Pharmacologic Properties of Polyethylene Glycol-Modified 
Bacillus thiaminolyticus Thiaminase I Enzyme

Shuqian Liu, Younsoo Bae, Markos Leggas, Abigail Daily, Saloni Bhatnagar, Sumitra Miriyala, Daret K. St. Clair, and Jeffrey A. Moscow

Department of Pediatrics (S.L., A.D., S.B., J.A.M.) and Graduate Center for Toxicology (S.M., D.K.S.C.), College of Medicine, and Department of Pharmaceutical Sciences, College of Pharmacy (Y.B., M.L.), University of Kentucky, Lexington, Kentucky

Received January 20, 2012; accepted March 16, 2012

ABSTRACT

We have previously shown that the bacterial enzyme thiaminase 1 has antitumor activity. In an attempt to make thiaminase 1 a more effective pharmaceutical agent, we have modified it by adding polyethylene glycol (PEG) chains of various lengths. We were surprised to find that 5k-PEGylation eliminated thiaminase cytotoxic activity in all cell lines tested. Both native thiaminase and 5k-PEGylated thiaminase efficiently depleted thiamine from cell culture medium, and both could use intracellular phosphorylated thiamine as substrates. However, native enzyme more effectively depleted thiamine and thiamine diphosphate in RS4 leukemia cell cytosol, and native thiaminase depressed cellular respiration, whereas PEGylated thiaminase did not. Despite the lack of in vitro cytotoxicity, PEGylation markedly increased the in vivo toxicity of the enzyme. Pharmacokinetic studies revealed that the half-life of native thiaminase was 1.5 h compared with 34.4 h for the 5k-PEGylated enzyme. Serum thiamine levels were depleted by both native and 5k-PEGylated enzyme. Despite superior pharmacokinetics, 5k-PEGylated thiaminase showed no antitumor effect against an RS4 leukemia xenograft, in contrast to native thiaminase, which showed antitumor activity. PEGylation of thiaminase I has demonstrated that depression of mitochondrial function contributes, at least in part, to its anticancer activity. PEGylation also enhances plasma retention time, which increased its vivo toxicity and decreased its activity against a leukemia xenograft, the opposite of the desired effects. These studies suggest that the mechanism of anticancer cytotoxicity of thiaminase requires acute depression of cellular respiration, whereas systemic toxicity is related to the duration of extracellular thiamine depletion.

Introduction

Thiamine is a vitamin cofactor in enzyme complexes that play key roles in central carbon metabolism (Richardson and Moscow, 2010). Extracellular thiamine is transported into the cell by two specific transporters and is phosphorylated into its biologically active forms. Thiamine pyrophosphate, the diphosphate form, is the cofactor form of thiamine and is required for pyruvate dehydrogenase complex, which facilitates the catalysis of glucose-derived pyruvate and formation of acetyl CoA. The latter enters the tricarboxylic acid cycle for the efficient extraction of energy from its chemical structure. In addition, thiamine pyrophosphate is a cofactor for transketolase, another key step in a different pathway that extracts the carbon from glucose for production of biomass. Other intracellular forms of phosphorylated thiamine include thiamine monophosphate, thiamine triphosphate, and adenosine thiamine triphosphate (Gangolf et al., 2010). Gangolf et al. (2010) have recently shown wide variation in intracellular concentrations of thiamine and phosphorylated thiamine pools in various tissues.

The enzyme thiaminase (42 kDa) catabolizes thiamine into two molecules: one is a pyrimidine conjugated to a nucleophile (for type 1 thiaminases) and the other is a thiazole that may be phosphorylated, depending on the thiamine substrate that enters the reaction. Several forms of thiaminase exist in nature, including plant, animal, and bacterial forms of the enzyme. Because thiamine is an essential vitamin and because thiamine in excess is not known to be toxic, the physiologic role of this enzyme is not known (Costello et al., 1996).

The dysregulation of central carbon metabolism in cancer has been the target of many different anticancer strategies...
(Vander Heiden et al., 2009). We have focused on the disruption of thiamine metabolism in cancer, a strategy that was derived from initial observations that tumors down-regulate thiamine uptake transporters (Liu et al., 2003, 2004). We have found that recombinant thiaminase enzyme is cytotoxic in breast cancer cells (Liu et al., 2010) and a chemically modified 1k-polyethylene glycol (PEG)ylated form of thiaminase has gain inhibitory effects at very low concentrations in leukemia cell lines (Daily et al., 2011).

