCK to cytosolic 5′-nucleotidase. The latter acts as a deactivator of nucleoside analogues by dephosphorylating the triphosphate metabolites and enabling their transport out of the cell. The importance of dCK for the efficacy of clofarabine was confirmed by our recent finding that the cells deficient in this activity are more resistant to the cytotoxic effect of this drug [18]. Furthermore, the concentration of dCK is typically highest in lymphoid tissues, as well as considerably higher in tumor tissues than in normal tissues [19]. The studies with recombinant human dCK and kinases in the crude leukemic cell extracts have revealed that the efficiency (V_{max}/K_m) of clofarabine phosphorylation by these enzymes is close to, or greater than, that of the natural substrate deoxycytidine, as well as significantly greater than that of cladribine or fludarabine [20,21]. The interactions between 2-Cl and its surrounding hydrophobic residues contribute to the high catalytic efficiency of dCK for clofarabine [22].

Although dCK activity is rate-limiting for the accumulation of fludarabine triphosphates, this is not the case with clofarabine [15]. Accumulation of clofarabine appears to be determined by the rate of phosphorylation of its monophosphate to diphosphate by purine nucleotide monophosphate kinase, similarly to cladribine [15,23]. One explanation might be that nucleoside analogues

bearing a 2-chloroadenine nucleobase are relatively poor substrates for the monophosphate kinase.

2.3. Mechanisms of anti-cancer activity

The anti-cancer activity of clofarabine involves three major mechanisms: inhibition of DNA synthesis, inhibition of ribonucleotide reductase, and direct induction of apoptosis (Fig. 2). This broad range of activities explains the efficacy of the drug against both rapidly growing and quiescent tumors [14].

Clofarabine competes potently with dATP for binding to DNA polymerase-α and -ε [24]. At a low ratio of clofarabine triphosphate to dATP, clofarabine is incorporated primarily into internal phosphodiester linkages inhibiting DNA repair; whereas, at higher ratios, clofarabine is detected mainly at terminal sites inhibiting DNA elongation [15]. The most pronounced inhibition of DNA elongation is observed at clofarabine triphosphate to dATP ratios greater than one [25]. Incorporation of clofarabine monophosphate into DNA impairs its elongation and repair by causing chain termination and strand breaks. However, clofarabine triphosphate is not a potent inhibitor of DNA polymerase-β, mitochondrial DNA polymerase-γ, or DNA primase [24].

In a recent article Chen et al. suggested that clofarabine action may as well involve an RNA-directed mechanism. Clofarabine triphosphates were shown to cause chain termination in the absence of ATP [26]. With increasing amounts of clofarabine triphosphates, there was a concentration-dependent effect on polyadenylation due to blockage of the ability of yeast poly(-A)polymerase (yPAP) to extend the poly(A)-tail by the analogues' incorporation into RNA [26].

