Linear chain PEGylated recombinant *Bacillus thiaminolyticus* thiaminase I enzyme has growth inhibitory activity against lymphoid leukemia cell lines

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Abstract

Cancer cells acquire abnormalities in energy metabolism, collectively known as the Warburg effect, affecting substrate availability of thiamine-dependent enzymes. To investigate a strategy to exploit abnormal cancer-associated metabolism related to thiamine, we tested the cytotoxicity of native Bacillus thiaminolyticus thiaminase I enzyme, which digests thiamine, in the NCI60 cell line drug cytotoxicity screening program, and found that leukemia cell lines were among the most sensitive to thiaminase I. We obtained additional lymphoid leukemia cell lines and confirmed that native thiaminase I and linear chain polyethylene glycol-modified thiaminase I (LCPTE) have cytotoxic activity in these cell lines. In addition, the IC₅₀ of three of the five leukemia cell lines (Reh, RS4, and Jurkat) were at least 1000-fold more sensitive than Molt4 cells which, in turn, were among the most sensitive in the NCI60 panel. The three LCPTE-sensitive leukemia cell lines were also sensitive to removal of thiamine from the medium, thus suggesting the mechanism of action of LCPTE involves extracellular thiamine starvation. Surprisingly, rapamycin showed a protective effect against LCPTE toxicity in the three LCPTE-sensitive cell lines but not in the other two cell lines, suggesting involvement of an mTOR-dependent pathway. Immunoblot analysis of the LCPTE-sensitive cell lines after LCPTE exposure revealed changes in mTOR pathway phosphorylation. Nude mice bearing RS4 leukemia xenografts showed both tumor growth delay and prolonged survival after a single dose of LCPTE. Therefore, disruption of thiamine-dependent metabolism may be a novel therapeutic approach to target altered energy metabolism in leukemia and other cancers.
Introduction
The altered energy metabolism of cancer cells, known as the Warburg effect, involves increased and dysregulated glucose uptake. In this altered metabolism, glucose is shunted away from Kreb’s cycle entry via the thiamine-dependent pyruvate dehydrogenase (PDH) enzyme complex while still being required for biomass synthesis via another thiamine-dependent enzyme, transketolase (TK), in the pentose phosphate shunt. (1). Cells require two specific transport proteins, THTR1 and THTR2, for thiamine uptake. We have previously shown that RNA levels of THTR1 and THTR2 are downregulated in tumors compared to adjacent non-malignant tissue (2, 3). We have hypothesized that the downregulation of thiamine uptake may be a result of decreased PDH activity, however the increased need for TK-dependent biomass production may make tumor cells more sensitive to thiamine starvation.

To explore the therapeutic potential for disruption of thiamine-dependent enzymes, we developed methods to produce and purify recombinant thiaminase I, an enzyme cleaving thiamine into thiazole and pyrimidine catabolites. We originally tested native thiaminase I as an agent useful in breast cancer, and have demonstrated that thiaminase I is active against breast cancer cell lines and synergistic with doxorubicin and paclitaxel (4). The focus on leukemia as a target of thiamine-depleting strategy was prompted by results of the NCI60 screening panel results indicating that leukemia cell lines may be more sensitive to thiaminase I treatment than other cell lines. Upon further testing of other leukemia cell lines, we unexpectedly found that some lymphoblastic leukemia cell lines were extremely sensitive to thiaminase I.

Every new anticancer agent must demonstrate a therapeutic window, that is a dose having antitumor activity which can also be tolerated by normal tissues. While true of every cancer drug, this may be of greater immediate concern in the development of new antimetabolite anticancer agents as all cells in the body, both normal and malignant, have the same metabolic machinery. Nevertheless, antimetabolite therapy
has developed into an important component of the anticancer arsenal, especially in lymphoblastic leukemia where antifolate drugs such as methotrexate and asparaginase play important roles in antileukemia treatment regimens. The extreme sensitivity of several lymphoblastic cell lines to recombinant thiaminase I suggested that a therapeutic window might exist for this novel antimetabolite therapy in some malignancies.

