Therapeutic Discovery

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 PEGylated thiaminase I enzyme (LCPTE) have cytotoxic activity in these cell lines. In addition, the IC₅₀ of 3 of the 5 leukemia cell lines (Reh, RS4, and Jurkat) were at least 1,000-fold more sensitive than Molt-4 cells, which in turn, were among the most sensitive in the NCI60 panel. The 3 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 3 LCPTE-sensitive cell lines but not in the other 2 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. *Mol Cancer Ther*, 10(9); 1563–70. ©2011 AACR.

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 enzyme complex, while still being required for biomass synthesis via another thiamine-dependent enzyme, transketolase, in the pentose phosphate shunt (1). Cells require 2 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 with adjacent nonmalignant tissue (2, 3). We have hypothesized that the downregulation of thiamine uptake may be a result of decreased pyruvate dehydrogenase activity; however, the increased need for transketolase-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 shown 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 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 show a therapeutic window that is a dose having antitumor activity that can also be tolerated by normal tissues. Although 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 in which 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.

Materials and Methods

Enzyme production

Native recombinant thiaminase I was purified from bacterial culture by 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 was purified from the cytosol by using a HisTrap FF column (GE Healthcare). After elution, buffer exchange and concentration, lipopolysaccharide (LPS) was removed by successive rounds of purification by 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 the 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 on the basis of 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, 1 kDa, 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 to modify primary amino groups on the enzyme. NHS-activated PEG reagents were dissolved in anhydrous dimethyl sulfoxide at 250 mmol/L, whereas thiaminase I was dissolved in 10 mmol/L HEPES buffer at 10 mg/mL. PEGylation reactions were initiated by mixing these solutions at room temperature. The reactions were allowed to proceed for 3 hours, 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.

Linear chain PEGylated thiaminase I enzyme physical characterization

Dynamic light scattering 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 degree backscattering settings.

Cytotoxicity assays

Leukemia cell lines were obtained from the American Type Culture Collection (ATCC; Molt-4) or were generously provided by Dr. Terzah Horton, Baylor College of Medicine (Reh, RS4, Jurkat, JM1, and HS2). Cell line authentication was done after all studies had been completed by PCR amplification of 9 short tandem repeat (STR) loci (Research Animal Diagnostic Laboratory) and by 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, Molt-4, 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 loci 2. 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 25 mmol/L HEPES) medium containing 10% FBS at final densities between 3 × 10^4 to 8 × 10^4 cells per well (optimal conditions for each cell line were determined by assay). Medium containing thiaminase I or linear chain PEGylated thiaminase I enzyme (LCPTE) was added to cells and incubated for 4 days. Following incubation, an MTT Cell Proliferation Assay (ATCC) was done according to the ATCC protocol (optimal conditions were initially determined for each cell line). The IC_{50} was calculated from the dose–response curve as the concentration of drug producing a 50% decrease in the mean absorbance compared with the untreated wells by using Prism GraphPad software. The cytotoxicity experiments were repeated a minimum of 3 times in triplicate. For synergy experiments, cells were plated at 4 concentrations of LCPTE representing the approximate IC_{20}, IC_{40}, IC_{60}, and IC_{80} concentrations for each cell line and then coincubated in increasing concentrations of vincristine, doxorubicin, methotrexate, and cytarabine. The experiments were repeated 3 times in triplicate and the results analyzed with Calcsyn software. For thiamine starvation experiments, cells were plated in triplicate in 96-well microtiter plates in custom thiamine-free medium (RPMI-1640 with 25 mmol/L HEPES; Invitrogen) containing 10% FBS 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 300 μmol/L (3 μmol/L is standard medium concentration) in thiamine–HCl-free medium and coincubated with various concentrations of LCPTE. Final concentrations of LCPTE ranged from an IC_{20} to an IC_{80} for each cell line. After 4 days of incubation, an MTT Cell Proliferation Assay was done according to ATCC protocol to determine cell growth. For combination studies with rapamycin, cells were treated by coincubating with LCPTE and rapamycin over a range of concentrations. LCPTE concentrations ranged from an IC_{20} to an IC_{80} for...
Thiaminase concentration in tissue culture medium

LCPTe was added over a range of concentrations to RPMI-1640 medium containing 10% FBS and incubated for 24 hours 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 Agilent C18, 5-μm particle size, 4.6 × 250 mm column was used for reverse-phase chromatography. The mobile phase was dibasic sodium phosphate (25 mmol/L, pH 7.0): methanol 50:50, vol/vol; analytes were eluted at a flow rate of 1.00 mL/min. Injection volume was 5 μL. Thiochrome was detected at an excitation wavelength of 375 nm and emission wavelength of 435 nm. Peak area was measured, and a linear standard curve was constructed relating area under curve to known thiamine concentrations.