These observations led to attempts to optimize thiaminase enzyme with a series of modifications in the hope of improving its “drugability.” We studied linear chain, polyethylene glycol-modified 1k, 5k, and 10k forms of thiaminase with most of the focus on the 5k-PEGylated form with respect to cytotoxic potency, pharmacokinetics, and pharmacodynamics. We intended to arrive at a formulation that would have superior tolerability and efficacy that could be taken forward as a lead agent for further preclinical development. In addition, by studying different modifications, we hoped to gain a better understanding of the mechanism of action of the drug. As expected, PEGylation improved that plasma retention time, with the 5k-PEGylated form seeming to be superior to 1k- and 10k-PEG modifications. We were surprised to find, however, that PEGylation with the longer chains abrogated in vitro cytotoxic activity of thiaminase was less tolerable and eliminated thiaminase activity against an RS4 leukemia xenograft model. In short, 5k-PEG modification of thiaminase had the exact opposite of the desired effect, but did result in insight into the mechanism of the antitumor action of thiaminase enzyme.

Materials and Methods

Enzyme Production. Native recombinant thiaminase I was purified from bacterial culture by using the Escherichia coli BL21 (DE3) thiaminase I overexpressing strain provided to us by the Begley laboratory (Cornell University, Ithaca, NY) (Costello et al., 1996) and purified as described previously (Liu et al., 2010).

Enzyme Activity Assays. Two methods were used to determine enzyme activity. One assay, using aniline as a nucleophile, was used to measure enzyme activity of purified products (Costello et al., 1996). We found that this method was not suitable for pharmacokinetic studies, because hemoglobin in serum interfered with detection. Therefore, a different method, using 2-nitrothiophenol as a nucleophile, was used for enzyme detection in pharmacokinetic studies (Hanes et al., 2007). For determination of substrate utilization, thiamine monophosphate and thiamine diphosphate were substituted for thiamine at the same concentrations.

Fluorescent Microscopy. MDA231 cells were seeded onto 15 mm diameter glass No. 1 coverslips (Thermo Fisher Scientific, Waltham, MA), inside a 24-well tissue culture petri dish, at a density of ~5 × 10^4 cells/well. The following day, cells were treated with Alexa Fluor 568 labeled native thiaminase for 24 h. Then the media was aspirated and exchanged for pre-warmed, fresh media containing 100 nM of LysoTracker Green DND-26 (Invitrogen, Carlsbad, CA). After half an hour of incubation with LysoTracker, cells were rinsed with PBS, pH 7.4 three times. Coverslips were mounted onto slides with PBS, pH 7.4. Cells were imaged on a Nikon Eclipse Ti microscope (Nikon Instruments Inc., Melville, NY). The excitation and detection conditions for LysoTracker and Alexa Fluor568 being adjusted to the channels for GFP and Cy3.

Immunoblot of Thiaminase Uptake. Cells were treated with 0.2 units/mL of native thiaminase for 24 h. Cells were lysed with a triple-detergent lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 0.02% sodium azide and protease inhibitors). Equal amounts of protein were loaded into each well and separated by 10% SDS-PAGE gel, followed by transfer onto nitrocellulose membranes. The membranes were blocked, incubated with anti-thiaminase antibody at room temperature for 1 h, and the appropriate horseradish peroxidase-conjugated secondary antibody was added for 1 h at room temperature. Immunoblots were developed by use of the enhanced chemiluminescence (ECL) detection system (Thermo Fisher Scientific) according to the manufacturer’s protocol and autoradiography. Anti-thiaminase antibody was custom made by Proteintech Group Inc. (Chicago, IL). The secondary antibody was purchased from Sigma-Aldrich (St. Louis, MO).

