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Preclinical examination of clofarabine in pediatric ependymoma: Intratumoral concentrations insufficient to warrant further study

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Abstract

Clofarabine, a deoxyadenosine analog, was an active anticancer drug in our in vitro high-throughput screening against mouse ependymoma neurospheres. To characterize the clofarabine disposition in mice for further preclinical efficacy studies, we evaluated the plasma and central nervous system (CNS) disposition in a mouse model of ependymoma. A plasma pharmacokinetic study of clofarabine (45 mg/kg, IP) was performed in CD1 nude mice bearing ependymoma to obtain initial plasma pharmacokinetic parameters. These estimates were used to derive D-optimal plasma sampling time-points for cerebral microdialysis studies. A simulation of clofarabine pharmacokinetics in mice and pediatric patients suggested that a dosage of 30 mg/kg, IP in mice would give exposures comparable to that in children at a dosage of 148 mg/m². Cerebral microdialysis was performed to study the tumor extracellular fluid (ECF) disposition of clofarabine (30 mg/kg, IP) in the ependymoma cortical allografts. Plasma and tumor ECF concentration-time data were analyzed using a nonlinear mixed effects modeling approach. The median unbound fraction of clofarabine in mouse plasma was 0.79. The unbound tumor to plasma partition coefficient ($K_{pt,uu}$: ratio of tumor to plasma $AUC_{u,0-inf}$) of clofarabine was 0.12 ± 0.05 . The model predicted mean tumor ECF clofarabine concentrations were below the in vitro 1-hr IC₅₀ (407 ng/mL) for ependymoma neurospheres. Thus, our results show the clofarabine exposure reached in the tumor ECF was below that associated with an antitumor effect in our in vitro washout study. Therefore, clofarabine was de-prioritized as an agent to treat ependymoma, and further preclinical studies were not pursued.

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INTRODUCTION

Ependymoma, the third most common tumor of the central nervous system (CNS) in children, has a relatively poor prognosis compared with other brain tumors in children [1]. Current treatment for pediatric ependymoma includes surgical resection followed by radiation and chemotherapy as adjuvant therapy; however, current chemotherapies show dismal response in disease control [2]. The recent development of a mouse model of a genomic subgroup of ependymoma has enabled the identification of drugs active in this disease, but with relatively less toxicity to the normal cell of origin [3].

Using this ependymoma model, clofarabine (Clolar™, Genzyme) was identified through high-throughput drug screening of FDA approved compounds as an active and selective anticancer drug to undergo further evaluation for the treatment of pediatric ependymoma. Clofarabine is a deoxyadenosine analog approved by the US Food and Drug Administration (FDA) in 2004 for treatment of acute lymphoblastic leukemia (ALL) in pediatric patients who failed to respond to at least two other treatment regimens, or whose ALL recurred. Unlike other deoxyadenosine analogs, clofarabine causes a cytotoxic effect via multimodal mechanisms of action [4, 5]. Clofarabine is a prodrug which enzymatically converts to active clofarabine triphosphate in the cytoplasm. Clofarabine triphosphate incorporates in DNA and inhibits chain elongation by causing single strand breaks [5]. Clofarabine triphosphate is also a potent inhibitor of ribonucleoside reductase that depletes the intracellular pool of deoxynucleotides, which ultimately potentiates formation of clofarabine triphosphate [6]. Clofarabine triphosphate also disturbs the transmembrane potential of mitochondria leading to programmed cell death by release of cytochrome C and apoptosis inducing factors [7].

Several dose-finding and efficacy studies of clofarabine performed in various patient populations suggest a large variability in response and tolerability [8–11]. For example, a phase I study of clofarabine administered daily for 5 days every 3 to 6 weeks identified a maximum tolerable dosage (MTD) of 2 mg/m² in adult patients with solid tumors and 40 mg/m² in patients with hematological malignancies. Dose limiting toxicities (DLT) varied as well, and included myelosuppression and hepatotoxicities in solid tumors and hematological malignancies, respectively. Pediatric leukemia patients had an increased tolerance to clofarabine with a MTD of 52 mg/m² compared to adult patients (MTD - 40 mg/m²), when given daily for 5 days [9–11]. When clofarabine was given at a different schedule of once a week for three weeks in a 4 weeks cycle, it was possible to escalate the dosage up to 148 mg/m² in adult patients with solid tumors [8].

