Forodesine, an inhibitor of purine nucleoside phosphorylase, induces apoptosis in chronic lymphocytic leukemia cells.

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Abstract

Purine nucleoside phosphorylase (PNP) deficiency in humans results in T-lymphocytopenia. Forodesine, a potent inhibitor of PNP, was designed based on the transition-state structure stabilized by the enzyme. Previous studies established that forodesine in the presence of deoxyguanosine (dGuo) inhibits the proliferation of T-lymphocytes. A phase I clinical trial of forodesine in T-cell malignancies demonstrated significant antileukemic activity with an increase in intracellular dGuo triphosphate (dGTP). High accumulation of dGTP in T-cells may be dependant on the levels of deoxynucleoside kinases. Because B-cell chronic lymphocytic leukemia (B-CLL) cells have high activity of deoxycytidine kinase (dCK) we hypothesized that these lymphocytes would respond to forodesine. This postulate was tested in primary lymphocytes during in vitro investigations. Lymphocytes from 12 patients with CLL were incubated with forodesine and dGuo. These CLL cells showed a wide variation in the accumulation of intracellular dGTP without any effect on other deoxynucleotides. This was associated with DNA damage-induced p53 stabilization, phosphorylation of p53 at ser15, and activation of p21. The dGTP accumulation was related to induction of apoptosis measured by caspase activation, changes in mitochondrial membrane potential, and PARP cleavage. Based on these data a phase II clinical trial of forodesine has been initiated for CLL patients.
**Introduction**

The major role of mammalian purine nucleoside phosphorylase (PNP) is to catalyze the cleavage of inosine, deoxyinosine, guanosine, and deoxyguanosine (dGuo) to their corresponding base and sugar 1-phosphate by phosphorolysis.\(^1\) PNP deficiency in humans produces a relatively selective depletion of T-cells.\(^2\) PNP deficient children exhibit profound impairment in the T-cell component of their immune systems, but have normal B-cell function.\(^3\) This rare condition provided a model for the development of specific inhibitors of PNP, either to enable selective suppression of T-cell function that has been useful in the treatment of T-cell-mediated diseases or as potential T-cell selective chemotherapeutic agents.\(^4\)

The aza-C-nucleosides, Immucillin-H and Immucillin-G, are transition state analogue inhibitors of PNP.\(^5\) Immucillin analogues modified at the 2', 3', or 5'-positions of the azasugar moiety or at the 6-, 7-, or 8-positions of the deazapurine, as well as methylene-bridged analogues, have been synthesized and tested for their inhibition of human PNP.\(^6\) Forodesine (BCX-1777 / Immucillin-H) is a potent inhibitor of PNP (Fig. 1). Forodesine in the presence of dGuo inhibited the proliferation of CEM-SS (T-acute lymphoblastic leukemia) cells with an IC\(_{50}\) of 0.015 μM. This inhibition by forodesine and dGuo was accompanied by a 154-fold and 8-fold elevation of endogenous dGuo triphosphate (dGTP) and deoxyadenosine triphosphate (dATP) pools, respectively.\(^7\) Forodesine, in the presence of dGuo, (3-10 μM), inhibited human
lymphocyte proliferation activated by various agents such as interleukin-2 (IL-2), mixed lymphocyte reaction and phytohemagglutinin with an IC₅₀ values that ranged between 0.1 and 0.38 µM. Forodesine is a 10-100-fold more potent inhibitor of human lymphocyte proliferation than other known PNP inhibitors like PD141955 and BCX-34. Previous studies demonstrated that the cytotoxic activity of forodesine in the presence of dGuo was selective to T-lymphocytes. High kinase and low nucleotidase levels make these cells more sensitive to inhibition by forodesine and dGuo.

Based on these observations, we conducted a phase I clinical trial of forodesine in patients with advanced T-cell malignancies. Significant antileukemic activity was correlated with an increase in plasma forodesine (median 5 µM) and dGuo (median 14 µM), and an accumulation of intracellular dGTP. As reported in cell lines, it was postulated that high accumulation of dGTP in T-cells may be dependant on activity of deoxynucleoside kinases such as deoxycytidine kinase (dCK) which is a primary enzyme for the conversion of dGuo to dGMP (dGuo monophosphate), which is then converted to dGTP.