PEGylation. Linear chain methoxy-PEG (1 kDa, Thermo Fisher Scientific; and 5 and 10 kDa, NanoCS, New York, NY) was used for PEGylation of thiaminase I. All PEG chains were activated with N-hydroxysuccinimide esters to modify primary amino groups on the enzyme. N-hydroxysuccinimide-activated PEG reagents were dissolved in anhydrous dimethyl sulfoxide at 250 mM, whereas thiaminase I was dissolved in 10 mM 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 h, followed by repetitive ultrafiltration using 10-kDa molecular mass cut-off membranes to remove dimethyl sulfoxide. Concentrated PEGylated enzymes were purified by fast protein liquid chromatography [Bio-Rad Biologic equipped with Superose 12 10/300 GL column (Bio-Rad Laboratories, Hercules, CA); 1× phosphate-buffered saline]. Twenty linear chain PEGylated thiaminase enzyme (LCPTE) with high purity was isolated by fraction collection. Buffer solutions were subsequently replaced with deionized water. Purified LCPTE was sterilized with 0.22-μm membrane filtration and freeze-dried for storage at −20°C.

LCPTE 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, Worcestershire, UK). Samples (2 mg/ml) were placed in disposable cuvettes, and measurements were conducted with 173° backscattering settings. Particle size was measured in triplicate.

Cytotoxicity Assays. The human breast cancer cell lines MCF7 and MDA-231, human prostate cancer cell line PC-3, human lung cancer cell line H460, human glioma cell line LN18, and leukemia cell line Molt-4 were obtained from the American Type Culture Collection (ATCC; Manassas, VA). The human ovarian cancer cell line IGROV1 was obtained from the National Cancer Institute (Bethesda, MD). The leukemia cell lines Reh, RS4, and Jurkat were generously provided by Dr. Terzah Horton (Baylor College of Medicine, Houston, TX). Cell line authentication was performed after all studies had been completed by polymerase chain reaction amplification of nine short tandem repeat (STR) loci (Research Animal Diagnostic Laboratory, St. Louis, MO), and the profile was compared with the profile of the MCF-7, MDA-231, PC-3, H460, LN-18, Reh, and Jurkat cell lines was identical to the ATCC profile. The Reh cell line matched all alleles in the ATCC Reh profile plus one extra allele at two loci. Cells were plated in triplicate in 96-well microtiter plates in RPMI 1640 (with 25 mM HEPES) medium containing 10% fetal bovine serum at final densities between 3 × 10^4 cells/well (optimal conditions for each cell line were determined by assay). Medium containing native thiaminase or 5k-PEGylated thiaminase at a concentration range of 1 × 10^−6 to 4 units/ml was added to cells and incubated for 4 days. After 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 with the untreated wells by using Prism software (GraphPad Software Inc., San Diego, CA). The cytotoxicity experiments were repeated a minimum of three times in triplicate.
Thiamine Assay. For quantitative determination of thiamine, we implemented a previously published HPLC/fluorescence assay for indirect detection of thiamine, by derivatizing thiamine to thiochrome by using potassium ferricyanide, with slight modifications (Lu and Frank, 2008). An Agilent C18, 5-μm particle size, 4.6 × 250-mm column (Agilent Technologies, Santa Clara, CA) was used for reversed-phase chromatography. An isocratic elution was used for medium and serum, and the mobile phase consisted of dibasic sodium phosphate (25 mM, pH 7.0) and methanol, 50:50(v/v) (1 ml/min). The injection volume was 5 μl. Thiochrome was detected at an excitation wavelength of 375 nm and emission wavelength of 435 nm. Quantitative relationships were determined based on peak area. The limit of quantitation was 0.3 nM. For determination of intracellular thiamine catabolism, RS4 cells were incubated with native thiaminase and 5k-PEGylated thiaminase at a concentration of 1 unit/ml and harvested at 24 and 48 h. The cells were lysed in 100 μl of triple detergent lysis buffer, protein was precipitated by 200 μl of 10% trichloroacetic acid, and supernatant was washed two times with 750 μl of water-saturated methyl-ter-butyl ether to remove thiochrome, followed by derivatizing thiamine and its phosphate esters by using potassium ferricyanide. A previously described HPLC column was used (Lu et al., 2008). Mobile phase A was 25 mM dibasic sodium phosphate, pH 7.0/methanol (90:10 v/v), and mobile phase B was 25 mM dibasic sodium phosphate, pH 7.0/methanol (30:70 v/v). Gradient steps were programmed as follows: 10 to 15% B in 1 min, 30% B in 2 min, ramped to 50% B in 5 min, held at 50% B for 4 min, returned to initial conditions for 2 min, and equilibrated for 5 min. Injection volume and detection wavelengths were the same as described above. Protein concentrations were determined by the DC Protein Assay (Bio-Rad Laboratories) and read at 750 nm. The results were adjusted for protein concentration and normalized to unincubated control specimen (pmol/mg protein). The experiment was repeated twice in duplicate.