A crucial challenge in drug development for CNS tumors is the ability to reach adequate drug exposure (i.e., product of concentration and time) in the target tissue, which is limited by selective permeability of the blood-brain barrier and blood-cerebrospinal fluid barrier. Like many anticancer agents, limited information is available about the clofarabine CNS disposition. [12]. Hence, as part of our drug development process we characterized clofarabine disposition directly in the target tissue (i.e., tumor ECF). To do this, cerebral microdialysis was performed in mice bearing ependymoma allografts. The objectives of the current study were to 1) determine the relation of clofarabine dose and exposure time to its

cytotoxicity in an in vitro cell culture model, 2) determine the percentage of clofarabine bound to mouse plasma proteins and brain homogenate constituents, 3) simulate clofarabine exposure in a pediatric population to determine pediatric equivalent mouse dosage, 4) characterize the plasma and tumor ECF disposition of clofarabine in CD1 nude mice bearing ependymoma allografts, and 5) compare in vivo tumor ECF disposition of clofarabine with its in vitro cytotoxic effect to determine the next series of studies (if any) for clofarabine in our preclinical screening process.

MATERIALS AND METHODS

Animals and tumor implantation

Female CD1 nude mice (Charles River, Wilmington, WA) were orthotopically implanted with ependymoma (*Ink4a/Arf*-null + *RTBDN* + *Luci*) allografts [13] using the procedure described previously [3]. Animals were kept under controlled environment where temperature, humidity, and 12-hour day and night cycles were maintained artificially. All animal studies performed were approved by the St. Jude Children's Research Hospital Institutional Animal Care and Usage Committee (IACUC).

Chemicals and drugs

Clofarabine (purity, >99%) and cladribine (purity, 99%) were purchased from Selleck Chemicals (Houston, TX, USA). Acetonitrile, ethyl acetate, and formic acid were purchased from Fisher Scientific (Fair Lawn, NJ, USA). All solvents used were HPLC grade. Water was purified using Milli-Q Advantage A10 system (Millipore, Billerica, MA, USA). Mouse plasma (CD1 strain) in sodium heparin was purchased from BioChemed (Winchester, VA, USA). Artificial cerebrospinal fluid (aCSF) consisting of NaCl 148 mmol/L, KCl 4 mmol/L, MgCl₂ 0.8 mmol/L, CaCl₂ 1.4 mmol/L, Na₂HPO₄ 1.2 mmol/L, NaH₂PO₄ 0.3 mmol/L, and dextrose 5 mmol/L, and pH adjusted to 7.4 using 0.1 N NaOH was prepared in lab [14].

In vitro washout study

Mouse ependymoma cells (2000 cells/well) were seeded in 96-well plates (Corning) in 100 μ L of neurobasal media using an automated microplate dispenser. Cells were treated after 24 hrs with a range of clofarabine concentrations using an automated workstation. Cells were incubated with clofarabine for indicated time points (1, 3, 6, 10, 24, and 72 hrs) after which drug containing media was replaced with fresh media and incubated to a total of 72 hrs. Cell viability was determined after 72 hrs using CellTiter-Glo (Promega, Madison, WI) and luminescent signal was quantified using an Envision plate reader (Perkin-Elmer, Waltham, MA). Dose response curves were plotted to determine EC₅₀ values at the various time points using Prism software [15].

Plasma and brain homogenate binding study

A plasma protein and brain homogenate binding study of clofarabine was performed using equilibrium dialysis [16]. Spiked aCSF samples (n=3) were prepared at a concentration of 500 ng/mL. CD1 nude mice bearing orthotopic ependymoma allograft (n=3) were dosed with clofarabine (45 mg/kg, IP injection) and plasma samples were collected at 4 hr post-dose. After plasma sample collection, animals were perfused with normal saline and brain

samples were collected and stored at -80°C. Brain samples were homogenized in aCSF (tissue to aCSF ratio = 1:5) using FastPrep®-24 (MP Biomedicals, Solon, OH). Blank aCSF (200 µL) was transferred to the buffer-side of a 96-well Equilibrium Dialyzer™ (molecular weight cut-off (MWCO): 5K Daltons; Harvard Apparatus, Holliston, MA). Plasma, aCSF, and brain homogenate samples (200 µL each) were transferred to the sample-side of a 96-well Equilibrium Dialyzer™. The dialysis plate was inserted in the plate rotator (Harvard Apparatus, Holliston, MA) and kept in the incubator set to 37°C for 24 hr. After 24 hr, samples from each side of 96-well Equilibrium Dialyzer™ were collected in separate tubes and stored at -80°C until further analysis. Fraction unbound of clofarabine in plasma and brain homogenate was calculated as ratio of unbound clofarabine concentration in buffer-compartment to total clofarabine concentration in sample-compartment of 96-well Equilibrium Dialyzer™. Non-specific binding of clofarabine to the equilibrium dialysis apparatus was calculated using spiked samples prepared in aCSF.