Methods
Enzyme Production: Native recombinant thiaminase I was purified from bacterial culture using the *E. coli* BL21 (DE3) thiaminase I overexpressing strain provided to us by the Begley laboratory (5). The expression vector pET22b(+) is IPTG-inducible and has an N-terminal polyhistidine tag allowing detection and efficient purification of the expressed recombinant enzyme. After IPTG-induction, cells were collected and lysed, and the recombinant enzyme purified from the cytosol using a HisTrap FF column (GE). After elution, buffer exchange and concentration, LPS was removed by successive rounds of purification using ToxinEraser Endotoxin removal kit (GenScript) until the LPS concentration at the highest concentration of thiaminase I used in vitro (4 units/ml), measured with LAL endotoxin assay kit (GenScript), would equal the concentration of LPS in serum. Enzyme activity of the final native thiaminase I product was determined by a spectrophotometric assay based on a method developed by Lienhard and modified by Costello and colleagues (5). The assay is based on a change of absorbance at 252 nm resulting from the conjugation of pyrimidine catabolite with the nucleophile aniline.

PEGylation: Methoxy-poly(ethylene glycol) (MS-PEG, 1kDa, linear chain, Thermo Scientific) and Trimethyl succinimidyl-poly(ethylene glycol) (TMS-PEG, 2 kDa, branched chain, Thermo scientific) were used for PEGylation of thiaminase I. Both MS-PEG and TMS-PEG were activated with N-Hydroxysuccinimide (NHS) esters in order to modify primary amino groups on the enzyme. NHS-activated PEG reagents were dissolved in anhydrous dimethyl sulfoxide (DMSO) at 250mM while thiaminase I was dissolved in 10mM HEPES buffer at 10mg/ml. PEGylation reactions were
initiated by mixing these solutions at room temperature. The reactions were allowed to proceed for 3h, followed by repetitive ultrafiltration (molecular weight cut-off 10 kDa). Concentrated PEGylated enzymes were sterilized by 0.22 μm filtration and stored at -20°C until use.

LCPTE Physical Characterization: Dynamic light scattering (DLS) measurements were conducted to determine hydrodynamic diameters of native and PEGylated enzymes by using a particle size detector (Zetasizer Nano-ZS, Malvern). Samples (2 mg/ml) were put in disposable cuvettes and measurements were conducted with 173° backscattering settings.

Cytotoxicity assays: Leukemia cell lines were obtained from ATCC (Molt-4), or were generously provided by Dr. Terzah Horton, Baylor College of Medicine (Reh, RS4, Jurkat, JM1 and HSB2). Cell line authentication was performed after all studies had been completed by PCR amplification of 9 short tandem repeat (STR) loci (Research Animal Diagnostic Laboratory, St. Louis), and comparing the profile to the ATCC STR database. All cell lines were confirmed to be of human origin. The STR profile of cell lines RS4, JM1, MOLT4 and JSB2 were identical to the ATCC profile. The Reh cell line matched all alleles in the ATCC Reh profile plus one extra allele at 2 loci. The Jurkat cell line matched all ATCC Jurkat STR alleles except one, and had one extra allele at one loci. Cells were plated in triplicate in 96-well microtiter plates in RPMI-1640 (with 25mM HEPES) medium containing 10% fetal bovine serum at final densities between 3 to 8 x 10⁴ cells/well (optimal conditions for each cell line were determined by assay). Medium containing thiaminase I or LCPTE was added to cells and incubated for 4 days. Following incubation, an MTT Cell Proliferation Assay (ATCC) was performed according to the ATCC protocol (optimal conditions were initially determined for each cell line). The IC₅₀ was calculated from the dose response curve as the concentration of drug producing a 50% decrease in the mean absorbance compared to the untreated wells using Prism GraphPad software. The cytotoxicity experiments were repeated a minimum of three times in triplicate. For
synergy experiments, cells were plated at four concentrations of LCPTE representing the approximate IC20, IC40, IC60 and IC80 concentrations for each cell line, and then co-incubated in increasing concentrations of vincristine, doxorubicin, methotrexate and cytarabine. The experiments were repeated three times in triplicate and the results analyzed with CalcuSyn software. For thiamine starvation experiments, cells were plated in triplicate in 96-well microtiter plates in custom thiamine-free medium (RPMI-1640 with 25mM HEPES, Invitrogen) containing 10% fetal bovine serum at optimal cell densities for each cell line as previously determined. Thiamine-HCl was added at various concentrations for final concentrations of 0, 0.3, 3, 30 and 300μM (3μM is standard medium condition) in thiamine-HCl free medium and co-incubated with various concentrations of LCPTE. Final concentrations of LCPTE ranged from an IC20 to an IC80 for each cell line. After 4 days of incubation, an MTT Cell Proliferation Assay was performed according to ATCC protocol to determine cell growth. For combination studies with rapamycin, cells were treated by co-incubating with LCPTE and rapamycin over a range of concentrations. LCPTE concentrations ranged from an IC20 to an IC80 for each cell line. Rapamycin concentrations were 0.0001, 0.001, 0.1, 1, and 10μM. Cells were incubated for 4 days post-treatment, followed by determination of cell growth by the MTT Cell Proliferation Assay according to ATCC. The experiments were repeated 3-5 times in triplicate.