Pharmacokinetic Studies. All animal studies were approved by the University of Kentucky Institutional Animal Care and Use Committee. Pilot pharmacokinetic studies were performed with native thiaminase and thiaminase enzyme conjugated with 1k, 5k, and 10k linear chain PEG. Using pilot data to guide time point selection, pharmacokinetic studies were performed with native thiaminase and 5k-PEGylated thiaminase. Four animals were used for each time point. The pharmacokinetics of thiaminase activity (units/ml) in plasma were modeled by using WinNonlin version 5.3 (Pharsight, Mountain View, CA). Native thiaminase was modeled by using a linear chain PEG. Using pilot data to guide time point selection, quantitative relationships were determined based on peak area. The limit of quantitation was 0.3 nM. For determination of intracellular thiamine catabolism, RS4 cells were incubated with native thiaminase and 5k-PEGylated thiaminase at a concentration of 1 unit/ml and harvested at 24 and 48 h. The cells were lysed in 100 μl of triple detergent lysis buffer, protein was precipitated by 200 μl of 10% trichloroacetic acid, and supernatant was washed two times with 750 μl of water-saturated methyl-ter-butyl ether to remove thiochrome, followed by derivatizing thiamine and its phosphate esters by using potassium ferricyanide. A previously described HPLC column was used (Lu et al., 2008). Mobile phase A was 25 mM dibasic sodium phosphate, pH 7.0/methanol (90:10 v/v), and mobile phase B was 25 mM dibasic sodium phosphate, pH 7.0/methanol (30:70 v/v). Gradient steps were programmed as follows: 10 to 15% B in 1 min, 30% B in 2 min, ramped to 50% B in 5 min, held at 50% B for 4 min, returned to initial conditions for 2 min, and equilibrated for 5 min. Injection volume and detection wavelengths were the same as described above. Protein concentrations were determined by the DC Protein Assay (Bio-Rad Laboratories) and read at 750 nm. The results were adjusted for protein concentration and normalized to unincubated control specimen (pmol/mg protein). The experiment was repeated twice in duplicate.

Immunohistochemistry. Mice were euthanized by exposure to CO2, and tissues were harvested at the end of the pharmacokinetic study observation periods (24 h for native thiaminase and 168 h for 5k-PEGylated thiaminase). Tissues were fixed in 10% formalin. Tissue sections were mounted on glass slides and stained in hematoxylin solution 3 (Thermo Fisher Scientific) followed by 1% Eosin Y solution (Thermo Fisher Scientific), then washed with ethanol and mounted. For immunohistochemical detection of thiaminase, the tissue sections were blocked in a solution of telostein gelatin and Triton X, followed by 20 min in normal blocking solution, then incubation with the primary antibody for 1 h and the secondary biotinylated antibody for 20 min. The slides were developed with avidin-biotinylated complex reagent, washed, deparaffinized, and mounted.

Mitochondrial Bioenergetics Measurements. Real-time basal oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) for RS4 cells with and without native thiaminase and PEGylated thiaminase were determined by using the Seahorse Extracellular Flux (XF-96) analyzer (Seahorse Bioscience, Chiopecoe, MA). The XF-96 measures the concentration of oxygen and free protons in the medium above a monolayer of cells in real time. Thus the rates of oxygen consumption and proton production can be measured across several samples at a time. To allow comparison between experiments, data are expressed as OCR in pmol/min/104 cells or ECAR in mpH/min/104 cells. RS4 leukemia cells were seeded at 125,000 cells/well into gelatin-coated Seahorse Bioscience XF microplates, cultured in the presence or absence of 2 g/liter β-glucose, and then centrifuged to adhere to the bottom of the wells before OCR and ECAR measurements.