Because B-chronic lymphocytic leukemia (B-CLL) has high activity of dCK, we hypothesized that this disease would be sensitive to forodesine and dGuo treatment. To test our hypothesis we conducted the present investigation using primary leukemic lymphocytes obtained from patients with CLL. We demonstrate the cytotoxic effect of forodesine with dGuo in CLL cells from 12 patients using pharmacologically achievable levels of forodesine and dGuo at different time periods. Accumulation of dGTP and effect on other deoxynucleoside
triphosphates (dNTPs) were analyzed and related to induction of cell death. Molecular mechanisms such as DNA damage-induced p53 stabilization, phosphorylation of p53 at ser15, and activation in the expression of p21 proteins were determined. Based on our current data, we have initiated a phase II study of forodesine in patients with fludarabine-refractory CLL.
Patients and methods

Drugs and chemicals

Forodesine for laboratory use was provided by BioCryst Pharmaceuticals, Inc. (Birmingham, AL) and dGuo was purchased from Sigma (St. Louis, MO). For quantitation of deoxynucleotides, dNTPs were obtained from Amersham Biosciences (Piscataway, NJ) and were used as standards. [³H]dATP and [³H]dTTP were purchased from Perkin Elmer Life Sciences, Inc. (Boston, MA) and MP Biomedicals, LLC (Irvine, CA), respectively.

Patients

Present in vitro studies were carried out in leukemic lymphocytes obtained from patients with CLL (n = 12). The percentage of leukemic lymphocytes (B-Cell population) in 7 patients was measured by flow cytometry method using CD19 FITC antibody obtained from BD Biosciences. Blood samples obtained from these 12 patients were used for different pharmacological, biochemical, and molecular end points. All patients signed a written informed consent to participate in this laboratory protocol, which was approved by institutional research board.

Clinical laboratory endpoints

Determination of IgVH gene mutation status and ZAP-70 analysis for the patients in our study were conducted as described previously¹² and provided by the Chronic Lymphocytic Leukemia Research Consortium (University of California, San Diego and the University of Texas, M. D. Anderson Cancer Center). Fluorescent in situ hybridization (FISH) analysis data were provided by the clinical cytogenetics laboratory, Department of Hematopathology at M.D.
Anderson Cancer Center. FISH technique was used to detect the chromosome 17p deletion for p53 gene in CLL cells. The detailed methodology for the assay has been described before.13

Chromosomal cytogenetics was determined using CLL lymphocytes at M.D. Anderson Cancer Center.

**Isolation of lymphocytes**

Whole blood was collected in heparinized tubes 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, Inc., Grand Island, NY). The blood was then centrifuged at 433 x g for 20 min, and mononuclear cells were removed from the interphase.14 Cells were washed twice with cold PBS and resuspended in 10 ml of 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 then resuspended at a concentration of 1 x 10⁷ cells/ml and fresh cells were used for all experiments.

**Source of normal lymphocytes**

Normal lymphocytes were isolated from buffy coat of healthy donor obtained from transfusion services at M D Anderson Cancer Center. B-cell and T-cell lymphocytes were isolated using EasySep B-cell enrichment cocktail and EasySep human T-cell negative selection cocktail obtained from Stemcell Technologies, (Vancouver, Canada). EasySep is an immunomagnetic cell selection procedure that uses specific antibodies and tiny FACS-compatible
magnetic nanoparticles in a column-free magnetic system. Cells are targeted for
selection or depletion using monoclonal antibodies directed against specific cell
surface antigens. These labeled cells are then crosslinked to EasySep magnetic
nanoparticles provided by manufacturer, in a standard FACS tube. The tube is
then placed directly in the specially designed EasySep magnet. This handheld
magnet is gently inverted to remove the cells that are not bound to the magnetic
nanoparticles. The % B- and T-cell populations in the isolated fractions were
measured by flow cytometry using CD19 FITC antibody and the CD3 FITC
antibody from BD Biosciences (San Jose, CA) respectively.

Measurement of dNTP pools

The primary CLL lymphocytes were incubated on the same day of their isolation
with or without 2 µM forodesine and 10 µM dGuo for 0, 4, 8, 16 and 24 hr. These
concentrations were selected based on plasma pharmacology data during phase
I study of forodesine\textsuperscript{10}. Cultures were maintained and aliquots (1 x 10\textsuperscript{7} cells/ml)
were removed at the end of incubation times. The nucleotides in the leukemia
cells were extracted by 60% methanol as described\textsuperscript{15} and the dNTPs were
quantitated by DNA polymerase assay as modified by Sherman and Fyfe\textsuperscript{16} in
these cell extracts.