Plasma pharmacokinetic study

CD1 nude mice (n=9) bearing orthotopic ependymoma allografts were dosed with 45 mg/kg clofarabine via IP injection. The clofarabine dosing solution was formulated at a concentration of 4.5 mg/mL in 8% DMSO prepared in normal saline. A population based study design was implemented where multiple samples were collected per mouse. Mice were divided into three groups for plasma sample collection. Mice from group 1 were sampled at 0.08, 4, and 8 hr post-dose. Mice from group 2 were sampled at 0.25 and 1 hr, and mice from group 3 were sampled at 0.5 and 2 hr post-dose. Blood samples (~ 70 µL) were collected by retro-orbital eye bleed techniques using heparin coated capillaries (Fisher Sci, Pittsburgh, PA), except the terminal samples, which were collected by cardiac puncture using a 1 mL syringe pre-coated with heparin. Immediately after collection, blood samples were centrifuged and plasma was separated. Plasma samples were stored at -80°C until further analysis.

Simulation of clofarabine plasma exposure in pediatric patients

Clofarabine plasma exposures (i.e., AUC) in the pediatric population were simulated using a population pharmacokinetic model developed by Bonate et al [8]. Plasma concentration-time data were simulated for 2100 pediatric patients (100 simulations per dosage) with age randomly selected from a uniform distribution of age between 4 to 17 years. Patient body weight was estimated based on age and median weight correlation given in a growth-chart obtained from a Centers for Disease Control and Prevention (CDC) database [17]. A clofarabine dosage was sampled from a range of IV infusion dosages (11.25 to 148 mg/m²) used in studies incorporated in the model development, and assigned to each hypothetical patient [8]. Patient body surface area (BSA) was calculated using the following equation, which was a relationship with body weight (BDWT) established in the population used in model development [18].

$$BSA=0.2603+0.02860 * BDWT-0.0000858 * BDWT^2$$

Pediatric clofarabine plasma concentration-time profiles were simulated for 24 hr after one 2 hr infusion of clofarabine at an assigned dosage. Population mean parameters, between subject variability, and covariance structure were implemented as shown in following equations adapted from Bonate et al [8].

$$\begin{aligned}CL(L/hr) &= 32.7 * \left(\frac{BDWT}{70 \text{ kg}}\right)^{0.75} * \left(\frac{\text{Age}}{30 \text{ years}}\right)^{-0.181} * \exp(\eta_{CL}) \\V1(L) &= 71.5 * \left(\frac{BDWT}{70 \text{ kg}}\right) * \left(\frac{\text{Age}}{30 \text{ years}}\right)^{-0.560} * \exp(\eta_{V1}) \\Q(L/hr) &= 40.6 * \left(\frac{BDWT}{70 \text{ kg}}\right)^{0.75} * \exp(\eta_Q) \\V2(L) &= 147 * \left(\frac{BDWT}{70 \text{ kg}}\right) * \exp(\eta_Q)\end{aligned}$$

Between subject variance for CL, V1, corr(CL, V1), Q, corr(CL, Q), V2, and corr(V2, Q) adapted from Bonate et al. were 0.0731, 0.350, 0.469, 0.338, 0.577, 0.338, and 0.497, respectively [8]. Further, for simulation purposes, residual variability and between occasion variability terms were assumed to be zero [19]. Simulations were implemented with NONMEM 7.2 (ICON Development Solutions, Ellicott City, MD). Area under concentration-time curve (AUC₀₋₂₄) for an individual patient was calculated by integrating concentration-time profile from time zero to the indicated time-point. Plasma exposure of unbound clofarabine was calculated as the product of total AUC₀₋₂₄ and the fraction unbound of clofarabine in human plasma ($f_{u, \text{human}} - 0.53$) [20].

Cerebral microdialysis study

Cerebral microdialysis studies were performed using CD1 nude mice bearing orthotopic allografts of ependymoma (n=7) to characterize clofarabine disposition in tumor ECF. Ependymoma tumor cells and microdialysis guide cannulae (MD-2255, BASi, West Lafayette, IN) were implanted into the cerebral cortex of mice using a stereotactic instrument as described previously [3, 21]. Tumor growth was measured weekly by bioluminescence imaging using a Xenogen (Caliper Life Science, Waltham, MA) system. Once the tumor reached a predetermined size (tumor bioluminescence $\sim 10^7$ photons/sec) mice were used for a microdialysis experiment. On the day of the microdialysis experiment, a 1 mm microdialysis probe with 38 KDa MWCO membrane (MD-2211, BASi) was inserted into the guide cannula, and infused with aCSF at a flow-rate of 0.5 $\mu\text{L}/\text{min}$ for 1 hr to equilibrate with the in vivo environment. Once the probe was equilibrated, the mice were dosed with clofarabine (30 mg/kg, IP) and this time was considered as time-zero. Microdialysate samples were collected over 1 hr intervals for 5 hrs. Blood samples from each mouse were collected at the limited sampling model derived time points (0.25, 2.5, and 5 hr, post-dose). Immediately after collection, blood samples were centrifuged and plasma was separated. Plasma and dialysate samples were stored at -80°C until further analysis.