Xenograft Studies. All animal studies were approved by the University of Kentucky Institutional Animal Care and Use Committee. RS4 leukemia cells (1 × 107) were injected subcutaneously into the flanks of 5- to 6-week-old female Crl:NU-Foxn1 nude mice (Charles River Laboratories, Inc., Wilmington, MA). When palpable tumors had formed, mice were treated with native thiaminase (850 units/kg s.c.) twice weekly for 4 weeks (n = 10) or a single dose of 5k-PEG thiaminase at its maximum tolerated dose (MTD) (5 units/kg) (n = 10) at a site distant from the formed tumor. The predetermined endpoints were a tumor volume of 1500 mm³ or death. All of the events in the control and 5k-PEG thiaminase cohorts were tumor progression. In the native thiaminase cohort, one of the events was unexplained death. The control group of mice was injected with RS4 cells and left untreated, and results were combined with a previous control group of the same xenograft (Daily et al., 2011). Kaplan-Meier plots were generated to determine survival curves of the groups. The differences in survival were tested using the log-rank test. A p value < 0.05 was considered statistically significant. All conclusions are based on statistical analysis of the data. 

Fig. 1. Coomassie-stained SDS gel showing PEGylation of thiaminase 1 was completed by using 1k-, 5k-, and 10k-LCPTE. Decreased and diffused SDS mobility is expected with polyethylene glycol modification.
lysate. Fig. 3B demonstrates immunoreactive thiaminase in cellular mechanism of cell uptake is endocytosis. An immunoblot in take and co-localizes with lysozomes, indicating that the fluorescence-labeled native thiaminase achieves cellular up- thiaminase required a concentration of 0.5 units/ml.

than 1 nM at a concentration of 0.02 units/ml, whereas 5k- further depletion, decreasing thiamine concentrations to less was more efficient at the lower thiamine concentrations in thiamine from 3
Both forms of the enzyme were equally effective at reducing thiamine in cell culture medium is shown in Fig. 2. Medium containing 10% fetal bovine serum was incubated at 37°C for 1 day to recapitulate cell culture conditions. Both forms of the enzyme were equally effective at reducing thiamine from 3 μM to 10 nM. However, the native enzyme was more efficient at the lower thiamine concentrations in further depletion, decreasing thiamine concentrations to less than 1 nM at a concentration of 0.02 units/ml, whereas 5k- thiaminase required a concentration of 0.5 units/ml.

Because thiaminase is a 42 kDa protein, we determined whether it could be taken into cells. Fig. 3A shows that fluorescence-labeled native thiaminase achieves cellular uptake and co-localizes with lysosomes, indicating that the mechanism of cell uptake is endocytosis. An immunoblot in Fig. 3B demonstrates immunoreactive thiaminase in cellular lysate.

The cytotoxic potency of native versus 5k-PEG thiaminase in nine different cell lines is shown in Fig. 4. We were surprised to find that 5k-PEG thiaminase was inert in the six solid tumor monolayer cell lines. In contrast, 5k-PEG thiaminase and native thiaminase were equally potent and achieved growth inhibition in Reh, RS4, and Jurkat leukemia cell lines at enzyme concentrations that depleted thiamine in the growth medium (Fig. 2). However, at higher enzyme concentrations cell killing seemed to occur with native thiaminase but not with 5k-PEG thiaminase. It is noteworthy that cytotoxicity in all cell lines occurred at native thiaminase concentrations far higher than the concentration needed to deplete medium of thiamine.

To determine whether 5k-PEGylation decreased cytotoxic effect by decreasing cellular thiamine catabolism, we first determined that native and 5-PEGylated enzymes were equally efficient at catabolizing the major forms of intracellular thiamine pyrophosphate (thiamine diphosphate) and thiamine monophosphate (Table 1). Both phosphorylated thiamine derivatives were substrates for both forms of the enzyme by spectrophotometric assay, with thiamine monophosphate only marginally less efficient than thiamine as substrate (mol/unit activity), 92% and 95% for native thiaminase and LCPTE compared with thiamine, respectively, whereas thiamine pyrophosphate was slightly less efficient, with catalytic rates of 83% for both forms of the enzyme compared with thiamine. Next, we examined intracellular thiamine and thiamine diphosphate (thiamine pyrophosphate) in RS4 cells exposed to equal concentrations of native thiaminase and 5k-PEGylated thiaminase and found that native thiaminase was much more efficient at depleting both intracellular thiamine (Fig. 5A) and thiamine pyrophosphate (Fig. 5B) than the 5k-PEGylated form of the enzyme. Because two thiamine-dependent enzymes, pyruvate dehydrogenase and α-ketoglutarate dehydrogenase, are involved in energy metabolism, we determined whether thiaminase-induced changes in thiamine pools had an effect on cellular respira- tion by analyzing OCR, a measure of mitochondrial respira- tion, and ECAR, a measure of glycolysis, after treatment with native and PEGylated thiaminase. As can be seen in Fig. 5C, native thiaminase treatment dramatically decreased the OCR (26 versus 1 pmol/min/10^4 cells; p < 0.001), whereas PEGylated thiaminase had little effect (21 pmol/min/10^4 cells) compared with untreated control cells, demonstrating that native thiaminase produced downstream metabolic effects from thiamine depletion that were not produced by PEGylated thiaminase.