Immunoblot analysis

Cells were treated with LCPTe at 8 × 10⁻⁵ units/mL for Reh and RS4 cells and 1 × 10⁻⁵ units/mL for Jurkat cells and/or rapamycin (0.001 μmol/L) for a time course of 0 to 48 hours or at a single time point of 96 hours. Cells were lysed with a triple detergent lysis buffer [50 mmol/L Tris pH 8.0, 150 mmol/L NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 0.02% sodium azide, 100 μg/mL phenylmethylsulfonyl fluoride, 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 1 hour 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. The secondary antibodies were purchased from Jackson ImmunoResearch Labs. An anti-β-actin antibody was used as a control for protein loading (Sigma).

Animal studies

All animal studies were approved by the University of Kentucky Institutional Animal Care and Use Committee. RS4 leukemia cells (1 × 10⁷) were injected s.c. into the flanks of 5- to 6-week-old female Crl:NU-Foxn1 nude mice (Charles River Laboratories). 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 i.p. The predetermined endpoint was a tumor volume of 1,500 mm³.

Results

We submitted thiaminase I to the NCI60 Drug Screening Program, in which novel agents are screened against 60 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 (Supplementary Fig. S1). The X-axis in Supplementary Fig. S1 refers to a serial dilution of a stock solution of native thiaminase I.

At the same time, we modified native thiaminase with a 1-kDa linear chain PEG (LCPTe), as well as with a 2-kDa branched chain PEG. Figure 1 shows that PEGylation of thiaminase I was successfully conducted by using both 1-kDa (MW = 1,214) linear chain and 2-kDa (MW = 2,420) branched chain PEG. Hydrodynamic diameter (5.615 nm) of the LCPTe decreased slightly in comparison with that of native thiaminase (8.721 nm), indicating PEGylation suppresses intermolecular aggregation between enzymes (Fig. 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 (Fig. 1B). In comparison with native enzymes that underwent aggregation over time, both linear and branched PEGylated thiaminase I dispersed 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 1-kDa PEGylated form, LCPTe. First, we confirmed the NCI60 results with native thiaminase I by examining Molt-4 cells along with additional leukemia cell lines (Fig. 2A). In comparison with Supplementary Fig. S1, 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 with 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 with Molt-4 cells, which were among the most sensitive cells in the NCI60 screen. These 3 lymphoid cell lines were the most sensitive of all cell lines tested. The IC₅₀ values of native thiaminase I were 1 × 10⁻⁵ units/mL in RS4 cells, 2 × 10⁻⁶ units/mL in Reh cells, and 8 × 10⁻⁷ units/mL for Jurkat cells, whereas the IC₅₀ was 0.3 units/mL for Molt-4 cells. For reference, our previously reported range of IC₅₀ values for native thiaminase in 6 breast cancer cell lines ranged from 0.012 to 0.22 units/mL (4).