Besides DNA polymerase inhibition, clofarabine triphosphate potently inhibits RR activity (50% at 65 nM) [24] by binding to the allosteric site on the regulatory subunit, incorporating in this manner anti-cancer properties of fludarabine. Inhibition of RR depletes the dNTP pool, with the most pronounced reduction in the dCTP pool, followed by dATP and dGTP, and only a minor effect on the dTTP pool. Through depletion of the dNTP pool, clofarabine self-potentiates the incorporation of clofarabine triphosphates into DNA, thus explaining the increased effectiveness of clofarabine in DNA synthesis inhibition [27].
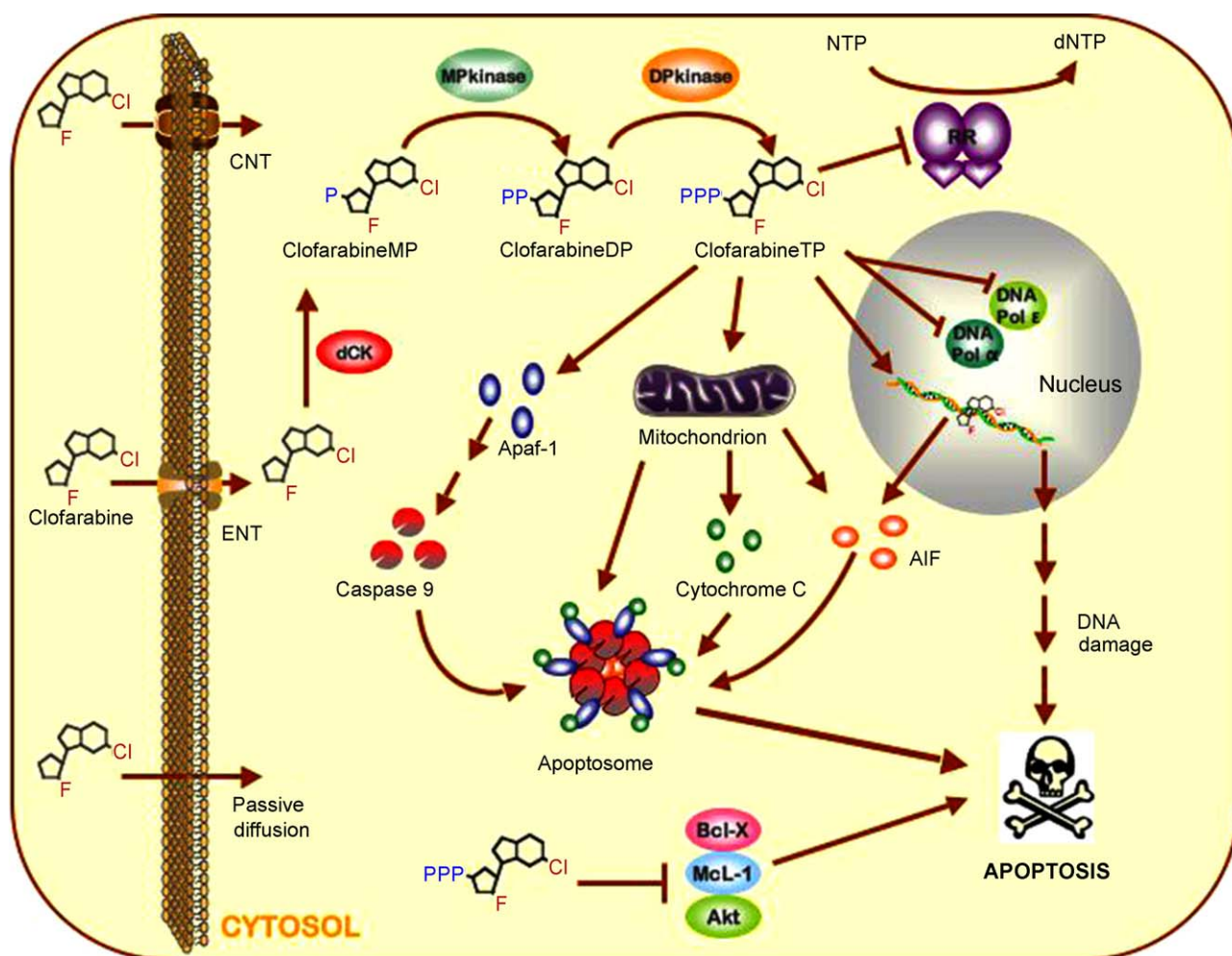

Fig. 2. Clofarabine's mechanism of action. Clofarabine is transported into the cell by either passive diffusion or facilitated/active transport by concentrative and equilibrative nucleoside transporters (ENTs and CNTs); in addition, there is some evidence of mitochondrial transport [16]. Upon entering the cell, clofarabine is phosphorylated stepwise by deoxycytidine kinase (dCK), monophosphate kinase (MPkinase), and diphosphate kinase (DPkinase) to its triphosphate active form (clofarabineTP). ClofarabineTP acts as an inhibitor of DNA polymerase-α (DNA pol α) and -ε (DNA pol ε) by competing with the natural substrate dATP. When incorporated into DNA clofarabine leads to DNA damage, which signals activation of apoptotic pathways. It can further inhibit ribonucleotide reductase (RR), causing dNTP pool reduction and thus reinforcing its own incorporation into DNA. By directly affecting the mitochondrial transmembrane potential, clofarabine releases cytochrome c and apoptosis-inducing factor (AIF) [30]. In addition, it binds cytosolic apoptotic protease-activating factor 1 (APAF-1) and thus mediates caspase activation [30]. Consequently, caspase-9, together with other pro-apoptotic factors, forms apoptosome, leading to cell apoptosis. Moreover, clofarabine may mediate pro-apoptotic signals by inducing down-regulation the death suppressor proteins Bcl-X(L) and Mcl-1 and dephosphorylation of the anti-apoptotic kinase Akt [31].

