Phase II and pharmacodynamic study of oral forodesine in patients with advanced and/or fludarabine-treated chronic lymphocytic leukemia

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Running Title: Forodesine in fludarabine refractory CLL

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ABSTRACT

Prognosis of patients with fludarabine-refractory CLL is poor and novel therapies are needed. Forodesine is a new and potent purine nucleoside phosphorylase inhibitor. CLL patients with primary resistance to fludarabine-based therapy or with progressive disease were eligible for oral forodesine (200mg/day) for up-to 24 weeks. Eight patients with median lymphocyte count 35.9x10^9/L and median serum β2 microglobulin 6.45 mg/L were treated. Six had Rai stage III-IV and were previously heavily treated (median prior therapy = five). Two had transient decrease in lymphocyte count to normal, while in five, disease progressed. Adverse events were mild. Steady-state level of forodesine ranged 200-1300 nM and did not reach desired 2 µM level. PNP inhibition ranged from 57 – 89% and steady-state dGuo concentration was median 1.8 µM. Intracellular dGTP increase was very modest; from median 6 µM to 10 µM. Compared to in vivo, in vitro incubations of CLL lymphocytes with 10 or 20 µM dGuo + forodesine (2 µM) resulted in accumulation of higher levels of dGTP (40 - 250 µM) which resulted in increase in apoptosis. Forodesine has biological activity and pharmacodynamic parameter suggests alternate dosing schedule and/or higher doses to achieve greater intracellular dGTP may be beneficial in this patient population. This study is registered at http://clinicaltrials.gov as NCT00289549.
INTRODUCTION

The prognosis of patients with fludarabine-refractory chronic lymphocytic leukemia (CLL) is poor and this appears, at least in part, to be related to a more resistant disease phenotype as well as an increased infection risk related to the effects of the disease and prior therapy. Current salvage regimens, although effective in some patients, produce low complete remission (CR) rates and are unlikely to improve survival in this population. As such, these patients are candidates for phase I/II clinical trials to discover new effective agents and strategies for the treatment of CLL.

Purine nucleoside phosphorylase (PNP), is an enzyme that catalyzes the phosphorolysis of purine nucleosides such as deoxyinosine and 2'-deoxyguanosine (dGuo) to their respective bases and to deoxyribose-1-phosphate. Genetic PNP deficiency syndrome results in an accumulation of dGuo in plasma and deoxyguanosine triphosphate (dGTP) in T cells, thereby leading to dGTP-directed inhibition of DNA synthesis and cell death with T-cell-selective depletion as the major phenotype. As the PNP enzyme is abundant in large body organs, weak inhibitors of PNP enzyme do not exhibit manifestations of T-cell deficiency and do not accumulate circulating dGuo. Therefore, nearly complete inhibition of PNP (>95%) must be achieved to increase the dGuo concentration to the level required for T cell toxicity.

Forodesine (also known as BCX-1777 and immucilin H) was developed as a novel PNP transition-state inhibitor. It is the most potent inhibitor of PNP, with a low picomolar Ki value in human PNP enzyme assays. In-vitro, in CEM-SS [T-acute
lymphoblastic leukemia (T-ALL)] cells, forodesine in the presence of dGuo inhibited the proliferation of T-cells with an IC$_{50}$ of 0.015 µM, which was accompanied by a 154-fold accumulation of dGTP compared to a 15-fold accumulation in human lymphocytes. Similar to the accumulation kinetics, the elimination profile of dGTP was favorable with a slow elimination in CEM cells (18 hr) and fast degradation in normal T lymphocytes (4 hr)$^{8,10}$. T cell cytotoxicity is due to phosphorylation of dGuo via dCK (deoxycytidine kinase) to dGuo monophosphate which gets accumulated as dGTP. Perturbation of dGTP pool leads to inhibition of DNA synthesis and cell proliferation$^{11}$.