Microdialysis recovery for each probe was determined using in vitro recovery studies prior to its use in the vivo microdialysis study. Each probe was placed in a stirred clofarabine solution prepared in aCSF at a concentration of 1 $\mu\text{g}/\text{mL}$ and maintained at 37°C . The probe was perfused with blank aCSF at a flow-rate of 0.5 $\mu\text{L}/\text{min}$. After equilibration for 1 hr, three consecutive fractions each of 1 hr interval were collected. Dialysate samples were

stored at -80°C until further analysis. Probe recovery was calculated using the ratio of clofarabine concentration in the dialysate sample to that in the bulk sample. Unbound concentration in tumor ECF was calculated by dividing concentration observed in dialysate samples collected during microdialysis study with the recovery ratio.

Bioanalytical method

Plasma and aCSF samples collected during the study were analyzed for clofarabine using a selective and specific LC-MS/MS method. Plasma and aCSF dialysate samples (25 μL) were mixed with 10 μL of internal standard (100 ng/mL cladribine, IS) as the first step of sample processing. Plasma samples were processed by protein precipitation using 80 μL of 0.1% formic acid containing acetonitrile. After protein precipitation, samples were centrifuged and clear supernatants were transferred to autosampler vials. Dialysate samples were processed using liquid-liquid extraction performed by vortexing with 700 μL of ethyl acetate, followed by centrifugation and separation of organic layer into a clear glass vial. The organic layer was concentrated by drying, and then reconstituted in 80 μL of 20:80 water:acetonitrile containing 0.1% formic acid. Processed plasma or aCSF samples (3 μL) were then injected into the chromatographic system. Chromatographic separation was achieved using mobile phases consisting of (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile, in a gradient elution pumped at flow-rate of 0.25 mL/min, and a Phenomenex Luna C18 column. Clofarabine and cladribine (IS) were quantified using an API-4000 LC-MS/MS mass spectrometer system (AB SCIEX, Framingham, MA). Mass spectrometric analysis was performed in the positive ionization mode with a dwell time of 250 ms. Multiple reaction monitoring (MRM) of the precursor-product ion transitions m/z 304.10 > 170.00 for clofarabine and m/z 286.00 > 170.00 for cladribine (IS) was used for quantification. Further details on the analytical methodology are presented in the Supplementary Materials. The developed method was linear and reproducible with typical correlation coefficient value for calibration curve of > 0.99, in both matrices (plasma and aCSF). The intra- and inter- day assay coefficients of variations were found to be 5.1% and accuracies were found to be between 86% – 109%.

Pharmacokinetic model

Clofarabine plasma concentration-time data were analyzed using non-linear mixed effect modeling (NONMEM 7.2, ICON development solutions). A two-compartment model consisting of a central and a peripheral compartment, and an IP dosing compartment linked to central compartment was fitted to total (bound + unbound) plasma concentration-time data. The model was parameterized using a first-order absorption rate constant (K_A) for the IP dosing compartment, central compartment clearance (CL_T), inter-compartmental clearance (CL_D), and volume of central (V_C) and peripheral (V_P) compartments. The first order conditional estimation method with interaction (FOCE-I) was used to derive population mean pharmacokinetic parameters and between animal variability. Standard errors of parameter estimates were generated using the importance sampling method (IMP) with interaction by performing only the expectation step (EONLY=1) [22]. Area under plasma concentration-time curve (AUC_{0-t}) for each individual animal was estimated by integration of concentration-time profile from time zero to the indicated time-point (e.g., t) implemented in NONMEM control stream, whereas $AUC_{0-\text{Inf}}$ was estimated as a ratio of

dosage to individual systemic clearance. Plasma exposure of unbound clofarabine (AUC_u) was calculated as the product of the plasma AUC and the clofarabine fraction unbound in mouse plasma. Population mean and 95% confidence interval of AUC_u estimates were generated by simulating concentration-time profile for 1000 animals using the final parameter estimates.