Furthermore, depletion of dNTP pools can lead to enhanced nucleoside kinase activity and thereby increased phosphorylation of nucleoside analogues, more pronounced accumulation and prolonged retention of the triphosphate metabolites, and, consequently, a more potent anti-cancer effect [25]. The damage caused by incorporation of clofarabine monophosphate into DNA initiates a chain of events that result in activation of pro-apoptotic pathways. Clofarabine can replace dATP and directly affects cytosolic apoptotic protease-activating factor 1 (APAF-1), thus causing caspase activation [28,29]. Clofarabine also directly affects mitochondria by altering the transmembrane potential and, as a result, releasing cytochrome c and apoptosis-inducing factor (AIF) [30]. Mitochondrial damage caused by clofarabine may be explained by direct binding of the triphosphate metabolite to proteins in the mitochondrial membrane, leading to an alteration in membrane potential as well as intra-mitochondrial accumulation of this triphosphate [30]. The combined actions of cytochrome c, AIF, APAF-1, and caspase-9 lead to the formation of the apoptosome complex responsible for programmed cell death induction.

Moreover, clofarabine has been shown to induce dose- and time-dependent down-regulation of the death suppressor proteins Bcl-X(L) and Mcl-1 and dephosphorylation of anti-apoptotic kinase Akt and its downstream effectors (Bad, FKHRL1) [31]. An interesting observation was made in the T-ALL CEM cell line when treated with clofarabine and caffeine [31]. The addition of caffeine inhibited the down-regulation of Cdc25A, usually observed when cells are treated with clofarabine alone, and increased the cleavage of PARP. Therefore, a combinatorial effect on apoptosis could be expected if clofarabine is administered together with inhibitors of chk1, a checkpoint homologue that phosphorylates Cdc25 and prevents cells entering mitosis.

2.4. Mechanism of resistance

The effectiveness of nucleoside analogue treatment against cancer cells is principally limited by the primary or acquired drug resistance. This commonly happens due to alteration in the activity of one or more actors in the metabolic pathway of a drug, thus leading to its reduced effectiveness. A detailed knowledge of the molecular mechanisms underlying such resistance would enable the use of appropriate dosing schedules and combination therapies in the clinical setting to overcome this limitation.

There are few studies that have initiated the investigations into potential mechanisms of resistance. In one such study, the resistance to clofarabine acquired by HL-60 and CCRF-CEM cell lines is directly correlated with reduced activity of the nucleoside phosphorylating enzyme dCK, as has been previously found to be the case with cladribine [20,32]. We detected dCK deficiency at the level of enzymatic activity, the protein level, and also as mRNA expression for the cell lines resistant to clofarabine; these cells did not consistently form detectable levels of clofarabine triphosphates. However, no mutations in the coding region of the dCK gene were detected. The suggestion that DNA methylation is responsible for the decreased level of dCK in resistant cancer cells has not been confirmed [32,33].

The problem of resistance due to reduced dCK and/or dGK activity might be overcome by introducing the drug as a pronucleotide, thereby releasing the deoxynucleoside 5′-monophosphate directly into the cell. In addition, determination of dCK and/or dGK activities might help predict how well a patient will respond to a certain cytotoxic drug and which dose and combination of drugs will be most effective.

Clofarabine-resistant cell lines showed cross-resistance to other antimetabolites (i.e. fludarabine, AraC, difluorodeoxycytidine, dfdc) phosphorylated by dCK [20,32]. Thus, clofarabine-resistant cells also accumulate lower levels of cladribine and other analogue nucleotides. On the other hand, cells resistant to fludarabine exhibited cross-resistance to cladribine, but not clofarabine.

No alteration in nucleoside transport or expression of the classical MDR (multidrug-resistance) protein was detected in clofarabine-resistant cells, suggesting that the mechanism of acquired resistance to clofarabine is a specific, rather than a general, phenomenon [20,32]. Clofarabine-resistant cells also exhibit the highest number of structurally rearranged chromosomes, indicating a high potential of clofarabine to rearrange the genome [32]. However, this was not the cause of dCK deficiency.

Expression of a small subunit of RR (R2) was substantially reduced and the enzyme's activity was halved with no alteration of allosteric activity in the clofarabine-resistant cell lines, as opposed to fludarabine-resistant cell lines, where RR was substantially higher than in the wild type cell line [32]. Since the resistant cell line with inhibited dCK and RR activity still produced dNTPs and kept on proliferating, it was hypothesized that p53R2, a small subunit of RR directly induced by p53, replaces R2 to form active ribonucleotide reductase. A truncated form of p53R2 was detected that may replace R2 in the production of dNTPs in the resistant cell lines.