The picomolar potency of PNP inhibitors$^{12}$, T-cell selective toxicity in cell lines$^9$, and primary cells, and efficacy during in vivo animal studies$^{13}$ provided rationales for the use of forodesine in T cell malignancies. The proof-of-principle was the first clinical study with forodesine in patients with T-cell leukemias. Patients received intravenous forodesine (40 mg/m$^2$) which resulted in a median peak forodesine level of 5.4 µM, which increased plasma dGuo levels to a median of 15 µM. There was a 2-40 fold increase in intracellular dGTP which correlated with anti-leukemia activity$^{14}$. A phase II clinical trial in patients with T-ALL showed efficacy with 25% overall response rate$^{15}$. Similarly, an oral formulation of forodesine demonstrated clinical activity with an overall response rate of 39% in a phase I/II study of refractory cutaneous T-cell lymphoma (CTCL)$^{16}$.

This unique sensitivity of T cells to PNP inhibition is attributed to the relatively high levels of dCK, the rate-limiting step for accumulation of intracellular dGTP. Given that
CLL-B cells are known to possess high dCK activity\textsuperscript{17} we investigated forodesine \textit{in vitro} with freshly isolated CLL primary cells. Treatment of these cells with forodesine and dGuo at physiologically achievable concentrations led to an accumulation of intracellular dGTP, without any effect on other deoxynucleotides. The dGTP accumulation led to p53 stabilization and p21 activation in the leukemia cells, followed by the induction of apoptosis, demonstrated by PARP cleavage and caspase activation. These hallmark features of apoptosis were directly related to dGTP accumulation\textsuperscript{18}. In CLL cells lacking functional p53, forodesine induced transcriptional up-regulation of p73 (a p53-related protein) and was able to overcome the resistance to apoptosis of CLL cells\textsuperscript{19}. Forodesine mediated apoptosis was associated with a decrease in the levels of anti-apoptotic Mcl-1 protein and induction of the pro-apoptotic protein Bim\textsuperscript{19}.

Based on the encouraging reports in patients with T cell leukemia\textsuperscript{14} and cytotoxicity in CLL lymphocytes during \textit{in vitro} investigations\textsuperscript{18,19}, a phase II clinical trial was initiated in fludarabine refractory CLL. Our goals were i) to investigate the efficacy of forodesine in treating patients with advanced, fludarabine-treated CLL, ii) to evaluate the toxicity, duration of response, disease-free survival and overall survival associated with treatment with forodesine, and iii) to correlate pharmacokinetic and pharmacodynamic data of forodesine in CLL with its clinical activity.
MATERIALS AND METHODS

Patient selection

This open-label, nonrandomized, single center study was conducted at the University of Texas – M. D. Anderson Cancer Center from August 2005 to November 2008. Patients, aged 18 years and older, diagnosed with CLL by peripheral blood and/or bone marrow examination, with primary resistance to fludarabine-based therapy (no complete or partial remission) or with progressive disease after response to prior fludarabine based regimens were eligible. Patients with Rai stage III/IV or earlier stages with massive symptomatic lymphadenopathy requiring therapy were included. All prior investigational treatments were required to be completed at least one week before the start of study drug. Additional eligibility requirements included: adequate renal function (creatinine <2.0 unless related to the disease), adequate hepatic function (bilirubin <3.0 and transaminase levels less than 3 times upper limit of normal unless related to the disease), ECOG performance status of ≤3. Patients were excluded from the study if they were pregnant or nursing, had severe ongoing co-morbid medical conditions, had active serious infection which was not controlled with antibiotics, had known infection with HIV, or if they had active Hepatitis B and/or Hepatitis C infection. All patients signed informed consents in accordance following the Declaration of Helsinki. The study was approved by the institutional review board of M. D. Anderson Cancer Center.

Patient follow-up, treatment schedule, response criteria

Patients had history and physical examination with laboratory studies including bone marrow examination done at baseline. CT scanning was at the discretion of the treating
physician. Follow-up was done once a week for the first month, then before each subsequent cycle and on an as-needed basis. Forodesine capsule was administered at a dose of 200 mg oral once daily for 4 weeks to complete one cycle of therapy, with the intent to administer forodesine continually without interruptions between cycles, up to a maximum of 6 cycles. The National Cancer Institute-Sponsored Working Group (NCI-WG) Guidelines for CLL were used for assessment of response\textsuperscript{20}. Toxicity was defined by National Cancer Institute Common Terminology Criteria for Adverse Events Version 3.