We tested 2 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 3 sensitive cell lines were equally sensitive to both native and LCPTE forms, but the more resistant cell lines were more resistant to LCPTE than native enzyme, with JM1, HSB2, and Molt-4 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 seem to influence sensitivity, as sensitive RS4 and the more resistant JM1 cells are both B cell lines. The sensitive Jurkat and the more resistant Molt-4 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 (Fig. 2B). As shown in Fig. 2B, LCPTE decreased the thiamine concentration from the baseline of 3 to 0.03 µmol/L at 2 × 10⁻⁴ units/mL after 24-hour incubation at 37°C. The concentration of LCPTE required to deplete thiamine in medium to a submicromolar 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 thiaminase 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 (3 µmol/L). As seen in Fig. 3, the 3 LCPTE-sensitive cell lines showed a significant decrease in growth relative to normal medium, although the 2 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 4 drugs commonly used in the treatment of lymphoid leukemias, vincristine, methotrexate, doxorubicin, and cytarabine. The results are shown in Supplementary Table S1. 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, whereas 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 Molt-4, showed additivity with the exception of the combination of doxorubicin and LCPTE in Molt-4, 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 4 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 Supplementary Table S1, expecting that rapamycin might show additive or synergistic cytotoxicity as it inhibits the prosurvival mTOR pathway. To our surprise, rapamycin showed dose-dependent protection against LCPTE toxicity in the 3 LCPTE-sensitive leukemia cell lines (Fig. 4), which shows growth inhibition of cells incubated with LCPTE compared with growth in the same concentration of rapamycin alone. The Calcusyn calculated CIs 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 µmol/L produced CIs of 0.95, 0.92, and 0.96, respectively; at 0.001 µmol/L, CIs of 0.81, 0.79, and 0.79, respectively; and at 0.1 µmol/L, CIs of 0.61, 0.65, and 0.68, respectively. Rapamycin did not affect LCPTE cytotoxicity in the 2 more resistant leukemia cell lines (data not shown).

We therefore explored mTOR pathway regulation in response to both LCPTE and rapamycin in the 3 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.
Although there seems 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 seems to be an increase in total and nonphosphorylated 4E-BP1 in all 3 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 3 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 Fig. 6A. Again, rapamycin seems to decrease early phosphorylation at T37/40 at 6 hours and T70 at hours 12 to 48 in RS4 cells, and at S65 at 12 hours and T70 at hours 6 to 24 in Reh cells.

4E-BP1 serves to sequester and inhibit the function of the transcription factor eIF-4E and phosphorylation of 4E-BP1 releases eIF-4E to form a complex initiating cap-dependent translation (8). 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, hypoxia-inducible factor 1α (HIF-1α), or cyclin D1.

We also looked for common apoptotic pathway activation among the LCPTE-sensitive leukemia cell lines. Supplementary Figure S2 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 2 cell lines.

All cancer therapy must show selective toxicity toward tumor cells in comparison with 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 i.p. injection. The MTD of native thiaminase I is 300 units/kg i.p. daily for 2 weeks, so PEGylation seems to greatly enhance the plasma retention time and in vivo potency. The RS4 palpable s.c. flank tumors were treated with a single dose of LCPTE at 50 units/kg (n = 8) or saline control (n = 7) once by i.p. injection. Supplementary Figure S3 shows growth of the xenografts after implantation (Fig. S3A) and survival analysis (Fig. S3B). Three of 8 mice in the treated groups showed complete tumor regression at day 90 postimplantation (day 64 posttreatment), whereas no regression occurred in the controls. An event was predefined as the tumor xenograft reaching a volume of 1,500 mm$^3$. The estimated median event-free survival at day 64 posttreatment, calculated by using GraphPad Prism software, was 31 days in the treated group versus 15 days in the control group (Log-rank Mantel-Cox, P = 0.03).

Discussion

This article 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, in which leukemia cell lines seemed 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 also found 3 other cell lines, Reh, RS4, and Jurkat, which were over 1,000-fold...
more sensitive to native thiaminase than Molt-4 cells, among the most sensitive in the NCI60 panel.

Thiamine is transported into the cell by either of 2 thiamine transport proteins, where it is quickly phosphorylated into its active cofactor forms (9). Thiamine phosphates play a central role in enzymes regulating how both energy and glucose carbon are used by the cell (10). It is well established that energy metabolism is altered in cancer cells, and although 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), 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 3 LCPTE-sensitive cell lines had qualities in common, suggesting that mechanism of action of LCPTE in these cell lines is extracellular thiamine depletion. First, the 3 LCPTE-sensitive cell lines showed 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 3 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 (Fig. 4). Third, the IC<sub>50</sub> values of the 3 sensitive cell lines occur in the range of enzyme concentrations that deplete medium of thiamine (Fig. 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 3 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 shown to play a prosurvival role (11). 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 versus 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 (4). 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 and colleagues 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 (12). 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 seems to have prolonged plasma retention time in nude mice as compared with native thiaminase I. The 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 2 weeks at the same dose was lethal, suggesting that the 1-kDa 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 (13) 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 showing 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 preclinical 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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

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