Other mechanisms of resistance to clofarabine are thought to involve changes in Ca^{2+}-sensitive mitochondrial phenomena and are probably not influenced by the Fas pathway, as in the case of cladribine [34].

3. Pharmacokinetics and anti-cancer activity

3.1. In vitro investigations

As expected from its potent inhibition of DNA synthesis, clofarabine exerts potent growth inhibition and cytotoxic activity (IC_{50} values = 0.028–0.29 μM) in a wide variety of leukemia and solid tumor cell lines in vitro [35]. Thus, of 60 human tumor cell lines in the National Cancer Institute's Developmental Therapeutics Program panel, clofarabine potently inhibits the growth (GI_{50} values = <0.0001–0.45 μM) of 35. The cell types sensitive to clofarabine include those of non-small-cell lung, colon, central nervous system, melanoma, ovarian, renal, prostate, and breast tumors [36].

Cariveau et al. also demonstrated that clofarabine exhibits a strong radiosensitizing effect on murine tissues in vitro and in vivo, by interfering with the repair of DNA damage [37]. Another mechanism driving the radiosensitization activity of a combination of clofarabine with ionizing radiation (IR) was found to be the activation of dCK by IR, which consequently enhances clofarabine cytotoxicity [38].

In vitro exposure of mononuclear cells from chronic lymphocytic leukemia (CLL) and AML patients to identical concentrations of clofarabine and cladribine leads to higher intracellular accumulation of the former's triphosphate metabolites [20,39]. In these cells, the major metabolite detected was clofarabine monophosphate, with clofarabine diphosphate representing less than 10% of the total nucleotide metabolite present, thereby confirming that dCK is not the rate-limiting step for the formation of clofarabine triphosphate. Interestingly, no correlation was found between the level of phosphorylated metabolites, enzymes (dCK, dGK), and the cytotoxicity of clofarabine, suggesting that additional factors determine its cytotoxic activity [39].

In isolated perfused rat liver, clofarabine and cladribine have a similar first-pass metabolism of 50% [40]. The rate of elimination of both of these substances is strongly dependent on the initial concentrations. However, the elimination rate of clofarabine is much slower and its concentration declines without an increase in

the concentration of the degradation product, 2-chloroadenine, most likely due to the binding of clofarabine to components of hepatic tissue. The elimination kinetics of clofarabine 5′-mono-, di-, and triphosphate exhibit tri-phasic behavior, with a β-half-life of 8–24 h and a very long γ-half-life (29 h), indicating prolonged cellular retention [15].

In a study on polarized human kidney proximal tubule cells (hRPTC11), apical-to-basolateral fluxes of clofarabine as well as fludarabine and cladribine across the cell layer were observed following mediation by the coupling of apical hCNT3 to basolateral hENT2 [41]. Such directionality of transepithelial fluxes of these three nucleoside analogues resembles that of adenosine, which is reabsorbed in human kidney proximal tubules. These observations could explain the delayed elimination of clofarabine. A deeper insight into the mechanisms of hNTs transport of clofarabine could lead to strategies aimed at improving its dosing with maximum efficacies and minimum toxicity.

The major metabolite detected in rat and dog hepatocytes after exposure to clofarabine is P11, which accounts for 1.2% and 2.5%, respectively, of the total radioactivity recovered [42]. P11 is suggested to be a carboxy- or methoxy-clofarabine, but the exact location of the change remains unknown. The only metabolite observed in the case of human hepatocytes is P14, which accounts for 0.2% of the total radioactivity recovered and has been suggested to be the sulfates' conjugate of clofarabine at the 5′-carbon.

The in vitro efficacy of clofarabine was compared to those of nelarabine and AraC in a panel of acute lymphoblastic leukemia (ALL) cells [43]. The concentration of clofarabine that inhibits growth by 50% is 188 times lower than the corresponding concentration for nelarabine in all cases. Clofarabine appears to be marginally more effective in B-lineage than in T-lineage ALL and B-lineage is also several times more sensitive to clofarabine than AraC. There is a potential for cross-resistance of clofarabine to many ALL therapeutics, but not methotrexate or thiopurines. This distinct resistance profile may prove useful in combination with other compounds.