**Drug and other chemicals**

Forodesine for clinical use was provided by BioCryst Pharmaceuticals (Birmingham, AL). For quantitation of deoxynucleotides, deoxynucleotide triphosphates (dNTPs) were obtained from Amersham Biosciences (Piscataway, NJ) and were used as standards. \[^3\text{H}\]deoxyadenosine triphosphate (dATP) and \[^3\text{H}\]deoxythymidine triphosphate (dTTP) were purchased from Perkin Elmer Life Sciences (Boston, MA) and MP Biomedicals (Irvine, CA), respectively.

**Collection of samples for clinical pharmacology**

Blood samples were collected on the day before and days 1 through 4 and day 27 after the start of therapy on cycle 1 and pharmacokinetic and pharmacodynamic parameters were determined and correlated with \textit{in vivo} responses to therapy. \textit{In vitro} incubation with forodesine and dGuo were also performed in CLL lymphocytes to compare the
treatment effects in vivo and in vitro investigations. For the in vitro incubation a pretreatment blood sample was collected.

**Measurement of plasma dGuo and forodesine**

Blood samples (10 ml) before and after treatment at indicated days were obtained in green stopper Vacutainer tubes containing heparin and 50 μM BCX-34\(^{21}\) (BioCryst Pharmaceuticals) as an internal control. The plasma was removed after centrifugation and stored at -70°C and analyzed at BioCryst Pharmaceuticals.

Plasma dGuo and forodesine was analyzed as described previously\(^{14}\) using high-performance liquid chromatography (HPLC or LC) with tandem mass spectrometry detection (MS/MS). Briefly, BCX-1777 was extracted from plasma using a phenylboronic acid (PBA) affinity solid phase extraction (SPE) cartridge and dGuo was extracted from plasma using a Waters Oasis “HLB” affinity solid phase extraction (SPE) cartridge. The mass of BCX-1777 plus H\(^+\) (267.1 m/z) and the mass of dGuo plus H\(^+\) (268.1 m/z) are monitored in quadrupole one (Q1). The BCX-1777 product ion 148.0 m/z and the dGuo product ion 157.0 m/z are monitored in quadrupole three (Q3). The concentrations of forodesine and dGuo were determined by weighted (1/x) quadratic regression analysis of peak areas produced from the standard curve.

**Measurement of PNP inhibition**

PNP inhibition was measured by the spectrophotometric assay. Erythrocytes were separated from whole blood and PNP extracted from red blood cells by cell hemolysis.
Addition of the substrate inosine results in conversion of inosine to hypoxanthine by PNP. Hypoxanthine is catalyzed by xanthine oxidase to form a stable product, uric acid. The accumulation of uric acid is quantitatively determined by measuring the increase in absorbance at a wavelength of 293 nm. The observed data are then normalized by measuring the absorbance at a wavelength of 405 nm, which represents the number of erythrocytes used to prepare the PNP extract. The inhibitory effect was calculated using the equation: (1-(PNP activity post dose / PNP activity pre dose)) X 100.

Isolation of lymphocytes

Whole blood was collected in heparinized tubes for indicated time points and diluted 1:3 with cold PBS (0.135 M NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, 8 mM Na₂HPO₄ [pH 7.4]) and layered onto Ficoll-Hypaque (specific gravity, 1.086; Life Technologies, Grand Island, NY). The blood was then centrifuged at 433g for 20 minutes, and mononuclear cells were removed from the interphase²². Cells were washed twice with cold PBS and resuspended in 10 ml RPMI 1640, supplemented with 10% fetal bovine serum. A Coulter channelyzer (Coulter Electronics, Hialeah, FL) was used to determine cell number and the mean cell volume. The lymphocytes were used for dNTP and cell viability assays.