Quantitation of caspase 8, 9 and 3 activity

Cells that have been induced to undergo apoptosis are collected by
centrifugation and the pellet is lysed by the caspase lysis buffer. The cell lysate is
then tested for protease activity by the addition of a caspase-specific peptide that
is conjugated to the fluorescent reporter molecule 7-amino-4-trifluoromethyl
coumarin (AFC). These peptide substrates were DEVD-AFC, IETD-AFC, and LEHD-AFC for caspase 3, 8 and 9, respectively. The cleavage of the peptide by the caspase releases the fluorochrome that, when excited by light at 400 nm wavelength, emits fluorescence at 505 nm. The level of caspase enzymatic activity in the cell lysate is directly proportional to the fluorescence signal detected with a fluorescent micro plate reader according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

**Determination of mitochondrial membrane potential**

Mitochondrial membrane potential was determined in some of these samples by flow cytometry using a lipophilic cationic $\Delta\psi_m$-dependent fluorescent dye JC-1 (5,5',6,6'-tetrachloro-1,1',3,3' tetraethylbenzimidazol carbocyanineiodide) from Molecular Probes (Eugene, OR) which was analyzed in fluorescence detection channel 2 (FL-2). Lymphocytes with intact mitochondria excite an intense red fluorescence due to the formation of the dye aggregates whereas the monomer dye fluoresces green in cells with a disrupted mitochondrial membrane. All aliquots were incubated at room temperature for 20 minutes in the dark with 2 µl of the dye diluted in PBS at a concentration of 2.5 µg / ml and was analyzed immediately with a FACSCALIBUR cytometer (Becton–Dickinson, UK). Data, from at least 10,000 events per sample, were recorded and processed using the CellQuest software (Becton–Dickinson, UK).

**Apoptotic analysis using dual annexin V-FITC/ propidium iodide**

The analysis of annexin V binding was carried out with a Detection Kit I (PharMingen, San Diego, CA) according to the manufacturer's instructions.
Briefly, 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 × 10^6 cells/ml. 5 μl of annexin V-FITC was added and the cells were incubated in the dark for 15 min 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, UK). Data, from at least 10,000 events per sample, were recorded and processed using the CellQuest software (Becton–Dickinson, UK). The results were expressed after subtracting endogenous level of annexin positivity in untreated timed-control samples.

**Immunoblot analysis**

Cells were lysed on ice for 20 min in lysis buffer containing 25 mM HEPES, pH 7.5, 300 mM NaCl, 1.5 mM MgCl2, 0.5% sodium deoxycholate, 20 mM -glycerophosphate, 1% Triton X-100, 0.1% SDS, 0.2 mM EDTA, pH 8, 0.5 mM dithiothreitol, 1 mM sodium orthovanadate, pH 10 and protease inhibitor. Cells were centrifuged at 14,000 g for 15 min at 4°C, and the supernatant was stored at -80°C until use. Protein content was determined using DC protein assay kit according to the manufacturer’s instructions (Bio Rad Laboratories, Hercules, CA). Aliquots (30 μg) of total cell protein were boiled with Laemmli sample buffer and loaded onto 8-12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (GE Osmonics Labstore, Minnetonka, MN). Membranes were blocked for 1 hr in PBS Tween containing 5% nonfat dried milk and then incubated with primary antibodies for 2 hr followed by species-specific horseradish peroxidase (HRP)-conjugated secondary antibody (diluted 1: 5000)
for 1 hr. The blots were visualized by enhanced chemiluminescence according to the manufacturer’s instructions (Pierce Biotechnology, Inc., Rockford, IL) and normalized to the actin levels (antibody to actin was obtained from Sigma) in each extract. Mouse monoclonal antibody against p53 (OP43; Oncogene Research Products, Boston, MA), mouse monoclonal anti-p21 (Sc6246, Santa Cruz Biotechnology, Santa Cruz, CA), rabbit phospho-Ser15 of p53 (9284, Cell Signaling Technology, Beverly, MA) and mouse monoclonal antibody to PARP from BD Pharmingen International (San Diego, CA) were used to detect these proteins in each sample.