One such combination study was undertaken with alkylating agents and clofarabine or fludarabine in lymphocytes isolated from CLL patients [44]. DNA damage repair mechanisms were first initiated in the CLL lymphocytes by treatment with 4-hydro-xycyclophosphamide. This DNA repair was then inhibited by DNA chain termination in an equivalent manner by treatment with either fludarabine or clofarabine. However, clofarabine tripho-sphates exhibited increased rates of intracellular accumulation, thus leading to maximal inhibition at 1/10 the concentration of fludarabine triphosphates.

Exposure to such agents as cladribine, clofarabine, fludarabine, or AraC leads to a 2–4-fold increase in dCK activity (most probably by direct inhibition of ribonucleotide reductase) in the HL-60 cell line, as well as in peripheral blood mononuclear cells [45]. This phenomenon has been exploited by treating cells with one deoxynucleoside analogue initially in order to induce dCK activity and thereafter administering a second analogue; and, as expected, clofarabine pretreatment to induce dCK enhances subsequent accumulation of AraC triphosphate in myeloid cell lines [46]. Similarly, pretreatment of HCT-116 colon, K562 leukemia, and RL lymphoma cell lines with clofarabine enhanced the metabolism of T-araC (1-β-D: -[4-thio-arabinofuranosyl] cytosine), a new cyto-sine analogue with superior anti-cancer activity as compared to AraC [47].

Caution must be observed, however, when combining AraC with clofarabine since these compounds have been shown to have cross-resistance [48]. Alternatively, valproic acid (VPA), a histone deacetylase inhibitor used as a drug against epilepsy, exhibits a synergistic effect when combined with clofarabine in AML cell lines and primary cells. A possible explanation could be that VPA

induces apoptosis by activating both extrinsic and intrinsic apoptotic pathways.

A recent study demonstrated the hypomethylation property of clofarabine in human lymphoid tumor cells as well as its ability to up-regulate the expression of cancer-testis (CT) antigens (Sp17 and SPAN-Xb) aberrantly expressed in tumor cells [49]. If the same property is proven to be effective in vivo, it could be possible to administer low-dose clofarabine to patients to up-regulate the expression of CT antigens to increase the susceptibility of the tumor cells to the cytotoxic effect of antigen-specific cytotoxic T cells prior to administering specific tumor vaccines targeting CT antigens by hypomethylating. Furthermore, hypomethylating DNA clofarabine at low doses could potentially enhance the expression of tumor suppressor genes that are often hypermethylated, and thus inhibited, in cancer cells, leading to activation of pro-apoptotic genes.

Silencing of 5′-nucleotidase cNT1 by siRNA interference in a panel of leukemic cell lines has significantly augmented the cytotoxicity of clofarabine. This investigation could prove the combination of clofarabine nucleotidases inhibitors useful for cancer treatment [50].

3.2. In vivo investigations

In addition to numerous studies on the effects of clofarabine in cells, a considerable amount of in vivo data on various animal models is available.

The dose and schedule-dependent cancer activity of clofarabine was demonstrated by testing different dosing schedules in an NCI H460 lung tumor murine model with the greatest anti-tumor activity seen when the daily clofarabine dose was subdivided into three equal doses and administered at 4-h intervals each day for 30 days [36,51].

Since nucleoside transporters are expressed in many tissues, clofarabine is expected to be widely distributed in both tumor and normal tissues. Indeed, this has been found to be the case in mice and rats, where the tissue distribution is rapid and widespread with the highest concentrations occurring in highly perfused organs [42,52].

The tissue-to-plasma ratios of clofarabine in the liver and myocardium were considerably high (4.8 and upward) [42]. This is most probably due to the fact that the active clofarabine phosphates must be dephosphorylated in order to be exported from the cell as well as to the lipophilicity of the parent drug. These observations are consistent with pharmacokinetic studies which reveal that, following intravenous administration, clofarabine is extensively distributed throughout the body, with a volume of distribution at steady state (Vdss) of approximately 1.4–2.6 L/kg in mice, 3.2–3.6 L/kg in rats, and 0.9–1.2 L/kg in dogs [36].

Intravenous administration of [^{14}C]clofarabine 25 mg/kg/day to rats confirmed the non-linear pharmacokinetics, showing three exponential phases of elimination, with half-lives of 0.3, 1.3, and 12.8 h [42]. Close to 80% of the dose administered was recovered in the urine and 10% in the feces. The major metabolite, 6-ketoclofarabine, is believed to be formed extrahepatically via adenosine deaminase.