In vitro incubations

The primary CLL lymphocytes isolated from pretreatment sample were incubated with or without 2 µM forodesine and 20 µM dGuo. These concentrations were selected based on plasma pharmacology data during phase I study of forodesine¹⁴. Cultures
were maintained and aliquots (1 x 10^7 cells/ml) were removed at the end of incubation times. A Coulter channelyzer (Coulter Electronics, Hialeah, FL) was used to determine cell number and the mean cell volume. These cells were used for dNTP and cell viability assays.

**Measurement of dNTP pool**

The nucleotides in the leukemia cells were extracted by 60% methanol as described\(^{18}\) and the dNTPs were quantitated in these cell extracts by DNA polymerase assay as described by Sherman and Fyfe\(^{23}\).

**Measurement of cell viability**

Percentage apoptosis was measured by annexin V binding assay with a Detection Kit I (PharMingen, San Diego, CA) according to the manufacturer’s instructions. Briefly, fresh cells were washed with PBS and resuspended in 200 µL of 1 x annexin binding buffer obtained from BD Biosciences, at a concentration of 1 x 10^6 cells/ml. Annexin V–FITC (5 µL) was added and the cells were incubated in the dark for 15 minutes at room temperature. The labeled cells were then added to 10 µL propidium iodide (50 µg/ml) and analyzed immediately with a FACSCALIBUR cytometer (Becton-Dickinson). Data, from at least 10,000 events per sample, were recorded and processed using Cell Quest software (Becton-Dickinson).

**Statistical analyses and graphing**
All data were graphed using GraphPad Prizm (GraphPad Software, Inc., San Diego, CA). Linear regression analyses were done to determine the relationship between levels of dGuo and forodesine. Non-linear regression curves were used to determine achievement of steady state levels. Statistical significance was determined by the 2-tailed paired student’s t-test.
RESULTS

Clinical activity of forodesine

Eight patients were treated with forodesine on this study. The patient characteristics at start of therapy are given in Table 1. Majority of the patients (63%) had Rai stage IV disease, and almost all were previously heavily treated (one patient had 10, three had 5-7, another three had 3-4, and one patient had 1 prior chemotherapy). One patient was not evaluable for response as he progressed before the completion of the first cycle of treatment; and he had received 4 prior therapies before enrollment. The median number of cycles of forodesine received was 2 (range, 1-4) and the median duration of therapy was 8.7 weeks (range 4-20 weeks) The clinical response to forodesine in this heavily pretreated population was limited; only two patients had a decrease in their peripheral blood absolute lymphocyte count to normal levels after the first cycle, but this was short lasting and the counts increased thereafter. In the other 5 patients, the white blood cell (WBC) counts increased progressively and in 3 patients there was also progression in lymph nodes. The adverse events observed were mild. The clinical response and adverse events observed are listed in Table 2.

PNP inhibition during therapy

PNP inhibition was measured in circulating RBCs during therapy. The enzyme inhibition before therapy was 0% (n=7), which increased significantly to median 82% inhibition (range 63-84%; n=7) after the first oral ingestion of forodesine. However after the second day, the percent inhibition did not increase further and was a median 81%
(range 70-89%; n=7) at day 3. This steady-state inhibition was maintained until day 27 (median 73%; range 65-81%; n=5; Fig. 2).

Plasma pharmacology during therapy

Forodesine levels were quantitated in all eight patients on day 0 through 4 and in four patients on day 27. The highest level was achieved either on day 2 or 3 after the start of therapy. On day one, 300 nM of forodesine was achieved (range 126 – 600 nM, n = 8). One patient (#5) showed more than 1 µM forodesine on day 3 and 4, but he was not evaluated for response because he died before day 27. Overall the plasma forodesine on day 2, 3, 4, and 5, ranged between 200-1300 nM (n=8; Fig.3A).

With these concentrations of plasma forodesine and PNP inhibition, an increase in endogenous dGuo was expected. The level of dGuo in pretreatment samples was below the level of detection. On day 1, the dGuo concentration was a median 1.2 µM (range, 0.57 – 2.2 µM, n=8; Fig. 3B). Similar to forodesine levels, plasma dGuo levels reached a steady-state level, generally on day 2 and 3. On day 27, the concentrations were 1.1, 2.3, and 2.3 µM in samples from patient 3, 4, and 7, respectively.