**Statistical analysis**

The relationship between different parameters such as caspase activation and PARP cleavage with that of increase in dGTP accumulation (given as rectangular hyperbola) was determined using GraphPad Prism (GraphPad Software, San Diego, CA).
Results

Accumulation of dGTP with forodesine and dGuo

A total of 12 patients with CLL were studied; the median white blood cell (WBC) count was 125 K / µL (range between 35-360 K / µl). Most of these patients (n=10) were previously untreated. Their Rai stage, ZAP-70 status and IgVH mutational status and cytogenetics information are provided in Table 1. Only one patient (#11) had deletion of chromosome 17. The endogenous dGTP level was low and varied significantly between 0.3 and 4.4 µM with a median 2.7 µM (Table 1). In order to test if CLL cells do accumulate dGTP after PNP inhibition, leukemic lymphocytes obtained from 11 patients were incubated with forodesine and dGuo and the accumulation of dNTP pool was measured by DNA polymerase assay. Similar to T-cell leukemic lymphocytes\textsuperscript{10}, primary CLL lymphocytes also accumulated dGTP with 2 µM forodesine in presence of 10 µM dGuo but showed heterogeneity. In 10 of 11 patient samples, during the drug incubation there was a gradual accumulation of dGTP. At 4 hr the triphosphate accumulation was median 2.5 µM (range, 3-50 µM) and at 8 hr it further increased to median 14 µM (range, 6-118 µM, Fig. 2A). Lymphocytes from one patient (Pt # 2) had no significant increase in dGTP. Compared to dGTP levels, there was no increase in other dNTPs such as dATP, deoxycytidine triphosphate (dCTP), and deoxythymidine triphosphate (dTTP). Data from one representative patient (#1) is provided in Fig. 2B for illustrative purpose. At start, CLL lymphocytes from this patient had 3.8 µM intracellular dGTP level which
increased to 30 μM (8-fold) at 24 hr (Fig. 2B). The starting levels of dATP, dCTP, and dTTP were 1, 4 and 2 μM, respectively and these values remained almost the same at the end of 24 hr. This was true in all samples (n=11) analyzed for all four dNTPs (data not shown).

**Stabilization of p53 and phosphorylation at Ser15**

P53 regulates the expression of genes involved in DNA repair, cell cycle arrest, and apoptosis. It is up regulated during cell stress via a phosphorylation-dependent process. When CLL lymphocytes (Pt # 1-5) were incubated with forodesine and dGuo, 4 of 5 patients (Pt # 1, 3, 4 and 5) showed an increase in p53 protein level along with an upregulation of downstream protein p21 (Fig. 3A and 3B). However one patient (Pt # 2) showed neither the stabilization of p53 nor the upregulation of p21 (data not shown). Stabilization of p53 protein may be due to its phosphorylation on ser15 (Fig. 3A and 3B). These results suggest that DNA damage induced by forodesine up regulates p53, via phosphorylation-dependent protein stabilization.

**Induction of apoptosis in CLL lymphocytes**

When CLL lymphocytes were incubated with forodesine and dGuo there was induction of apoptosis which was measured by different experimental approaches. First, the PARP cleavage was measured at different time points in CLL cells for 5 patients (Pt # 1-5). In all samples, the PARP cleavage was detected starting at 4 hr. Data from two representative patients, #1 and #4 (Fig. 4A and 4B) are provided. Collective data from these 5 patient samples at 4, 6, and 8 hr after treatment were plotted to seek a relationship between fold increase
in PARP cleavage and increase in dGTP (Fig. 4C). There was a strong and proportional relationship ($r=0.92$) between these parameters reaching a plateau after 50-fold increase in dGTP. Furthermore, to identify if PARP cleavage was associated with activation of caspases 9, 8 and 3, fluorometric assays by the addition of a caspase-specific peptides were done.

Data from two patients for caspase 8 and 9 (#2 and #3, Fig. 5A and 5B) demonstrate that cell lysate from one patient (#2) did not show much activation in caspases 8 and 9 (Fig. 5A). The intracellular dGTP values for this individual were 4, 6, 8 and 5 µM at 0, 2, 4 and 8 hr respectively. In contrast to these data, cell lysates from another patient (#3) showed 2 - 4 fold increase in caspases 8 and 9 activation (Fig. 5B). The dGTP values for this patient were 3, 40, 80 and 118 µM at 0, 2, 4, and 8 hr respectively. The increase in caspases 8 and 9 was observed in 7 of 12 patients.