Thiamine concentration in tissue culture medium: LCPTE was added over a range of concentrations to RPMI-1640 medium containing 10% fetal bovine serum and incubated for 24 hr at 37°C. We optimized an assay for fluorescent detection of thiamine, in which thiamine is derivatized using potassium ferricyanide to thiochrome, which was detected by fluorescence (6). An AgilentC18, 5μm particle size 4.6X250mm column was used for reverse-phase chromatography. The mobile phase was dibasic sodium phosphate (25mmol/L, pH7.0): methanol 50:50, vol/vol; analytes were eluted at a flow rate of 1.00 ml/min. Injection volume was 5μl. Thiochrome was detected at an excitation wavelength of 375nm and emission
wavelength of 435nm. Peak area was measured, and a linear standard curve was constructed relating AUC to known thiamine concentrations.

Immunoblot analysis: Cells were treated with LCPTE at 8 x 10⁻⁵ units/ml for Reh and RS4 cells and 1 x 10⁻³ units/ml for Jurkat cells and/or rapamycin (0.001µM) for a time course of 0-48 hours or at a single time point of 96 hours. Cells were lysed with a triple-detergent lysis buffer (50mM Tris pH8.0, 150mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 0.02% sodium azide, 100µg/ml PMSF, protease inhibitors (Roche) and phosphatase inhibitors (Thermo Scientific)). Equal amounts of protein were loaded into each well and separated by SDS-PAGE gel, followed by transfer onto nitrocellulose membranes. The membranes were blocked, incubated with the indicated primary antibodies at 4°C overnight, and the appropriate horseradish peroxidase–conjugated secondary antibody was added for 1h at room temperature. Immunoblots were developed by use of SuperSignal West Pico chemiluminescent substrate (Thermo Scientific) according to the manufacturer’s protocol and analyzed by FujiFilm LAS-4000 luminescent image analyzer (Multigauge software). All of the primary antibodies were purchased from Cell Signaling Technologies (Danvers, MA). The secondary antibodies were purchased from Jackson ImmunoResearch Labs (West Grove, PA). An anti-β-actin antibody was used as a control for protein loading (Sigma, St. Louis, MO).

Animal Studies: All animal studies were approved by the University of Kentucky Institutional Animal Care and Use Committee. RS4 leukemia cells (1 x 10⁷) were injected subcutaneously into the flanks of 5-6 week old female Crl:NU-Foxn1 nude mice (Charles River Laboratories, Wilmington, MA). When palpable tumors had formed at day 26, a single dose of LCPTE therapy (50 units/kg, n=8) or saline (n=7) was injected intraperitoneally. The predetermined endpoint was a tumor volume of 1500 mm³.
Results

We submitted thiaminase I to the NCI60 Drug Screening program, where novel agents are screened against sixty cancer cell lines (7). The assay is a robotic 48 hour cytotoxicity assay. The results from the NCI60 program revealed that 3 of 5 leukemia cell lines (HL-60, MOLT-4, and RPMI-8226) were much more sensitive to thiaminase I than any breast cancer cell lines tested (Supplemental Figure 1). The X-axis in Supplemental Figure 1 refers to a serial dilution of a stock solution of native thiaminase I.