Interestingly, one patient who achieved high plasma forodesine levels also accumulated high levels of dGuo (There was a 90% reduction in absolute lymphocyte counts for this patient [#5] during 19 days of therapy; day 0, 32,000/µl and day 19, 2,400/µl of blood). With this observation the association between the plasma dGuo levels and plasma forodesine levels were correlated. There was a direct and linear relationship between these parameters (p = <0.0001) with an r value of 0.83 in all patients (Fig. 3C).
contrast, the correlation between plasma forodesine and the inhibition of PNP was weak ($r=0.64$) in these samples (data not shown).

**Cellular pharmacology during therapy**

The starting level of intracellular dGTP was about 6 µM (median 5.95; range 0.9-7.84 µM; n=7) which increased to 10 µM on day 2 (range 0.5 to 43 µM) (Fig. 4A). In cells from 2 patients (# 5 and 6) there was no increase in dGTP after forodesine therapy. The dGTP increase in CLL lymphocytes was weakly related to dGuo increase in plasma ($r = 0.54$, $p = 0.006$; data not shown). In contrast to dGTP, there was no increase in the accumulation of other dNTPs such as dATP, dCTP, TTP (n=5; data not shown).

The baseline apoptosis values, measured in samples from 6 patients was a median 2.5% (range 1 – 9%). When we measured the percent apoptosis in these lymphocytes during therapy, one patient (#2) displayed very high percentage of apoptosis (90%) and mitochondrial membrane permeabilization (90%; data not shown) by day 5 after therapy (the absolute lymphocyte count decreased by 30% in this patient by day 21). Two patients (#5 and 7) did not show any increase in the amount of apoptosis. 3 patients (#4, 6 and 8) exhibited 18%, 13% and 22% of apoptosis, respectively (Fig. 4B). Since our previous *in vitro* studies demonstrated correlation between the accumulation of dGTP and the percentage induction of apoptosis, we plotted these two parameters to analyze if there is any correlation in this study. In samples during therapy, there was no correlation found between dGTP accumulation and apoptosis induction ($r = 0.217$; n =
In samples during *in vitro* incubations the correlation was better but still weak ($r = 0.46; n = 8; p = 0.2507; data not shown).

**In vitro studies with forodesine in CLL primary cells**

Our previous *in vitro* studies demonstrated that a high accumulation of dGTP may tip the balance between the dNTP pools and induce apoptosis. To determine if samples from these patients can accumulate high dGTP if incubated with greater concentrations of dGuo, CLL lymphocytes were incubated with 20 µM dGuo. The accumulation of dGTP is comparatively high in *in vitro* incubations with forodesine and dGuo compared to clinical samples for the same time point. The endogenous concentration of dGTP was 6 µM (range 5-23 µM, n=6) There was as high as a 17-fold increase in the accumulation of dGTP (median 115; range 40-250 µM; n=6; Fig. 5A). Comparison of dGTP values after 24 hr of therapy or *in vitro* treatment in CLL lymphocytes from six patients (Fig. 5B) clearly demonstrated that these cells have the capability of accumulating high dGTP when incubated with high dGuo. A paired two tailed t-test showed the dGTP levels were significantly higher ($p = 0.0157, n = 6$) during *in vitro* incubations.

The percentage apoptosis in untreated lymphocytes was a median 15% (range 9 – 28%, n= 5) as measured by the annexin binding assay. After *in vitro* incubation with forodesine and dGuo, apoptosis was median 34%, range 13-62%, at 24 hr (Fig. 5C). Three patient samples (#4, 5, and 7) were also incubated for 48 hrs, the amount of apoptosis increased further with time in these patients’ samples (data not shown). For
one patient, we cultured CLL lymphocytes either with forodesine alone, or dGuo alone or with both, and measured cell death. The percentage cell death was significantly lower when either the drug or the dGuo was not added (data not shown).
DISCUSSION