The fact that clofarabine is more stable in an acidic environment and more lipophilic than cladribine [13,40] leads to the assump-tion that the former would have more side effects, particularly neurotoxicity. However, in mice, the accumulation of clofarabine in brain tissue was found to be lower than that of cladribine [52], contradicting the assumption that clofarabine's more pronounced lipophilicity would render it a more effective drug for the treatment of lymphoproliferative disorders in the central nervous system (CNS).

On the other hand, in a study of non-human primates, clofarabine was found to penetrate into the cerebrospinal fluid,

although to a modest extent [53]. The concentrations obtained there may nevertheless approach those known to be cytotoxic *in vitro*.

A substantial anti-cancer effect has been observed against human colon tumors transplanted into nude mice, exhibiting schedule-dependent anti-tumor activity upon intraperitoneal, intravenous or, particularly, oral administration of clofarabine [54]. Fifty percent of tumor growth was inhibited at 0.26 μM in a 72-h exposure, a concentration significantly lower than that of fludarabine and cladribine. The most effective of three administration regimens tested (single, twice-weekly, 5-day once daily) was a 5-day daily administration schedule.

Administration of oral doses in rats equivalent to intravenous doses of 10 and 25 mg/kg provides bioavailability estimates of approximately 50%, indicating the feasibility of oral treatment [55]. Approximately 83% of clofarabine was recovered in plasma with only 13.3% binding to plasma proteins.

Orally administered clofarabine again showed superior anti-tumor activity with regressive or cytostatic growth curves compared to intraperitoneally administered clofarabine in an HT29 colon tumor mouse model [56]. Additionally, in an RL lymphoma tumor mouse model, prolonged administration (21 days) of oral clofarabine showed markedly enhanced anti-tumor activity as compared to intravenous administration on a daily ×5 days schedule, the dosing regimen commonly used in clinical trials [57].

The hematological toxicity effects of clofarabine in Fischer 344 rats were least severe, in terms of effects on the circulating white blood cell count (WBC), when administered in daily oral doses of 36 and 60 mg/m^2 for 21 days than in doses of 150 and 240 mg/m^2 administered orally or intravenously daily for 5 days. Intravenously dosed rats also exhibited decreased cellularity in the bone marrow [57].

In an attempt to further improve the pharmacological and toxicological properties of clofarabine, Heckl-Östreicher et al. have synthesized an EPD-clofarabine prodrug, which is activated by membrane-associated specific hydrolases releasing the free nucleoside into the respective cells [58]. In a comparative pharmacokinetic study, a prodrug revealed a 36 times higher plasma exposure and an increased terminal half-life but very low free clofarabine concentrations. EPD-clofarabine showed high efficacy in a variety of solid tumor xenograft models, demonstrating improved tolerability and increased and sustained anti-tumoral growth inhibition compared to clofarabine. Furthermore, this compound could be a potential candidate for the treatment of pancreatic cancer as monotherapy or in combination with gemcitabine as well as in the gemcitabine-resistant form of pancreatic cancer [59].

In summary, the *in vivo* studies demonstrated clofarabine to be an effective drug with a substantial anti-cancer effect and superior oral bioavailability, as well as a very promising candidate for combination therapies, thus successfully highlighting its potential in human studies.

3.3. Pharmacokinetic data in humans

The initial studies in humans have corroborated the potent nature of clofarabine as an anti-cancer agent observed in animal studies. A consistent decline has been observed (68–99%) in the WBC count in all acute leukemia patients treated with 40 mg/m^2 of clofarabine after 5 days, indicating its high potency [60].

DNA synthesis is inhibited by 75–95% at the end of an infusion of clofarabine at doses ranging from 22.5 to 55 mg/m^2. In the lower dose (22.5 and 30 mg/m^2) range a partial recovery of the inhibition of DNA synthesis was observed after 24 h, while at the higher doses (40 and 55 mg/m^2) this inhibition remained unchanged. This implies that there is a dose-dependent effect on the maintenance of inhibition of DNA synthesis by clofarabine treatment [60]. Specifically, the decrease in DNA synthesis was shown to be associated with the accumulation of clofarabine triphosphates in the blasts of adults with refractory leukemias [60,61].

Bonate and coworkers have analyzed pharmacokinetic data from one phase I [62] and two phase II studies [63]. Weight and WBC count were the only patient-specific factors identified as being important for the pharmacokinetics of clofarabine [64]. However, in a study conducted by the Genzyme Corporation in adults with advanced solid refractory tumors, neither clofarabine clearance nor clofarabine central volume correlated with weight or body surface area [56].