Figure 6 and Table 2 show the activity-time profiles and pharmacokinetic parameters after 24 or 168 h of a single subcutaneous dose of native thiaminase (300 units/kg) or 5k-PEG thiaminase (10 U/kg). Native thiaminase was rapidly cleared in vivo, and PEGylation greatly enhanced the
Fig. 4. Dose-response curves of nine cancer cell lines showing growth relative to control of cells treated with increasing concentrations of native or 5k-PEGylated thiaminase enzyme.
plasma retention time and in vivo potency. 5k-PEGylation increased the plasma retention time from 1.47 to 34.4 h, an increase of 23.4-fold, and similarly increased the AUC from 1.54 to 36.71 h/μl. The estimated maximum concentration was similar for both the 300 units/kg of native enzyme and 10 units/kg of 5k-thiaminase, at 0.41 and 0.54 units/μl, respectively.

Data from pilot pharmacokinetic studies were used to estimate the exposure after administration of 1k- and 10k-PEGylated thiaminase. The dose-adjusted AUCs (AUC/dose; units/kg) are compared in Table 3. The 5k-thiaminase modification produced both the highest dose-adjusted AUC and was the most potent with respect to the maximum tolerated dose of the three modifications tested. This difference in the dose-adjusted AUC between the 5k- and 10k-PEGylated thiaminase formulations was probably caused by a difference in bioavailability. It is possible that the larger effective particle size had reduced access to plasma after its subcutaneous administration. Clearance mechanisms could also play a role in the lower exposure, although such mechanisms are typically observed with much larger particles (e.g., 50–200 nm).

Figure 7 shows the depletion of thiamine in serum corresponding to time points obtained for pharmacokinetic sampling and represents the extracellular pharmacodynamic effect of thiaminase. In comparison with studies of thiaminase depletion in tissue culture medium, where the starting thiaminase formulations were substituted for thiamine as substrates, and the relative activity of native thiaminase and 5k-PEGylated thiaminase was determined and expressed as the fraction of activity relative to thiamine as substrate.

### TABLE 1

<table>
<thead>
<tr>
<th>Thiamine Monophosphate</th>
<th>Thiamine Pyrophosphate</th>
<th>Thiamine Diphosphate</th>
<th>Thiamine Mono- and Pyrophosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native thiaminase</td>
<td>100</td>
<td>83</td>
<td>92</td>
</tr>
<tr>
<td>5k-LCPTE</td>
<td>100</td>
<td>83</td>
<td>95</td>
</tr>
</tbody>
</table>

### TABLE 2

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>Estimate</th>
<th>S.E.</th>
<th>Coefficient of Variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native thiaminase, 300 units/kg s.c.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AUC</td>
<td>h·U/μl</td>
<td>1.54</td>
<td>0.15</td>
<td>9.98</td>
</tr>
<tr>
<td>$t_{1/2}$</td>
<td>h</td>
<td>1.47</td>
<td>0.14</td>
<td>9.56</td>
</tr>
<tr>
<td>CL/F</td>
<td>ml/h/kg</td>
<td>194.8</td>
<td>19.45</td>
<td>9.99</td>
</tr>
<tr>
<td>$T_{max}$</td>
<td>h</td>
<td>1.63</td>
<td>0.19</td>
<td>11.42</td>
</tr>
<tr>
<td>$C_{max}$</td>
<td>U/μl</td>
<td>0.41</td>
<td>0.06</td>
<td>15.07</td>
</tr>
<tr>
<td>V/F</td>
<td>ml/kg</td>
<td>413.86</td>
<td>64.49</td>
<td>15.58</td>
</tr>
<tr>
<td>5k-thiaminase, 10 units/kg s.c.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AUC</td>
<td>h·U/μl</td>
<td>36.71</td>
<td>2.67</td>
<td>7.28</td>
</tr>
<tr>
<td>$t_{1/2}$</td>
<td>h</td>
<td>34.43</td>
<td>4.56</td>
<td>13.23</td>
</tr>
<tr>
<td>CL/F</td>
<td>ml/h/kg</td>
<td>0.27</td>
<td>0.02</td>
<td>7.28</td>
</tr>
<tr>
<td>$T_{max}$</td>
<td>h</td>
<td>15.38</td>
<td>1.26</td>
<td>8.17</td>
</tr>
<tr>
<td>$C_{max}$</td>
<td>U/μl</td>
<td>0.54</td>
<td>0.02</td>
<td>4.42</td>
</tr>
<tr>
<td>V/F</td>
<td>ml/kg</td>
<td>13.53</td>
<td>1.21</td>
<td>8.97</td>
</tr>
</tbody>
</table>