Population mean pharmacokinetic parameters obtained from the plasma clofarabine disposition study were used to develop a limited plasma sampling model (LSM) for the subsequent microdialysis studies. Due to limitations upon total blood volume that could be withdrawn from each mouse and the volume of plasma required for bioanalysis, we were limited to only three plasma samples per mouse during a microdialysis study. The LSM was used to obtain statistically more informative time points for the plasma sample collection during the microdialysis study. The LSM was developed using the D-optimality method implemented in ADAPT 5 (BMSR, Los Angeles, CA) [23, 24]. When limited sampling data were analyzed with data obtained from the prior plasma PK study using a population based pharmacokinetic model, we were able to derive a full plasma profile in an individual mouse, which could be used to study tumor ECF disposition in that mouse. A compartmental model (Figure 1) consisting of a plasma, a peripheral, an IP dosing, and a tumor ECF compartment linked to a central compartment using influx (CL_{24}) and efflux (CL_{42}) clearances was simultaneously fitted to total (bound + unbound) plasma and/or unbound tumor ECF concentration-time data obtained during the plasma PK and microdialysis study. Volume of tumor ECF was fixed to 0.00033 L/kg based on literature value assuming that tumor contributes 15% of total brain volume [25]. Random effects associated with pharmacokinetic parameters were assumed to be log-normally distributed around population mean parameter value. A normally distributed residual error model consisting of two proportional error terms, one each for plasma (σ^2_{plasma}) and tumor ECF ($\sigma^2_{\text{tumor ECF}}$) concentrations, was implemented in the model. Estimation methods in NONMEM 7.2 used to derive final population mean PK parameters and between animal variability for the microdialysis studies were similar to that used for plasma disposition study (see above). The unbound tumor to plasma partition coefficient for clofarabine ($K_{p,uu}$) was estimated using following equation:

$$K_{p,uu} = \frac{CL_{24}}{CL_{42} * f_{u,p}}$$

Where, CL_{24} and CL_{42} represents influx and efflux clearance of tumor ECF compartment, respectively. $f_{u,p}$ represents fraction unbound of clofarabine in the mouse plasma.

RESULTS

In vitro washout study

To understand the relationship between drug exposure and time required to achieve cell death, we performed in vitro washout studies. Mouse ependymoma cells were incubated for a predetermined time duration (up to a total of 72 hrs) with a range of concentrations of clofarabine. Results showed that the IC_{50} values (concentration required for 50% cell death)

of clofarabine were 407 ng/mL and 304 ng/mL for 1 hr and 6 hr exposures, respectively (Supplementary Figure 1).

Plasma and brain homogenate binding study

The plasma protein and brain homogenate binding of clofarabine was assessed using equilibrium dialysis. Clofarabine was negligibly bound to the dialysis membrane contained in the Equilibrium Dialyzer™ dialysis plate, suggesting that equilibrium dialysis was a suitable technique for determining clofarabine protein binding. The fraction unbound of clofarabine samples prepared in aCSF after 24 hr was 1.0, indicating that equilibrium was achieved by 24 hr. The binding of clofarabine to plasma and brain homogenate proteins was determined by subjecting plasma and homogenized brain samples collected from mice dosed with 45 mg/kg clofarabine to equilibrium dialysis. The median (range) fractions unbound of clofarabine in plasma and brain homogenate samples were 0.79 (0.71 – 0.97) and 1.02 (0.98 – 1.04), respectively.

Plasma pharmacokinetic study

A plasma pharmacokinetic study of clofarabine given as a 45 mg/kg IP injection was performed using CD1 nude mice bearing intracranial ependymoma allografts. Total clofarabine was measured in the plasma, and these data were modeled using a population approach. Shown in Figure 2 are the unbound plasma concentration-time data and it was well represented by a 2-compartment model. Mean model predicted population parameter estimates (CV%) were 4.83 (6.48%) L/hr/kg for CL_T , 0.48 (5.73%) L/hr/kg for CL_D , 2.60 (37.3%) L/kg and 0.99 (22.2%) L/kg for volume of central (V_C) and peripheral (V_P) compartments, respectively. For modeling purposes, the first-order absorption rate constant for the IP dosing compartment (K_A) was fixed to 11 hr^{-1} . Between animal variability for CL_T and V_P was estimated to be 10.6% and 44.2%, respectively. Due to the limited number of animals used in the study, between-animal variability for K_A , CL_D , and V_C were fixed to zero. As shown in Figure 2 clofarabine was rapidly absorbed after IP injection, with a time to achieve maximum concentration (T_{max} , mean $\pm$ SD) of 0.33 ± 0.17 hr. We calculated plasma exposure of unbound clofarabine ($AUC_{u,0-\text{Inf}}$) by multiplying observed $AUC_{0-\text{Inf}}$ with clofarabine plasma fraction unbound ($f_{u,p}$) to compare clofarabine exposure between mice and that observed in the pediatric population. Plasma $AUC_{u,0-\text{Inf}}$ in mice was $7445 \pm 707\text{ }\mu\text{g/L}\cdot\text{hr}$. We used the plasma PK parameters to derive a limited sampling model (LSM) for use during the microdialysis study, and the time points were 0.25, 2.5, and 5 hr post-dose.