Clofarabine demonstrated a β-half-life of 6.4 h and a Vdss of 210 L (72% between-subject variability) in a 40-kg individual with a WBC count of 10^4/μL, indicating extensive tissue distribution as observed in animal models. It was concluded that the pharmacokinetic parameters for clofarabine are proportional to the dose with no indication of a decline in intracellular triphosphate concentration with time [64].

The total binding of clofarabine to plasma protein amounts to 47%, and only 27% is bound to human serum albumin (HSA), which could be a factor in producing a wide tissue distribution [65].

When given to male and female patients with ALL or AML, clofarabine exhibits balanced clearance, with a renal clearance of 10.8 L/h/m^2 (28.8 L/h/m^2 total systemic clearance) with 57% of the dose being excreted unchanged in the urine [36]. The pharmacokinetics of clofarabine were further examined in 52 adults with solid tumors involved in a dose-escalation phase I study with doses up to 129 mg/m^2 administered once a week for 3 weeks every 28 days [66]. It was shown to be time-invariant, with an average systemic clearance, Vdss, and half-life of 18.1 L/h (9.9 L/h/m^2), 72 L (39 L/m^2) and 4.0 h, respectively. It was also observed during this study that, although clofarabine is a purine analogue (as is fludarabine), its effects on lymphocyte subsets more closely resemble those of gemcitabine, a pyrimidine analog, in having a selective detrimental effect on the B-lymphocyte subset [66]. This observation should be considered when developing appropriate combination regimens involving clofarabine.

Under the Biopharmaceutics Classification System, clofarabine is classified as a Class III drug. This classification is based on the fact that the adult clearance is ~20 L/h while oral bioavailability is ~50% with a mean absorption time of 2.0 h (in adult patients with advanced solid tumors) [56].

4. Clinical studies

To date, numerous clinical studies on clofarabine have been conducted and some with promising results. A wide variability in responses among the patient populations has been observed, indicating the complexity of factors that need to be considered before administering clofarabine [67].

In the phase I trial the maximum tolerated dose (MTD) for patients with solid tumors was determined at 2 mg/m^2 with the dose-limiting toxicity (DLT) being myelosuppression, while for patients with hematological malignancies, the MTD was determined at 40 mg/m^2/day with the DLT being hepatotoxicity [67]. This discrepancy is explained by the accepted view that drugs for leukemia cause myelosuppression and cytopenias of limited duration and thus are not regarded as dose-limiting conditions.

A phase II study in 62 adult patients with relapsed or refractory acute leukemia and myelodysplastic syndrome who received clofarabine at 40 mg/m^2 IV once daily for 5 days every 3 to 6 weeks demonstrated a total response rate of 48% [68]. After the first clofarabine infusion, responders accumulated more clofarabine triphosphate in blasts than non-responders. This increased only in

responders after the second clofarabine infusion, indicating that cellular pharmacokinetics may have prognostic significance. However, another phase II trial in adult AML patients demonstrated a minimal response, most likely due to the fact that the patients were significantly more refractory [69].

Clofarabine showed promising results in a phase II trial in elderly refractory AML patients at doses of 30 mg/m^2/day for 5 days every 28–42 days with almost 50% of responders (40–50% CR) [70]. In particular, this agent appears to be effective for patients with adverse cytogenetics, with an associated remission rate of approximately 40%. If this finding is validated in further studies, this is an interesting and beneficial feature.

In the phase I trial involving pediatric patients with ALL and AML, the MTD was determined to be 52 mg/m^2 and the recommended phase II dose [62]. The DLT was reversible hepatotoxicity and skin rash. A total response of 30% was observed in phase II trials in pediatric patients suffering from refractory AML and ALL treated with clofarabine at doses of 52 mg/m^2/day for 5 days every 2–6 weeks [63].

Most studies to date show the benefit of oral high-dose treatment with clofarabine. However, it is of interest to establish a low-dose regimen of clofarabine in tablet form when given during a longer period of treatment.

The promising in vitro data and results from animal studies led to the development of combination treatments with clofarabine and other cytotoxic agents. In this context, a human study involving treatment with clofarabine (30–40 mg/m^2/day) in combination with AraC (20–100 mg/m^2/day) in order to enhance dCK activity, was undertaken and was found to be more effective in previously untreated patients with AML and MDS than single agent treatment with comparable toxicity [71,72].