At the same time, we modified native thiaminase with a 1kDa linear chain PEG (LCPTE), as well as with a 2kDa branched chain PEG. Figure 1 shows that PEGylation of thiaminase I was successfully conducted by using both 1k (MW = 1214) linear chain and 2k (MW = 2420) branched chain PEG. Hydrodynamic diameter (5.615 nm) of the linear chain PEGylated thiaminase I enzyme (LCPTE) decreased slightly in comparison to that of native thiaminase (8.721 nm), indicating PEGylation suppresses intermolecular aggregation between enzymes (Figure 1A). There was not significant change in size between LCPTE and branched PEG thiaminase I while branched PEG migrated more slowly on the electrophoresis gel (Figure 1B). In comparison to native enzymes that underwent aggregation over time, both linear and branched PEGylated thiaminase I disperse homogeneously in aqueous solutions (data not shown). PEGylation increases the activity of the enzyme by 5-fold on a units/mg protein basis. Time course particle size measurements suggest that PEGylation effectively inhibits spontaneous enzyme aggregation.

We chose to pursue further characterization of the 1k PEGylated form, LCPTE. First, we confirmed the NCI60 results with native thiaminase I by examining Molt4 cells along with additional leukemia cell lines (Figure 2A). In comparison to Supplemental Figure 1, the X-axis refers directly to the concentration of thiaminase activity (units/ml) and the incubation period in our assay was for 4 days in comparison to the 2 day incubation of the NCI drug screen protocol. These studies revealed that Reh, RS4, and Jurkat leukemia cell lines were exquisitely sensitive to thiaminase as
compared to Molt4 cells, which were among the most sensitive cells in the NCI60 screen. These three lymphoid cell lines were the most sensitive of all cell lines tested. The IC₅₀’s of native thiaminase I were 1 x 10⁻⁵ units/ml in RS4 cells, 2 x 10⁻⁵ units/ml in Reh cells, and 8 x 10⁻⁵ units/ml for Jurkat cells, while the IC₅₀ was 0.3 units/ml for Molt4 cells. For reference, our previously reported range of IC₅₀ values for native thiaminase in six breast cancer cell lines ranged from 0.012 to 0.22 units/ml (8).

We tested two other leukemia cell lines, HSB2 and JM1. The IC₅₀ for native thiaminase for HSB2 was 1 unit/ml and the IC₅₀ for JM1 was 0.02 unit/ml. Of interest, all three sensitive cell lines were equally sensitive to both native and PEGylated thiaminase I (LCPTE) forms, but the more resistant cell lines were more resistant to LCPTE than native enzyme, with JM1, HSB2 and Molt4 cells not achieving an IC₅₀ level of cytotoxicity at doses up to 4 units/ml. The type of leukemia from which the cell line was derived did not appear to influence sensitivity, as sensitive RS4 and the more resistant JM1 cells are both B cell lines. The sensitive Jurkat and the more resistant Molt4 cells are of T cell origin and the non-T, non-B cell lines Reh and HSB2 also varied greatly in sensitivity to thiaminase.

We determined the concentration of LCPTE required to catabolize thiamine in the tissue culture medium (Figure 2B). As shown in Figure 2B, LCPTE decreased the thiamine concentration from the baseline of 3 uM to 0.03 uM at 2 x 10⁻⁴ units/ml after 24 hour incubation at 37°C. The concentration of LCPTE required to deplete thiamine in medium to a sub-micromolar level therefore corresponds to the range required for growth inhibition in the sensitive leukemia cell lines.