Nine of 11 patient samples showed a 6 to 31-fold increase in the activation of caspase 3. The increase in caspase 3 was correlated with the accumulation of dGTP and there was a proportional relationship between these 2 parameters ($r=0.932$ and $p=0.005$; Fig. 5C). As another measure of apoptosis, primary CLL cells from fourteen patients (Pt # 6-12, and an additional seven patient samples) were incubated with forodesine and dGuo and the induction of apoptosis was measured by annexin V binding using flow cytometry. Overall from 14 patients’ samples tested, 7 showed annexin V positivity at 8 hr which ranged from 5% to 40% and 11 patients’ samples showed annexin V positivity at 24 hr (range 8% -
41%), confirming that these cells undergo apoptosis with forodesine (data not shown).

In limited patient samples the disruption in the MMP was measured by flow cytometry using JC-1 dye. For MMP change there was heterogeneity among patients in response to forodesine (data not shown).

**Effect of forodesine, dGuo or combination on CLL lymphocytes**

To test if forodesine alone has any toxicity to CLL lymphocytes or if both forodesine and dGuo are needed for a cytotoxic effect, CLL lymphocytes from 3 additional patients were incubated with these drugs individually or in combination. Leukemic lymphocytes from none of these patients showed an increase in dGTP with forodesine alone; however 2 of 3 patients accumulated and increased dGTP with dGuo alone. Samples from all three patients resulted in augmentation of intracellular dGTP when forodesine and dGuo were combined. When caspase 3 was measured in these lysates none of these samples showed activation of caspase 3 with forodesine alone; however 2 of 3 patients showed increase in caspase 3 with dGuo alone and all samples showed activation with the combination of both drugs (Table 2).

**Effect of forodesine on normal T- and B- cell lymphocytes**

Normal T- and B- lymphocytes were obtained from three healthy donors (Table 3) and incubated with forodesine and dGuo and the accumulation of dGTP and induction of apoptosis was measured by annexin V. In this limited sample analysis compared to T-cell lymphocytes, B-cell lymphocytes appear to
accumulate higher levels of dGTP. Neither T- nor B- lymphocytes underwent apoptosis after 8 hrs of treatment (Table 3).
Discussion

We initiated this work based on 2 premises; first, purine nucleoside analogs have been the most effective agents for treatment of CLL and minor modifications in the structure of these analogs have resulted in diverse activity. Second, enzymatic (adenosine deaminase, ADA) inhibitor that alters the deoxynucleotide pool in the leukemic lymphocytes has been successful for treatment of this indolent disease. The prime examples of these strategies are fludarabine, cladribine, and nelarabine for the former and pentostatin for the latter. 

Availability of forodesine, a potent PNP inhibitor, its clinical activity in T-cell diseases, demonstration of PNP inhibition, accumulation of dGuo in plasma, and dGTP in circulating leukemia cells prompted us to test this novel enzymatic inhibitor for CLL cells to perturb cellular milieu for dNTPs. In the present study, we demonstrate that physiological concentration of forodesine in presence of clinically achievable level of dGuo result in accumulation of dGTP albeit at varied levels in primary CLL cells (Fig. 2A).

In order to be biologically active, dGuo needs to be phosphorylated intracellularly, and its cytotoxicity depends on accumulation of the dGTP in the cells. When dGuo enters the cell through nucleoside transporters, it is phosphorylated to monophosphate by cytosolic dCK or mitochondrial dGuo kinase and dephosphorylated by 5’-nucleotidase. The variability in the accumulation of dGTP in these CLL lymphocytes may be due to differential activities of these enzymes. The increase in dGTP level was exclusive as there
was no significant accumulation observed for the other dNTPs such as dATP, dCTP, and dTTP (Fig. 2B). Perturbation of dNTP pool leading to a p53 response has been observed with agents such as hydroxyurea, a ribonucleotide reductase inhibitor that lowers dNTP pool\(^{20}\) and pentostatin, an ADA inhibitor that will increase dATP pool\(^{21}\) in both cycling and indolent leukemia cells. In addition, previous studies showed that in quiescent lymphocytes, dATP accumulation interferes with proper repair of DNA, accumulation of DNA strand breaks, and p53 expression\(^{22}\). The p53 network which is normally ‘off’ is activated only when cells are stressed or damaged. Wild-type p53 protein is an important regulator of cell fate by its ability to accumulate after DNA damage and other types of stress and to cause growth arrest or apoptosis. Because majority of the CLL patients in the present investigation were previously untreated, it would be expected that they harbour WT p53\(^{23,24}\). Hence, we examined whether dGTP accumulation in the CLL cells leads to changes in p53 content (Fig. 3A and 3B). Forodesine and dGuo treatment, stabilized p53 protein level in all except for one patient. In concordance with our hypothesis, leukemia cells from this patient did not show an augmentation in dGTP accumulation. DNA damage induced phosphorylation of p53 at Ser15, a residue that lies adjacent to the MDM2-binding site has been reported\(^{25-27}\). This post-translational modification is required for p53 stabilization and activation following cell stress and is a determinant of p53-mediated apoptosis\(^{28-32}\). As shown in figure 3A and B, p53 stabilization after forodesine and dGuo treatment was associated with ser15 phosphorylation and activation of
downstream p21. Taken together, these molecular events suggest that forodesine treatment results in post-translational modification of p53, its stability, and p53-dependent p21 activation. These data are consistent with other anticancer drugs that induce DNA damage, and p53-mediated down-stream signaling leading to cell growth arrest, DNA damage repair, or apoptosis.\textsuperscript{33}