Comparison of clofarabine plasma exposure between mice and simulated pediatric population

To effectively and rationally use preclinical data to inform the design of subsequent clinical usage, cerebral microdialysis studies were performed using a dosage that produces systemic exposures similar to that expected in a pediatric population at clinically tolerable systemic exposures. To identify a mouse clofarabine dosage for our cerebral microdialysis study that would achieve a similar plasma exposure anticipated in children, we required extensive information about clofarabine plasma exposure in pediatric subjects. Since clofarabine has not been studied in pediatric patients with solid tumors, we used a simulation approach to

derive anticipated clofarabine exposures in these patients using a population based pharmacokinetic model developed by Bonate et al [8]. Using this model we simulated the pediatric unbound plasma exposure ($AUC_{u,0-24}$) of clofarabine at dosages ranging from 11.25 to 148 mg/m². As shown in Figure 3, the mean clofarabine $AUC_{u,0-24}$ in CD1 nude mice bearing ependymoma at a dosage of 45 mg/kg IP ($7445 \pm 707 \mu\text{g/L}\cdot\text{hr}$) was ~2-fold higher than that in pediatric patients receiving the highest simulated clofarabine dosage (148 mg/m²) as a 2 hr infusion ($3843 \pm 1073 \mu\text{g/L}\cdot\text{hr}$). To achieve a dosage for use in our microdialysis studies, we used linear extrapolation to estimate a murine dosage that would attain an unbound clofarabine AUC of ~4000 $\mu\text{g/L}\cdot\text{hr}$. Thus we considered 30 mg/kg IP dosage as an optimistic pediatric equivalent dosage for our cerebral microdialysis studies in mice.

Tumor ECF penetration of clofarabine

Cerebral microdialysis was used to determine the tumor ECF disposition of clofarabine (30 mg/kg, IP injection) in CD1 nude mice bearing intracranial ependymoma allografts. Mean $\pm$ SD in vitro recovery of microdialysis probes used for cerebral microdialysis was $18.8 \pm 4.4\%$. A population based modeling approach which combined data obtained from the plasma pharmacokinetic study and limited plasma sampling in the cerebral microdialysis studies was used to derive the individual plasma pharmacokinetic profile for each microdialysis experiment. As shown in Figures 4a and 4b, plasma and tumor ECF concentration-time data were well represented by a two compartment model with an efflux and influx clearance term for the tumor ECF compartment. The model derived population mean estimates $\pm$ standard errors for the parameters, between animal variability, and residual errors are listed in Table 1. Further individual parameter estimates of CL_T and V_P from mice that received 45 mg/kg (plasma PK study) or 30 mg/kg (cerebral microdialysis) of clofarabine were not significantly different, with p-values of 0.55 and 0.62, respectively (Figures 4c and 4d), suggesting that the clofarabine pharmacokinetics in CD1 nude mice were independent of dosage in this range. The tumor ECF to plasma partition coefficient for unbound clofarabine ($K_{pt,u}$) was estimated to be 0.12 ± 0.05 . As shown in Figure 5, the model predicted mean tumor ECF concentrations of clofarabine were below the in vitro 1-hr IC_{50} of 407 ng/mL. These results suggest that the in vivo tumor ECF concentration of clofarabine in mice bearing ependymoma are not adequate to cause a cytotoxic effect at a clinically relevant dosage (30 mg/kg, IP).

DISCUSSION

In the current study, we have shown that clofarabine is an active anticancer drug in vitro against ependymoma. Further, we have systematically studied the plasma and tumor ECF disposition of clofarabine in CD1 nude mice bearing orthotopic ependymoma allografts. Pharmacokinetic modeling of data derived from our in vivo studies determined that the tumor ECF clofarabine concentration at a clinically relevant dosage of 30 mg/kg, IP is below the in vitro 1hr IC_{50} . These results suggest that intratumoral clofarabine exposures would not produce cytotoxic effects, thus clofarabine was not prioritized as a candidate for further preclinical efficacy testing.

We have previously implemented a preclinical drug development approach that uses plasma PK, cerebral microdialysis, and modeling and simulation to examine potentially effective treatments for pediatric brain tumors [26]. In our studies, we emphasize the importance of unbound target tissue concentration as a surrogate for drug efficacy, and highly recommend that clinically relevant systemic exposure be used for preclinical studies to rationally translate findings from the lab to the clinic.

Clofarabine was identified as an active drug in our high-throughput drug screen against an ependymoma cell line. In many instances, drugs shown to be active in vitro fail to show pharmacological effect in vivo due to inadequate exposure in target tissue, especially those drugs that need to cross the blood-brain barrier to exert their pharmacological effect [27, 28]. The preclinical pipeline we have established uses cerebral microdialysis to characterize the disposition of clofarabine in target tissue (i.e., tumor ECF). In this study, only the unbound (free) drug in tumor ECF is able to cross the dialysis membrane, which allows us to quantitate the free clofarabine concentration in ependymoma tumors.