To determine whether the mechanism of action of native thaminase and LCPTE might be due to extracellular thiamine starvation, we incubated the cells in thiamine-free medium and compared growth relative to the concentration found in normal medium (3µM). As seen in Figure 3, the three LCPTE-sensitive cell lines showed a significant decrease in growth relative to normal medium, while the two more resistant cell lines showed no change in response to acute thiamine withdrawal.
We previously found that native thiaminase showed synergistic cytotoxicity in breast cancer cell lines in combination with doxorubicin and paclitaxel. We therefore examined leukemia cell lines to determine whether a similar synergy would be found between LCPTE and the chemotherapy agents commonly used in the treatment of leukemia. We studied the combined cytotoxicity of LCPTE against four drugs commonly used in the treatment of lymphoid leukemias; vincristine, methotrexate, doxorubicin and cytarabine. The results are shown in Supplemental Table 1. The table shows the median Combination Index (CI) as calculated by the software program Calcusyn over a range of dose combinations and fractional effects giving an overall picture of potential drug interaction. A CI close to 1 indicates drug additivity, while a CI significantly higher than 1 indicates antagonism and a CI significantly less than 1 indicates synergy. Overall, the most resistant cell lines, JM1 and Molt4, show additivity with the exception of the combination of doxorubicin and LCPTE in Molt4 which was consistently synergistic. In contrast, the cell lines most sensitive to LCPTE as a single agent, Reh and RS4, showed mild antagonism in the combination of LCPTE and the four chemotherapy drugs.

We hypothesized that the energy-sensing mTOR pathway might play a role in mediating the cytotoxic effect of LCPTE. To probe the role of the mTOR pathway we conducted cytotoxicity experiments combining rapamycin with LCPTE, similar to the experiments represented by Supplemental Table 1, expecting that rapamycin might show additive or synergistic cytotoxicity as it inhibits the pro-survival mTOR pathway. To our surprise, rapamycin showed dose-dependent protection against LCPTE toxicity in the three LCPTE-sensitive leukemia cell lines (Figure 4), which shows growth inhibition of cells incubated with LCPTE compared to growth in the same concentration of rapamycin alone. The Calcusyn calculated CI’s for some rapamycin-LCPTE dose combinations were greater than 100, indicating a very strong antagonism. Rapamycin alone produced modest cytotoxic activity at the concentrations tested. For Reh, RS4 and Jurkat cell lines rapamycin at 0.0001 uM produced IC’s of 0.95, 0.92 and 0.96, respectively; at 0.001 uM, IC’s of 0.81, 0.79 and
0.79, respectively; and at 0.1 μM, IC’s of 0.61, 0.65 and 0.68, respectively. Rapamycin did not affect LCPTE cytotoxicity in the two more resistant leukemia cell lines (data not shown).

We therefore explored mTOR pathway regulation in response to both LCPTE and rapamycin in the three LCPTE-sensitive leukemia cell lines, looking for common changes in these cell lines. Figure 5A shows mTOR, phosphorylated mTOR, the mTORC1 pathway target S6 kinase and the mTORC2 pathway target Akt. While there appears to be an increase in mTOR phosphorylation at S2481 in response to LCPTE in RS4 and Jurkat cell lines, this is not clearly observed in the Reh cell line. There are no clear changes in Akt or S6 kinase in response to LCPTE exposure. Figure 5B explores the mTOR downstream target 4E-BP1. There appears to be an increase in total and non-phosphorylated 4E-BP1 in all three cell lines in response to LCPTE. This effect is abrogated by rapamycin, a decrease in phosphorylation of 4E-BP1 at T70 comparing LCPTE alone to LCPTE plus rapamycin, and decreased phosphorylation of 4E-BP1 at the S65 site in all three cell lines that is reversed by rapamycin. The phosphorylation of 4E-BP1 at earlier time points in response to LCPTE, rapamycin or both in RS4 and Reh cells is shown in Figure 6A. Again, rapamycin appears to decrease early phosphorylation at T37/40 at 6 hrs and T70 at hours 12-48 in RS4 cells, and at S65 at 12 hours and T70 at hrs 6-24 in Reh cells.