CL/F, apparent clearance; V/F, apparent volume.

### TABLE 3

<table>
<thead>
<tr>
<th>Dose-adjusted AUCs</th>
<th>AUC Relative to Native</th>
<th>MTD Relative to 1/MTD</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC units/kg</td>
<td>Relative to Native</td>
<td>MTD units/kg</td>
</tr>
<tr>
<td>Native thiaminase</td>
<td>0.005</td>
<td>1</td>
</tr>
<tr>
<td>1k-thiaminase*</td>
<td>0.025</td>
<td>5</td>
</tr>
<tr>
<td>5k-thiaminase*</td>
<td>3.7</td>
<td>740</td>
</tr>
<tr>
<td>10k-thiaminase*</td>
<td>0.54</td>
<td>108</td>
</tr>
</tbody>
</table>

* AUCs are estimated.
The concentration of thiamine in mouse serum after single doses of native thiaminase (300 U/kg) or 5k-PEG thiaminase (10 U/kg). The result shows thiamine was depleted in vivo after native thiaminase or 5k-PEG thiaminase treatment within 6 h and lasted through the end of the pharmacokinetic experiment for 5k-PEGylated thiaminase at hour 168.

Lethality experiments designed to estimate the MTD determined that the dose-limiting toxicity of all forms of thiaminase seems to be gastrointestinal toxicity, not cardiac or central nervous system toxicity as might be expected from thiamine starvation. Tissues harvested at the end of the pharmacokinetic studies of native and 5k-PEG thiaminase are shown in Fig. 8. Tissues were stained with hematoxylin and eosin (H&E) and by immunohistochemistry with the polyclonal antibody raised against a thiaminase peptide used in the Western blot of Fig. 3B. At the doses used, H&E staining did not show evidence of tissue damage at the end of the experimental period. In addition, there was no evidence of thiaminase in heart or brain tissues probed with the antithiaminase antibody. However, thiaminase staining did appear in colonic epithelium, suggesting that gastrointestinal toxicity may be the result of the accumulation of enzyme in the gastrointestinal tract.

We used an established RS4 xenograft model to examine the in vivo antitumor activity of native and 5k-PEG thiaminases. We have shown previously that 1k-PEGylated thiaminase has activity in this model (Daily et al., 2011). As shown in Fig. 9, 5k-PEG thiaminase had no activity compared with the control group, whereas native thiaminase increased the event-free survival (EFS) from 15 to 70 days ($p < 0.001$) in an experiment that was terminated at day 100 from the initiation of treatment. In comparison, we have shown previously that 1k-PEG thiaminase increases the EFS to 31 days (Daily et al., 2011). Thus, at their respective MTDs, PEGylated thiaminases are not as effective as native thiaminase in this tumor model. On the other hand, native thiaminase seems to have promising activity, with not only significantly increased median EFS, but also from the observation that 5 of 10 mice with established RS4 xenografts showed tumor regression through day 100.

**Discussion**

We have explored the concept of using the bacterial enzyme thiaminase to disrupt energy metabolism in cancer. To optimize the biological and pharmacologic properties of this enzyme, we studied the effects of modifying it with the addi-
31 days in the treated group (versus control, for comparison, the median EFS for 1k-PEGylated thiaminase was 31 days in an experiment stopped at day 100. In previously published data shown in Fig. 4, the native thiaminase group showed regression of tumor by day 100 after treatment. We reported previously the regression of tumor by day 100 after treatment.