To interpret and apply preclinical findings to the clinical setting it is important to use clinically relevant dosages of drugs in cerebral microdialysis experiments. Since only unbound drug is considered available to distribute to extravascular compartments, we used unbound plasma drug exposure when comparing drug efficacy or toxicity between different species. In our study, clofarabine was moderately (21%) bound to mouse plasma proteins. Clofarabine protein binding reported in humans was approximately 2.24-fold higher compared with mice [20]. Further, accurate determination of pediatric equivalent mouse dosage requires extensive plasma pharmacokinetic studies performed at a range of dosages in the targeted patient population. Clofarabine has been extensively studied in pediatric patients with hematological malignancies using a daily x 5 regimen, given every 4 to 6 weeks, either as a single agent or in combination therapy [10, 29–32]. However, to the best of our knowledge, clofarabine has never been studied in children with solid tumors. The clofarabine MTD varies widely between solid tumors and hematological malignancies. For example, in an adult phase I study, patients with hematological malignancies were able to tolerate higher dosage of clofarabine (40 mg/m²) compared to patients with solid tumors (2 mg/m²) when given as a 2 hr infusion daily for 5 days every 3 to 6 weeks. When clofarabine was administered using an alternate dosing regimen (0.5 to 2 hr infusion given once a week for 3 weeks in a 4 week cycle), it was possible to escalate the dosage up to 148 mg/m² in adult patients with solid tumors [8, 33]. Since clofarabine has not been studied in pediatric solid tumor patients, we utilized a simulation approach with a population PK model developed by Bonate et al to derive a dosage-plasma exposure relationship in pediatric solid tumor patients [8]. This model was useful for our purpose as it was developed with data obtained from diverse populations, including children, with different disease states, and clofarabine dosing regimens. All pediatric data (1 - phase I, and 2 - phase II studies) used for model development were gathered from patients with hematological malignancies who were treated with clofarabine dosages ranging from 11.25 to 70 mg/m² (recommended Phase II dosage - 52 mg/m²) given as 1 or 2 hr infusions daily x 5 days every 4 to 6 weeks. All adult data used for this model were from patients with solid tumors, who were treated with one of two different clofarabine regimens: 1) clofarabine dosages ranging from 4 to 148 mg/m² given as 0.5 to 2 hr infusions once a week for 3 weeks in a 28 day cycle, or 2) clofarabine

dosages ranging from 1.5 to 5 mg/m² given orally daily for 5 days in a 28 day cycle. Bonate et al. described clofarabine plasma disposition by a 2-compartmental model, and showed differences in clofarabine pharmacokinetic parameters between adult and pediatric patients that were well defined in a population model using body weight and age as covariates. Using this model we were able to simulate clofarabine plasma exposure at wide range of dosages (11.25 to 148 mg/m²) in pediatric patients. Further to give clofarabine the best possible chance to be effective for treatment of pediatric ependymoma, we considered the fact that pediatric leukemia patient were able to tolerate higher clofarabine dosages compare to adult leukemia population when we calculated our pediatric equivalent mouse dosage [9–11].

CNS drug distribution is limited by selective permeability of the BBB and blood-CSF barriers. Therefore, it is necessary to characterize drug distribution in the target tissue (e.g., brain/tumor tissue) when developing new therapies for CNS malignancies. In our cerebral microdialysis study, we found that approximately 12% of the unbound clofarabine penetrated into the tumor (as measured by clofarabine concentration in tumor ECF). Similar to other nucleoside analogs, clofarabine distribution across BBB and blood CSF barrier and into cells is mediated by human nucleoside transporters. Clofarabine was found to have affinity for sodium independent hENT1 and sodium dependent hCNT1 and hCNT3 [34]. However, orientation of these transporters at BBB and blood-CSF barrier is not well defined. Further, a K_{pt,uu} value for clofarabine of 0.12 indicates that clofarabine disposition in CNS may be limited by efflux transporters. Berg et al studied plasma and cerebrospinal fluid (CSF) disposition of clofarabine at a dosage of 2.3 mg/kg (46 mg/m²) given as 2 hr IV infusion in four non-human primates [12]. Median CSF to plasma partition coefficient of clofarabine in non-human primates was lower at 5% with high variability (ranged from 3 – 26%) compared to that observed in our mouse study. However, it is important to note that the CSF to plasma partition coefficient calculated by Berg et al considered total (unbound + bound) plasma clofarabine exposure compared to tumor ECF to plasma partition coefficient of clofarabine calculated in our study which measured unbound plasma exposure.