4E-BP1 serves to sequester and inhibit the function of the transcription factor eIF4E and phosphorylation of 4E-BP1 releases eIF4E to form a complex initiating cap-dependent translation (9). Figure 6B examines eIF-4E expression, phosphorylation, as well as proteins indicating its cap-dependent translation activity. These studies show an inconsistent change in eIF-4E phosphorylation in response to LCPTE, with decreased phosphorylation in RS4 cells, an apparent increase in phosphorylation in Jurkat cells and no consistent effect on the translation of cMyc, HIF-1α or cyclin D1.
We also looked for common apoptotic pathway activation among the LCPTE-sensitive leukemia cell lines. Supplemental Figure 2 shows evidence of both caspase-3 and caspase-7 cleavage in the RS4 cell line which is prevented by rapamycin, but these caspases were not activated in the other two cell lines.

All cancer therapy must show selective toxicity toward tumor cells in comparison to normal cells. To address the potential therapeutic window for LCPTE, we established RS4 leukemia cell xenografts for an initial trial of a single dose of LCPTE therapy. We first established that the maximum tolerated dose (MTD) of LCPTE in nude mice is 50 units/kg when administered as a single intraperitoneal (IP) injection. The MTD of native thiaminase I is 300 units/kg IP daily for two weeks, so PEGylation appears to greatly enhance the plasma retention time and in vivo potency. The RS4 palpable subcutaneous flank tumors were treated with a single dose of LCPTE at 50 units/kg (n=8) or saline control (n=7) once by IP injection. Supplemental Figure 3 shows growth of the xenografts after implantation (Figure 3S-A) and survival analysis (Figure 3S-B). Three of eight mice in the treated groups showed complete tumor regression at day 90 post implantation (day 64 post treatment), while no regression occurred in the controls. An event was pre-defined as the tumor xenograft reaching a volume of 1500 mm³. The estimated median event-free survival at day 64 post treatment, calculated using GraphPad Prism software, was 31 days in the treated group vs 15 days in the control group (Log-rank Mantel-Cox, p=0.03).

Discussion

This paper explores the potential development of LCPTE as a novel agent for the treatment of leukemia. The impetus for the exploration of this agent in leukemia arose from initial results of the NCI60 drug screen, where leukemia cell lines appeared to be among the most sensitive to native thiaminase enzyme. We obtained other leukemia cell lines, and not only confirmed the NCI60 results with native thiaminase, but found three other cell lines, Reh, RS4 and Jurkat, which were over
1000-fold more sensitive to native thiaminase than Molt4 cells, among the most sensitive in the NCI60 panel.

Thiamine is transported into the cell by either of two thiamine transport proteins where it is quickly phosphorylated into its active cofactor forms (10). Thiamine phosphates play a central role in enzymes regulating how both energy and glucose carbon are utilized by the cell (11). It is well established that energy metabolism is altered in cancer cells and while other strategies focus on targeting the signaling pathways regulating energy metabolism, such as mTOR and Akt, we have embarked on a strategy to directly affect energy metabolism by disrupting thiamine-dependent enzymes. We hypothesized that cancer cells would be less capable of adapting to disruption of thiamine-dependent enzyme inhibition than normal cells. The ability of native thiaminase and LCPTE to inhibit growth of leukemia cell lines, at concentrations that are orders of magnitude less than the concentrations needed to inhibit growth of other cell lines (4, 8), indicates the potential of a therapeutic approach aimed at thiamine metabolism in leukemia. The very large differences in sensitivity to LCPTE in leukemia also suggest that a therapeutic window may exist in the treatment of malignancies sensitive to this intervention.

The three LCPTE-sensitive cell lines had qualities in common suggesting the mechanism of action of LCPTE in these cell lines is extracellular thiamine depletion. First, the three LCPTE-sensitive cell lines demonstrated equal sensitivity to native thiaminase and LCPTE, whereas the more resistant cell lines showed a greater difference in cytotoxicity between these forms of the enzyme. This finding suggests that intracellular uptake may be necessary for toxicity in the more resistant cell lines and not in the LCPTE-sensitive cell lines. Second, the three LCPTE-sensitive cell lines showed decreased growth upon thiamine withdrawal from medium, whereas the resistant cell lines showed no effect of thiamine withdrawal on growth (Figure 4). Third, the IC50’s of the three sensitive cell lines occur in the range of enzyme concentrations that deplete medium of thiamine (Figure 2B). Unlike the vitamin folate, which can be transformed into a storage form by polyglutamylation, thiamine
is not stored to a great degree in cells thus requiring constant replenishment. Therefore, some leukemia cells may have diminished thiamine reserves increasing susceptibility to thiamine starvation. These observations provide a basis for further exploration of the relation of intracellular thiamine depletion to LCPTE toxicity.