Forodesine demonstrated anti-leukemia activity in *in vitro* and *in vivo* models of T-ALL with an association between the accumulation of dGTP and the cytoreduction in T-leukemia cells during therapy\(^{14}\). It is well established that PNP inhibition causes an accumulation of dGTP via dCK. Although forodesine was introduced as a T-cell targeted therapy, B-CLL cells that also have high dCK levels should accumulate high levels of dGTP when PNP is inhibited and dGuo is accumulated in plasma. In concordance, we\(^{18}\) and others\(^{19}\) have demonstrated that CLL lymphocytes and B-Cell lineage hematologic malignancies\(^{24}\) were sensitive to *in vitro* forodesine and dGuo treatment. Consistent with these data, intracellular accumulation of ara-G (arabinosylguanosine, a dGuo analog) triphosphate was directly related to the response to nelarabine therapy both in T-cells and in CLL B cells. In both cases, patients whose cells accumulated low ara-GTP (less than 50 µM) did not respond to therapy, while T lymphoblast and B CLL cells from responders, had median 157 µM\(^{25}\) and 440 µM ara-GTP, respectively\(^{26}\).

Considering these favorable preclinical evaluations on mechanism of action and cytotoxicity of forodesine and its oral bioavailability, we initiated the phase II study of oral forodesine in patients with fludarabine refractory CLL. Oral forodesine (200 mg daily) was given continuously to 8 patients and 7 were evaluable for response (one did not complete 1 month of therapy for purposes of evaluation). There was an initial transient decrease in the WBC and absolute lymphocyte count to normal in 2 patients but then the counts increased. In others, there was no response. Similarly there was no
response in lymph node disease areas with progression in the size of lymph nodes on therapy in 3 patients. The present investigation reports the clinical outcomes as correlated with the pharmacokinetics of forodesine during therapy, the pharmacodynamic end points of dGuo accumulation in plasma as a result of PNP inhibition, and its conversion to dGTP in B-CLL cells.

During therapy, the increase in dGTP was very modest. The median dGTP was 10 μM (Fig. 4A) and this may be the reason for the modest clinical effect. Primary reasons for dGTP accumulation are increase in the plasma dGuo, transport of dGuo to cells followed by the phosphorylation of plasma dGuo, and degradation of dGTP. During therapy, the plasma level of dGuo with this oral dosing schedule of forodesine rose to a median of 1.2 μM (Fig. 3B) which was linearly related to the concentration of forodesine in plasma (Fig. 3C; r = 0.83). Forodesine uses equilibrative nucleoside transporters (ENT1 and ENT2) while dGuo uptake was mostly dependent on concentrative nucleoside transporters (CNT)27. CLL cells have high expression of CNT28, hence transport of dGuo should not be a factor in dGTP accumulation in CLL cells.

Additionally, it has been reported that CEM cell lines that lack hypoxanthine-guanine phosphoribosyl transferase (HGPRT) are more sensitive to low concentrations of forodesine (partial inhibition of PNP) in presence of high concentrations of dGuo because they lack metabolism of guanine to GMP leading to GTP accumulation27. CLL cells have low levels of HGPRT29, and therefore once PNP is blocked, dGuo will be converted to dGTP by deoxycytidine and dGuo kinases. These observations underscore the need for complete inhibition of PNP through high levels of forodesine.
The doses of intravenous forodesine used in a phase I study in T-cell diseases\textsuperscript{14}, a phase II clinical trial in T-ALL\textsuperscript{15,30}, a phase I trial in patients with CTCL\textsuperscript{31} and in a study in B-ALL\textsuperscript{24} were 40, 40, 40 and 80 mg/m\textsuperscript{2} intravenously, respectively. At these dose levels, during intravenous infusions of forodesine, the plasma dGuo levels rose to a median of 15 µM during the phase I study in T-cell diseases, ranged between 3.4 – 88.5 µM during the phase II trial in T-ALL, and ranged between 1.7 -12.8 µM in patients with CTCL. From these pharmacokinetic data, it is evident that in the present study, the required concentration of plasma dGuo was not reached. Furthermore, in \textit{in vitro} studies, the incubation of CLL cells from the same patients with higher concentration of dGuo resulted in higher levels of accumulation of dGTP (Fig. 5B). These data indicate that circulating CLL lymphocytes are capable of accumulating high dGTP levels and therefore efforts to increase the level of dGuo in plasma are needed.