To determine if dGTP mediated p53 response would lead to apoptosis, cell death measurements were done in these lymphocytes. The enzymatic activity of caspase 8 and 9 using a fluorometric biochemical assay showed activation of these extrinsic and intrinsic cell death pathways only in those cells that showed an accumulation of dGTP (Fig. 5B). In contrast, no effect or minor increase in dGTP pool did not result in caspase activation (Fig. 5A). This differential activation of caspases based on dGTP accumulation was also seen for caspase 3. In fact, the increase in caspase 3 was directly related to the accumulation of dGTP in these 11 patients ($r=0.932$ and $p=0.005$; Fig. 5C), suggesting that the induction of apoptosis in these leukemic lymphocytes is in response to the accumulation of dGTP. Consistent with the activation of caspase 3 and as a downstream substrate of this caspase, there was PARP cleavage in most patient samples studied (Fig. 4A and 4B) which was strongly associated with increase in intracellular dGTP (Fig. 4C). Apoptosis induced by chemotherapeutic agent can be mediated either via DNA damage and p53 protein expression as shown above for forodesine, or directly via perturbation of mitochondrial function and change in mitochondrial permeability transition pore (mtPTP).\textsuperscript{34} The CLL lymphocytes in our study also showed change in mitochondrial membrane potential suggesting
that the mitochondria may be directly or indirectly involved in dGTP-induced cell death.

The mechanism by which intracellular dGTP would initiate apoptosis of these quiescent lymphocytes is not fully understood. However our data do suggest that fold-increase in dGTP serves as a primary determinant of apoptotic response (Fig. 4C and 5C). With that respect, our data would mimic what was observed with ADA inhibitor pentostatin. Pentostatin, a highly effective agent for CLL is a potent inhibitor of ADA ($K_i = 2.5$ pM); and induces accumulation of dATP. Elevated dATP levels would cause an imbalance in the cellular levels of dNTPs, resulting in the inhibition of DNA synthesis and repair. In addition, dATP is a required element of apoptosome, an assembly of Apaf-1, cytochrome $c$, and pro-caspase 9 that activates the caspase cascade leading to apoptosis. Moreover, increased levels of Ado or dAdo derivatives in non-dividing cells can lead to an unbalanced ratio of S-adenylmethionine and S-adenylhomocysteine, thus impairing the synthesis of methylated nucleosides. Similar to these actions of pentostatin, forodesine inhibits PNP and induces the accumulation of another dNTP, dGTP and causes an imbalance in the cellular levels of dNTPs, resulting in the induction of apoptosis.

These data provide rationale for use of forodesine for the treatment of CLL. First, in vitro studies on CLL suggested accumulation of intracellular dGTP. Second, there was DNA damage response as measured with p53 stabilization and phosphorylation and activation of downstream p21. Third, the measure of PARP cleavage, caspase activation and changes in mitochondrial membrane potential.
demonstrated that CLL cells undergo apoptosis with forodesine and dGuo. Finally, there seems to be a therapeutic index as normal T and B lymphocytes isolated from healthy donors did not undergo apoptosis with this combination. Based on these observations we have initiated a phase II study of forodesine in patients with fludarabine-refractory CLL. Laboratory end points during this ongoing trial would provide information on the efficacy of oral forodesine for this indolent leukemia.