In addition, the three LCPTE-sensitive cell lines were protected by rapamycin against LCPTE-induced cytotoxicity. This was a most unusual finding, as rapamycin usually enhances cytotoxicity as it inhibits mTOR, a protein demonstrated to play a pro-survival role (12). Extensive immunoblot exploration of the mTOR pathway revealed some interaction between LCPTE exposure and phosphorylation of downstream mTOR pathway. However, a clear common pathway for mTOR-dependent cytotoxic response was not revealed and possibly may not be shared by these cell lines. Nevertheless, if mTOR activity is important for LCPTE antitumor activity, then the upregulation of mTOR in tumors vs normal tissues may provide one mechanism of selectivity of this therapeutic strategy.

We did not find evidence for synergy between LCPTE and other drugs used in leukemia therapy as we did with native thiaminase and doxorubicin and paclitaxel in breast cancer cell lines (8). It may be that the metabolic reprogramming in cancer cell lines can result from disruption of different pathways all leading to the same end of dysregulated glucose uptake and lactate production. Alternately, cancer cells may have different access to alternative metabolic pathways. Recently, Zhao et al found that the BCR-ABL induced metabolic reprogramming of a chronic myeloid leukemia cell line, mediated by induction of HIF-1α, was resistant to imatinib alone but responsive to imatinib when combined with the thiamine antagonist oxythiamine (13). This finding provides further evidence for the importance of thiamine-dependent pathways in the altered energy metabolism seen in cancer cells, known as the Warburg effect.
LCPTE appears to have prolonged plasma retention time in nude mice as compared with native thiaminase I. The maximum tolerated dose (MTD) of native thiaminase I in nude mice was 300 unit/kg per day daily for 14 days. In contrast, the MTD of LCPTE was 50 units/kg as a single dose. Repeat dosing within two weeks at the same dose was lethal, suggesting that the 1k PEGylated modification increased plasma retention time. LCPTE showed modest antitumor activity that has the potential to be enhanced by modifications that further increase plasma retention time, tumor uptake, or both.

Antifolates were first noted to have antitumor activity by clinical experimentation (14) and then by development of tolerable forms in animal models. Since then, the gold standard to show the potential of a therapeutic window is the demonstration of antitumor activity at a tolerable dose in xenograft models. LCPTE has initially met this standard by demonstrating antitumor activity against an RS4 xenograft after a single dose at the MTD. Clearly, LCPTE is a prototype of a modification of thiaminase I and may not be the ultimate form advanced into further pre-clinical development.

Additional studies will define the role of thiamine depletion, thiamine catabolite toxicity, and mTOR signaling in LCPTE cytotoxicity. Comparison of sensitive and resistant cell lines may also help to identify predictors of response that can guide the development and deployment of this novel agent. Finally, further chemical modification of the thiaminase enzyme, such as changes in the size of the conjugated PEG chain, will optimize this enzyme as a novel therapeutic agent targeting the altered energy metabolism in leukemia and other malignancies.
Acknowledgements

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References


**Figure legends**

**Figure 1.** A. Particle size distribution of native, 1k-linear chain PEGylated thiaminase I (LCPTE) and 2k branched chain PEGylated thiaminase I. B. Coomassie-stained SDS gel showing purified native thiaminase I, 1k-linear PEG thiaminase I (LCPTE) and 2k-branched chain PEG thiaminase I. Decreased and diffused SDS mobility is expected with PEG modification.