The increase in plasma dGuo is due to an inhibition of PNP by forodesine. Data from all patients entered in this clinical trial showed a consistent result regarding the degree of PNP inhibition in the red blood cells which have a very high specific activity of this enzyme\textsuperscript{32}. In all cases, a steady-state inhibition of 80% was observed throughout the oral therapy (Fig. 2). Because the specific activity of PNP is high in human body, it is possible that greater than 80% inhibition may have resulted in higher plasma dGuo levels. dGuo is generated when cells die and liberate this nucleoside. Therefore, an alternative explanation for the low dGuo levels in plasma is that there was only a modest amount of cell death during the forodesine therapy. High levels of forodesine
(median 5 µM) were reached with intravenous infusion of this drug\textsuperscript{14} suggesting this route of administration may benefit patients with CLL. Increasing the dose, changing dosing schedule, or intravenous schedules should be feasible in CLL as forodesine is well-tolerated\textsuperscript{15,24,30,31}. Both forodesine and dGuo are needed during \textit{in vitro} incubations suggesting that endogenous levels of dGuo present in fetal bovine serum are not sufficient for a high accumulation of dGTP that could lead to a biological response. Similarly, dGuo without forodesine undergoes phosphorolysis by cellular PNP enzyme during \textit{in vitro} incubations\textsuperscript{18}. Thus for CLL cells to be sensitive to forodesine in \textit{in vitro} both forodesine and dGuo couplet were required.

Our preclinical and clinical data suggest that forodesine has biological activity in \textit{in vitro} CLL cell cultures. However, when administered orally to patients with CLL, the pharmacokinetic and pharmacodynamic endpoints did not meet the expectations. Because growth of the CLL clone depends on a variety of interactions with the microenvironment, variations in the requirements for these interactions on the part of the leukemia cells may be responsible for changes in the clinical course. These microenvironment sites may rescue CLL cells from apoptosis and facilitate cell survival preferentially in lymph-node pseudofollicles and bone marrow clusters. This goes in line with our findings where we recently demonstrated that CLL-B cells co-cultured in the presence of bone marrow stromal microenvironment exhibits reduced level of apoptosis with forodesine (manuscript under review). Thus, circulating CLL lymphocytes in the peripheral blood may be more susceptible to forodesine therapy while those in lymph nodes and bone marrow microenvironment may be resistant to this treatment.
In this clinical trial of heavily pretreated patients with CLL, forodesine failed to produce significant and lasting clinical responses in a limited number of patients. However, the drug is administered orally and is well-tolerated; therefore, its dose can be significantly increased and its schedule can be adjusted for a more favorable pharmacodynamic effect. The \textit{in vitro} biologic data and \textit{in vivo} pharmacodynamic and clinical data suggest that forodesine has biological activity. Therefore either increasing the dose of forodesine and/or alternated dose schedule to achieve more favorable pharmacodynamic parameters and/or combining it with agents that could interfere with other mechanisms of resistance should be explored in future CLL trials.
AUTHORSHIP

K.B. designed laboratory endpoint research, performed in vitro experiments, analyzed pharmacokinetic and pharmacodynamic results and wrote the paper.

D. V. analyzed the clinical data and wrote the paper.

S. O., M. J. K. and H. M. K. treated patients on the clinical trial and approved the final manuscript.

B.T. and Y.C. collected blood samples from patients, isolated and processed red blood cells for PNP assay, and separated plasma and leukemia cells for pharmacokinetic and pharmacodynamic endpoints.

S.B. collected clinical data and assisted in their analysis.

M.K. and S.B. supervised PNP assay and plasma pharmacology analyses.

V.G. participated in clinical protocol design, supervised laboratory endpoint research, analyzed pharmacokinetic and pharmacodynamic data, and wrote the paper.

F. R. designed and conducted the clinical trial, wrote the paper and reviewed and approved the final manuscript.

Conflict-of-interest disclosure:

M.K. and S.B. are employees of BioCryst.

F.R. and V.G. received research funding from BioCryst.