Another phase I study on elderly AML patients has revealed that clofarabine, in combination with idarubicine and AraC, can lead to clinical remission. Anthracyclines are commonly used to potentiate the activity of other nucleoside analogues, with idarubicin being the preferred anthracycline in induction and salvage regimens of patients with AML. The response to combinations of clofarabine with AraC, clofarabine with idarubicin, and clofarabine plus AraC and idarubicine in AML salvage studies was not significantly different with a total response of 39% (CR + CRp) [73].

A pilot phase I study was conducted on 13 patients with AML and ALL being treated with clofarabine in combination with cyclophosphamide and etoposide to determine the MTD and the dose-limiting toxicities of the combination [74]. This combination induced durable remission in children with relapsed or refractory acute leukemia. The recommended phase II doses of clofarabine, cyclophosphamide, and etoposide were 40, 440, and 100 mg/m^2/day, respectively, each given for 5 days in induction. The phase II portion of the study is now underway.

Treatment with 4-hydroperoxy-cyclophosphamide, an activated form of cytoxan, induces DNA repair patches, thus increasing the incorporation of clofarabine triphosphates into DNA and consequently enhances the cytotoxic effect [44]. Conversely, the use of clofarabine prior to cyclophosphamide appeared to augment both cyclophosphamide-induced DNA damage (by phosphorylated H2AX) and apoptosis (by sub-2N DNA) [75].

In a phase I/II trial, clofarabine was given in combination with a myeloablative dose of busulfan, an alkylating antineoplastic agent, in an attempt to develop a pre-transplant conditioning regimen suitable for patients with refractory, non-remissive hematologic malignancies at the time of transplant. As a result, all 20 patients engrafted rapidly, and this combination shows promising anti-tumor activity in this group of very high-risk patients [76].

In summary, the MTD in patients with solid tumors is 20 times lower than in patients with hematological malignancies. Myelo-suppression is the most common adverse affect in the case of solid tumors, whereas hepatotoxicity seems to be doselimiting in patients with acute leukemias. Clofarabine plasma concentrations were generally lower in the pediatric population than in the adult population when the same doses were administered [36]. Although it is currently approved only for pediatric patients, clofarabine showed promising results in usually unresponsive elderly patients. Furthermore, clinical data show that combination regimens are feasible, but the optimal regimen for clofarabine combined with other anti-cancer agents remains to be determined.

5. Conclusion

In a nutshell, clofarabine exhibits efficacy in hematologic malignancies such as ALL, AML, and myelodysplastic syndrome. Like cladribine and fludarabine, clofarabine is toxic to both non-proliferating human lymphocytes and rapidly proliferating cells, while being resistant to phosphorylitic cleavage and deamination and stable in acidic environments. It exhibits the highest lipophilicity among related purine analogues [13] and is resistant to degradation by E. coli nucleoside phosphorylase [14]. Clofarabine's high affinity for nucleoside transporters, dCK, and key enzymes involved in DNA synthesis, as well as the prolonged intracellular retention of its metabolites, contributes to its potent anti-cancer activity.

In clinical trials clofarabine shows the potential in the group with the poorest prognosis: older patients and those with adverse cytogenetics. It has also been shown to be active both as a single agent and in combination with other cytotoxic drugs. Although clofarabine acts as a myelosuppressive agent, its toxicity profile makes the drug potentially useful for patients excluded from intensive chemotherapy at diagnosis or who have relapsed after CR.

The process of approving a drug is often a very long and grueling procedure. Taking this into consideration and the fact that clofarabine has already been approved for the treatment of pediatric ALL with a second or higher relapse, new promising treatments with clofarabine could be implemented at an accelerated rate. Therefore, there is an urgent need to evaluate more thoroughly its potential in treating cancer. More recent clinical trials indicate that this drug has a broad-spectrum impact on a variety of leukemias, a unique property with a substantial potential for wider application.

The toxicities connected with clofarabine administration are manageable, thus allowing this drug to be combined effectively with other chemotherapeutic agents. However, an exact understanding of the clofarabine mechanism of action remains to be achieved, making it difficult to explain the broad-spectrum distribution of responses to this drug in ongoing clinical trials. Although the clinical data suggest a higher efficacy of clofarabine in hematological malignancies, the in vitro anti-angiogenesis activity [77] can prove the drug useful for treatment of solid tumors.

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