**Figure 2.** A. Dose-response curves of native (closed symbols) and linear chain PEGylated (LCPTE) (open symbols) recombinant thiaminase I enzyme against four lymphoid leukemia cell lines. The cells were plated in triplicate wells, incubated in increasing concentrations of thiaminase I for 96 hours, and then assayed with an MTT (tetrazolium)-based kit. The graphs show the average ± SD of two to four independent experiments. B. Thiamine concentration of RPMI-1640 medium with 10% fetal bovine serum after incubation for 24 hrs in increasing concentrations of LCPTE.

**Figure 3.** Growth of five leukemia cell lines in thiamine-free RPMI-1640 medium compared to standard RPMI-1640 medium. **p<.001, *p<.05.

**Figure 4.** LCPTE growth inhibition in Reh, RS4 and Jurkat leukemia cells at a fixed concentration of LCPTE and increasing concentrations of rapamycin as a percentage of growth in the same concentration of rapamycin alone. The graph shows the average ± SD of four determinations performed in triplicate.

**Figure 5.** A. Immunoblot analysis of mTOR pathway proteins in Reh, RS4 and Jurkat leukemia cells before and after treatment for 96 hours with LCPTE in the presence or absence of rapamycin (1 nM). B. Immunoblot analysis of 4E-BP1 pathway proteins in Reh, RS4 and Jurkat leukemia cells before and after treatment for 96 hours with LCPTE in the presence or absence of rapamycin (1 nM).

**Figure 6.** A. Immunoblot analysis of time course of 4E-BP1 phosphorylation in RS4 and Reh leukemia cell lines in the presence of 1k-LCPTE (LCPTE) (8 X 10^{-5} units/ml),
rapamycin (rap) (1 nM) or both. B. Immunoblot analysis of eIF-4E pathway proteins in Reh, RS4 and Jurkat leukemia cells before and after treatment for 96 hours with LCPTE in the presence or absence of rapamycin (1 nM).
Figure 1

A

Native Thiaminase 1
8.721 nm (39.1%)

Linear PEG Thiaminase 1
5.615 nm (38.9%)

Branched PEG Thiaminase 1
5.615 nm (40.7%)

B

Native thiaminase I
Linear PEG thiaminase I
Branched PEG thiaminase I
Marker

kd
- 175
- 83
- 47.5
- 32.5
- 25
Figure 2

A

Growth inhibition (percent of control)

Native thiaminase or LCPTE (units/ml)

B

Thiamine (μM)

LCPTE (units/ml)

- Jurkat-Native
- Molt4-Native
- REH-Native
- RS4;11-Native
- Jurkat-LCPTE
- Molt4-LCPTE
- REH-LCPTE
- RS4;11-LCPTE
Figure 3

Growth Inhibition (percent of control)

Cell line

- Ren
- RS4
- Jurkat
- JM1
- Molt4

* p < 0.05
** p < 0.01

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Figure 4

Growth inhibition (percent of control)

Rapamycin (µM)

- 0
- 0.0001
- 0.001
- 0.1

Cell Line (LCTPE concentration)

- Reh (8 x 10^-5 u/ml)
- RS4 (8 x 10^-5 u/ml)
- Jurkat (1 x 10^-3 u/ml)
Figure 5

A

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- p-mTor (S2481)
- b-actin
- p-p70 S6 Kinase (Thr389)
- p-p70 S6 Kinase (Thr421/Ser424)
- Akt
- p-Akt (S473)
- b-actin

B

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- 4E-BP1
- p-4E-BP1 (S65)
- p-4E-BP1 (T70)
- p-4E-BP1 (Thr37/46)
- NonP-4E-BP1 (T46)
- 4E-BP2
- b-actin
Figure 6

**A**

- RS4:
  - 4E-B1
    - LCPTE
    - LCPTE + Rap
  - p-4E-B1 (T37/46)
    - LCPTE
    - Rap
    - LCPTE + Rap
  - p-4E-B1 (S65)
    - LCPTE
    - Rap
    - LCPTE + Rap
  - p-4E-B1 (T70)
    - LCPTE
    - Rap
    - LCPTE + Rap
  - non-P 4E-B1
    - LCPTE
    - Rap
    - LCPTE + Rap

**B**

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Molecular Cancer Therapeutics

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Abigail Daily, Shuqian Liu, Younsoo Bae, et al.

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