Financial disclosure:

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Table 1. Patient characteristics at start of forodesine therapy

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Table 2. Clinical response and adverse events on forodesine therapy.

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<thead>
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<th></th>
<th>n=7</th>
<th>Number (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Therapy duration, weeks median [range]</td>
<td>8.7 [4-20]</td>
<td></td>
</tr>
<tr>
<td>Therapy duration, median no. of cycles [range]</td>
<td>2 [1-4]</td>
<td></td>
</tr>
<tr>
<td>↓ in ALC (absolute lymphocyte count) to Normal</td>
<td>2 (29)</td>
<td></td>
</tr>
<tr>
<td>Progressive ↑ in ALC</td>
<td>5 (71)</td>
<td></td>
</tr>
<tr>
<td>Lymphadenopathy progression</td>
<td>3 (43)</td>
<td></td>
</tr>
<tr>
<td>Adverse events (all grades)</td>
<td></td>
<td></td>
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<tr>
<td>Fatigue</td>
<td>3 (43)</td>
<td></td>
</tr>
<tr>
<td>Bronchitis</td>
<td>3 (43)</td>
<td></td>
</tr>
<tr>
<td>Diarrhea, mucositis</td>
<td>2 (29)</td>
<td></td>
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<tr>
<td>Fever - low grade</td>
<td>3 (43)</td>
<td></td>
</tr>
<tr>
<td>Transient neutropenia and/or thrombocytopenia)</td>
<td>3 (43)</td>
<td></td>
</tr>
<tr>
<td>Pneumonia</td>
<td>1 (14)</td>
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Figure legends:

Figure 1. Role of Purine Nucleoside Phosphorylase (PNP) in purine pathway
This mammalian enzyme is involved in phosphorolysis of substrates such as inosine/deoxyinosine, xanthosine/deoxyxanthosine, and guanosine/deoxyguanosine. With these conversions, bases such as hypoxanthine, xanthine, and guanine, respectively are formed.

Figure 2. PNP inhibition in red blood cells during therapy
Red blood cells from patients (all eight patients; except pt # 6) were isolated from whole blood starting day 0 to day 4 (n = 7) and day 27 (n = 5) and the cells were processed to determine level of PNP and its inhibition after start of therapy as described in methods. Data were fitted using non-linear rectangular hyperbola. PNP assay was done in quadruplicate and data are mean ± SD. The average specific activity of PNP was 3.7 mUnits/min where 1 mUnit is defined as amount of PNP crude lysate that catalyzes the phosphorolysis of 1 µmole of inosine per minute under standard assay condition.

Figure 3. Plasma forodesine and dGuo levels during therapy
Whole blood was collected from all 8 patients at indicated time points and plasma was separated. Forodesine (A) and dGuo (B) levels in each sample were determined using a LC/MS/MS liquid chromatography as described under methods section. Each symbol represents a patient. The correlation between plasma forodesine and plasma dGuo (C) was evaluated by plotting data from figure 3A and 3B and linear regression analysis was performed.
Figure 4. Accumulation of dGTP and induction of cell death during therapy

Blood samples were collected from all patients (except pt #1) at indicated time points before and after start of therapy and CLL lymphocytes were isolated. After methanol extraction, cells were processed to measure dGTP by DNA polymerase assay (A) and cell death by annexin binding procedure (B) as described under methods section.

Figure 5. In vitro accumulation of dGTP and induction of cell death. Primary CLL cells from 6 patients were incubated without drug or with 2 µM of forodesine and 20 µM of dGuo for 24 hr and the nucleotides were extracted and dGTP was measured by DNA polymerase assay in untreated (grey bars) or forodesine and dGuo treated (black bars) cells. (A). Data from figure 4 A are plotted with data from figure 5A to compare in vivo (grey bars) and in vitro (black bars) accumulation of dGTP (B). In the same cells, cell death was analyzed at 24 hr by annexin positivity procedure as described under methods in untreated (grey bars) and forodesine and dGuo treated (black bars) cells (C).
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Phase II and pharmacodynamic study of oral forodesine in patients with advanced and/or fludarabine-treated chronic lymphocytic leukemia

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