To achieve further cytotoxicity. We hypothesized that thiaminase worked through an extracellular mechanism of action, by catabolizing extracellular thiamine and causing acute thiamine starvation. Indeed, Fig. 2 shows that both native and 5k-PEG thiaminase are very potent in the catabolism of thiamine, achieving a 100-fold reduction of thiamine in medium at concentrations of 0.0001 units/ml. The potential for intracellular effect of thiaminase is shown in Fig. 3, where intracellular uptake of the enzyme is demonstrated, and the colocalization with lysosomes indicates that the mechanism of uptake involves endocytosis, and in Table 1, where native and PEGylated thiaminase is shown to have the capacity to catabolize the predominant intracellular forms of thiamine, thiamine diphosphate, and thiamine monophosphate.

The comparison of the cytotoxicity profiles of native and 5k-PEG thiaminase in Fig. 4 to the concentrations needed to deplete thiamine in medium demonstrated three surprising results. First, most solid tumor cell lines showed cytotoxicity at concentrations of thiaminase (0.1–2 units/ml) that were orders of magnitude greater than the concentration needed to deplete medium of thiamine. Second, in these cell lines 5k-PEG thiaminase was inert. Third, in leukemia cell lines (Fig. 8), suggesting that cellular uptake is required for cytotoxicity in vitro against cancer cell lines, it was appreciably more toxic in vivo, with an increase in its MTD in proportion to its dose-adjusted AUC. However, at tolerable doses, native thiaminase was far more active against an RS4 leukemia xenograft than 5k-PEG thiaminase. The lack of in vitro cytotoxicity correlated with the lack of in vivo efficacy.

The dose-limiting toxicity seems to be gastrointestinal; at lethal doses food accumulates in the stomach, and a picture of colitis becomes apparent. The toxicity of thiaminase for native enzyme, and all PEGylated forms, seems similar. The expected toxicity in the brain and heart, the most affected organs during thiamine starvation, is not observed. Unlike cancer cells, there is evidence that 5k-PEGylated thiaminase as well as native thiaminase can accumulate in gastrointestinal cells (Fig. 8), suggesting that cellular uptake is required for toxicity in most cells.

Thus, although PEGylation improved the pharmacokinetic properties of the enzyme, the modification resulted in an inferior product that had less in vitro activity, more toxicity, and reduced antitumor activity in a xenograft model. Despite the disappointing and unexpected result of PEGylation, the observations in regard to PEGylation of thiaminase help to elucidate its mechanism of action. The targets of thiaminase include two enzymes involved in respiration, pyruvate dehydrogenase and α-ketoglutarate dehydrogenase. We expected that thiaminase would therefore suppress respiration, and subsequently result in cytotoxicity. Indeed, native thiami-
nase does suppress respiration, and cause cytotoxicity, whereas the PEGylated form does neither. These studies indicate that suppression of respiration is a targeted effect of thiaminase that leads to the intended consequence of tumor cell death.

In addition, the antitumor potential of thiaminase therapy has been demonstrated, because administration of the native enzyme, a completely novel therapy, resulted in a significant tumor response, with regression of established tumors, in a subcutaneous RS4 leukemia xenograft model (Fig. 9). The results point to the necessity of improving cellular uptake for cytotoxic effect, while avoiding prolonged extracellular activity that seemed to cause toxicity to normal tissues. This presents a significant challenge for the development of a protein as a therapeutic agent.

The activity of native thiaminase against cell lines and established RS4 xenografts demonstrates the therapeutic potential of this novel approach. The challenge of improving cellular uptake to enhance cellular effect has been met with other proteins that are used as therapeutic agents, most notably the development of a modified β-glucosidase protein that is transported into macrophages for the treatment of Gaucher's disease (Cox, 2010).

Acknowledgments

We thank Tiffany L. Taylor for technical assistance in the preparation of the histology study specimens and Hui Yu for the careful preparation of recombinant thiaminase enzyme.

Authorship Contributions

Participated in research design: Liu, Leggas, Miriyala, St. Clair, and Moscow.

Conducted experiments: Liu, Daily, Bhatnagar, and Miriyala.

Contributed new reagents or analytic tools: Bae.

Performed data analysis: Leggas, Miriyala, St. Clair, and Moscow.

Wrote or contributed to the writing of the manuscript: Liu, Leggas, Daily, Miriyala, and Moscow.

References