Acknowledgement

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References


17. Salvioli S, Ardizzoni A, Franceschi C, Cossarizza A. JC-1, but not DiOC6(3) or rhodamine 123, is a reliable fluorescent probe to assess delta psi changes in intact cells: implications for studies on mitochondrial functionality during apoptosis. FEBS Lett. 1997;411:77-82.


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Tx, Therapy; T, Treated; UNT, untreated; ND, not done.
Table 2:
Comparison of dGTP accumulation either with forodesine, dGuo, or both.

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<td>8.2</td>
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<td>Caspase 3, % activation</td>
<td>74</td>
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<td>dGTP, µM</td>
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<td>5.0</td>
<td>17.0</td>
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<td>1578</td>
<td>1999</td>
<td>1962</td>
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<td>3</td>
<td>dGTP, µM</td>
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<td>51.7</td>
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<td>Caspase 3, % activation</td>
<td>111</td>
<td>98</td>
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Table 3:

Accumulation of dGTP in B- and T-lymphocytes isolated from healthy donors

<table>
<thead>
<tr>
<th>Donor</th>
<th>Cell-type</th>
<th>dGTP (µM)</th>
<th>Annexin V (%)</th>
<th>JC-1 (%)</th>
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<tr>
<td></td>
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<td>8 hr untreated</td>
<td>8hr treated</td>
<td>8hr untreated</td>
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<tr>
<td>1</td>
<td>T-cell</td>
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<td>4.6</td>
<td>18</td>
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<td>B-cell</td>
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<td>37.4</td>
<td>19</td>
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<td>T-cell</td>
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<td>10.9</td>
<td>36</td>
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<td>2</td>
<td>B-cell</td>
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<td>70</td>
<td>29</td>
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<tr>
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<td>T-cell</td>
<td>1.4</td>
<td>6.8</td>
<td>37</td>
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<tr>
<td>3</td>
<td>B-cell</td>
<td>1.4</td>
<td>6.8</td>
<td>37</td>
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</table>
Figure Legends:

Figure 1: Structure of forodesine

Figure 2: Accumulation of dGTP (A), and all 4 dNTPs (B) with the incubation of forodesine in primary CLL cells. The primary CLL cells from 11 patients were incubated with 2 µM of forodesine and 10 µM of dGuo at 4 and 8 hr and the nucleotides in the leukemia cells were extracted by 60% methanol and dNTPs were measured by DNA polymerase assay. The data of accumulation of dGTP for all patients (A) and the 4 dNTPs (dGTP, dATP, dCTP and dTTP) for one patient (B) are plotted.

Figure 3: Forodesine increases p53 stabilization and induces p53 phosphorylation on ser15, with the activation of p21. CLL primary cells were incubated with 2 µM of forodesine and 10 µM of dGuo at different time points and the expression of p53, and the phosphorylation of p53 at ser15 and the expression of p21 were measured by western blot and were normalized to actin. Western blot data of 2 patients (#1, Fig. 3A and #4, Fig. 3B) are presented.

Figure 4: Induction of apoptosis in primary CLL cells with forodesine. CLL cells when incubated with forodesine at different time points, there was induction of apoptosis that was measured by PARP cleavage and the western blots were normalized to actin. Data from 2 patients (#1, Fig. 4A and #4, Fig. 4B) are provided. The immunoblots of PARP for 5 patients were quantitated using densitometer and the cleaved PARP
was correlated with the dGTP accumulation and the relationship between accumulation of dGTP and the PARP cleavage for 5 patients at different time periods (4, 6, and 8 hr) are plotted (Fig. 4C).

**Figure 5: Relationship between intracellular dGTP and activation of caspases in primary CLL cells with forodesine.** CLL cells were incubated with 2 µM of forodesine and 10 µM of dGuo at different time points and the activation of caspases 8, 9 and 3 were measured by fluorometric assay. Data from 2 patients for caspases 8 and 9 (5A and 5B), and the relationship between accumulation of dGTP and the activation of caspase 3 (5C) for 11 patients are plotted.
Figure 1
Figure 2

A

B

Figure 2

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Figure 3
Figure 4
Figure 5

A
Activation, %

B
Hrs
0 2 4 8 24

C
caspase 3, fold increase
dGTP, fold increase

Figure 5
Forodesine, an inhibitor of purine nucleoside phosphorylase, induces apoptosis in chronic lymphocytic leukemia cells

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