Overall, the field of oncology has the highest clinical trial failure rate compared to other therapeutic areas [35]. In general key reasons for failure of phase II and III clinical trials includes suboptimal preclinical validation including target tissue exposure, target binding, and expression of functional pharmacological activity (together known as ‘three pillars of survival’) [36]. Considering the rarity of pediatric brain tumors, it is crucial to utilize preclinical studies for evaluating the use of chemotherapeutic agents. Based on our in vitro washout data, the tumor ECF concentration of clofarabine was sub-optimal to exhibit anticancer activity. The negative results established from our study show that clofarabine is not a suitable candidate for further preclinical and clinical studies in our search for new treatments of pediatric ependymoma and the significance of this cannot be overstated.

In conclusion, we have systemically characterized the plasma and tumor ECF disposition of clofarabine in CD1 nude mice bearing orthotopic ependymoma. Based on our finding we show that clofarabine resulted in sub-effective tumor exposure at clinically relevant dosage.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Schematic diagram of PK model

2-compartment model with a IP dosing and tumor ECF compartments linked to central compartment where, K_A - first order absorption rate constant; CL_T - total clearance of clofarabine; CL_D - distributional clearance of clofarabine; V_C and V_P - volume of central and peripheral compartments, respectively; CL_{42} and CL_{24} - clearance of clofarabine from central to tumor ECF and vice versa; V_T , V_C , and V_P - volume of tumor ECF, plasma and peripheral compartment, respectively; C_T , C_C , and C_P - concentration of clofarabine in tumor ECF, plasma and peripheral compartment, respectively

Figure 2. Clofarabine full plasma pharmacokinetic study

Clofarabine unbound plasma concentrations plotted against time (Open circle represents observed unbound concentrations, whereas dotted line represents model predicted population mean unbound concentrations)

Figure 3. Comparison of clofarabine plasma exposure between mouse and pediatric patient
 Plasma exposures of unbound clofarabine were plotted against pediatric dosage (open triangle represent clofarabine dosages studied in pediatric population with hematological malignancies as daily x 5 day regimen; open circles represent clofarabine dosages studied in adult populations with solid tumors as once a week x 3 weeks regimen; black horizontal dotted-line and gray shaded area represents mouse plasma exposure of unbound clofarabine with 95% confidence interval at a dosage of 45 mg/kg given as IP injection)

Figure 4. Population based pharmacokinetic modeling of plasma pharmacokinetic study and cerebral microdialysis study

Representative individual plasma and/or tumor ECF concentration-time profile of unbound clofarabine from plasma pharmacokinetic study (a) and cerebral microdialysis study (b) (open circles and triangles represent observed clofarabine unbound plasma and tumor ECF concentrations, respectively; Dotted and solid line represents model predicted individual unbound plasma and tumor ECF concentrations, respectively). Box-plot comparison of individual volume of peripheral compartment (c) and systemic clearance (d) between plasma pharmacokinetic study (45 mg/kg clofarabine) and cerebral microdialysis study (30 mg/kg clofarabine).

Figure 5. Cerebral microdialysis study

Clofarabine unbound concentrations were plotted against time (open circles and triangles represents observed unbound plasma and tumor ECF concentrations, respectively; dotted and solid line represents model predicted population mean unbound plasma and tumor ECF concentrations, respectively; horizontal dotted line represents in vitro 1-hr IC₅₀)

Table 1

Final model parameter estimates for clofarabine plasma and tumor ECF disposition in CD1 nude mice bearing ependymoma

Parameters	Unit	Estimates ± Standard errors	Between animal variability (%)
Absorption rate constant for IP dosing (K_A)	1/hr	11 FIXED	0 Fixed
Systemic Clearance (CL_T)	L/hr/kg	4.89 ± 0.587	11.4
Volume of central compartment (V_C)	L/kg	2.93 ± 0.921	0 Fixed
Inter-compartmental clearance (Q)	L/hr/kg	0.436 ± 0.566	0 Fixed
Volume of peripheral compartment (V_P)	L/kg	1.14 ± 0.89	33.5
Influx clearance for tumor ECF (CL_{24})	L/hr/kg	0.000062 ± 0.000035	56.0
Efflux clearance for tumor ECF (CL_{42})	L/hr/kg	0.000694 ± 0.000176	27.4
Volume of tumor ECF compartment (V_T)	L/kg	0.00033 FIXED	0 Fixed
Proportional residual error for plasma concentrations (σ^2_{plasma})	NA	0.0241 ± 0.006	NA
Proportional residual error for tumor ECF concentrations ($\sigma^2_{\text{tumor ECF}}$)	NA	0.0474 ± 0.019	NA