

Circulating Half-Life of PEGylated Recombinant Methioninase Holoenzyme Is Highly Dose Dependent on Cofactor Pyridoxal-5'-Phosphate

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ABSTRACT

Recombinant methioninase (rMETase) has been shown to target the elevated methionine (MET) dependence of tumor cells and arrest their growth as well as make tumors more sensitive to standard chemotherapy agents. Polyethylene glycol (PEG)-modified rMETase (PEG-rMETase) has reduced antigenicity compared with unmodified rMETase. However, PEG-rMETase has a limited active circulating half-life due to rapid *in vivo* dissociation of its cofactor pyridoxal-5'-phosphate (PLP), a surprising finding, because PLP is tightly bound to PEG-rMETase in buffer. The question asked in the current study was on the effect of increasing doses of PLP to extend the circulating half-life of active PEG-rMETase holoenzyme *in vivo*. rMETase was conjugated with methoxypolyethylene glycol succinimidyl glutarate 5000 (MEGC-PEG). Miniosmotic pumps containing various concentrations of PLP were implanted in BALB-C mice. PLP-infused mice were then injected with a single dose of 4000 or 8000 units/kg PEG-rMETase. Mice infused with 5, 50, 100, 200, and 500 mg/ml PLP-containing miniosmotic pumps increased plasma PLP to 7, 24, 34, 60, and 95 μM , respectively, from the PLP baseline of 0.3 μM . PLP increased the half-life of MEGC-PEG-rMETase holoenzyme in a dose-dependent manner. Pumps containing 500 mg/ml PLP increased the half-life of MEGC-PEG-rMETase holoenzyme 4.5-fold from 1.5 to 7 h. Infused PLP did not extend the half-life of MEGC-PEG-rMETase apoenzyme. With a dose of 4000 units/kg MEGC-PEG-rMETase in the mice infused with 5, 50, 200, and 500 mg/ml PLP, plasma MET was depleted from 50 μM to ≤ 5 μM for 8, 24, 72, and 72 h, respectively. Thus, PLP infusion could extend the period of MET depletion by MEGC-PEG-rMETase by ~ 10 -fold in a dose-dependent manner. The mice given 8000 units/kg MEGC-PEG-rMETase showed a similar plasma MET depletion time course, indicating that the limiting factor for MEGC-PEG-rMETase-mediated MET depletion *in vivo* was PLP. The extended time of MET depletion by MEGC-PEG-rMETase was due to the maintenance of active MEGC-PEG-rMETase holoenzyme by infused PLP. The infused PLP either bound to apo-MEGC-PEG-rMETase and/or inhibited dissociation of PLP from holo-MEGC-PEG-rMETase, thereby maintaining the holoenzyme form of MEGC-PEG-rMETase *in vivo*. The combination of MEGC-PEG-rMETase treatment with PLP infusion suggests an effective clinical strategy for long-term MET depletion to arrest cancer growth.

INTRODUCTION

Methionine (MET) dependence, the elevated minimal MET requirement for cell growth relative to normal cells, has been observed in many human cancer cell lines and cancer xenografts in animal models (1–3). MET dependence is a metabolic defect seen only in cancer cells and precludes the cells from growing in media in which methionine is depleted (4, 5).

Recombinant methioninase (rMETase), chemical name: L-methionine α -deamino- γ -mercaptomethane lyase or EC 4.4.1.11, is a pyridoxal-L-phosphate (PLP)-dependent enzyme with four subunits of 43

KDa each. rMETase is found in *Pseudomonas putida*, *Aeromonas*, and *Clostridium* but not in yeast, plants, or mammals (1–7). The enzyme has been cloned from *Pseudomonas putida* and produced in *Escherichia coli* (8, 9). rMETase has been demonstrated to be a powerful approach to methionine depletion *in vivo* (6, 7).

rMETase alone or in combination with chemotherapeutic agents such as cisplatin, 5-fluorouracil, and 1,3-bis(2-chloroethyl)-1-nitrosourea has shown efficacy and synergy, respectively, in mouse models of colon, lung, and brain cancer (10–13). A pilot Phase I clinical trial demonstrated that rMETase depleted plasma methionine levels without observed clinical toxicity over a period of ~ 24 h in patients with advanced cancer (14, 15). A recent study in primates, however, indicated that rMETase could cause anaphylactic reactions (16).

Conjugation of protein therapeutics with polyethylene glycol (PEG) has been shown to confer important therapeutic benefits, most importantly reduced antigenicity (17). The Food and Drug Administration has approved the PEGylated forms of several protein therapeutics for clinical use, including adenosine deaminase, asparaginase, α -IFN, and a growth hormone antagonist (18–22).

rMETase was coupled to methoxypolyethylene glycol succinimidyl glutarate-5000 (MEGC-PEG; Ref. 23). Pharmacokinetic evaluation in mice showed that MEGC-PEG-rMETase had decreased antigenicity, increased serum half-life, and increased methionine depletion time compared with unmodified rMETase. However, the half-life of active MEGC-PEG-rMETase holoenzyme was shown to be limited because of *in vivo* dissociation of PLP (23).

The present study demonstrates the dose dependence efficacy of infused PLP to markedly increase the serum half-life of the active holoenzyme form of PEGrMETase and the period of maintenance of serum MET depletion in mice, suggesting the clinical potential of the combination of MEGC-PEG-rMETase and PLP treatment.

MATERIALS AND METHODS

Materials. rMETase was produced by Shionogi Co., Ltd. (Osaka, Japan) as described previously (23). The rMETase was formulated in 50 mM sodium phosphate buffer (pH 7.2), containing 10 μM PLP, with protein concentration 31 mg/ml, and specific activity 50.7 units/mg. rMETase was $>95\%$ pure by high-performance liquid chromatography with a tetramer/oligomer ratio of 96.7/3.3 and endotoxin of 0.06 EU/mg. MEGC-PEG used for PEGylation of rMETase was provided by the NOF Corporation (Kawasaki-shi, Kanagawa, Japan). The average molecular mass was 5461 Da. PLP and other chemicals were purchased from Sigma (St. Louis, MO).

PEGylation. The protocol for preparation of PEGylated rMETase was previously described (23): the activated MEGC-PEG derivative was used at a molar excess (2-fold) of MEGC-PEG to free lysines in rMETase (32 per rMETase tetramer), which corresponds to a molar ratio of PEG to rMETase of 60/1. A given amount of the activated MEGC-PEG was added to the rMETase solution (100 mg/ml) with three stepwise additions at 20-min intervals. The PEGylation reaction was carried out at 37°C (pH 9.0) under gentle stirring for 60 min. The PEGylation reaction was terminated by adjusting the pH to 7.2 with 0.1 M sodium diphosphate (pH 4.5). Diafiltration was followed by purification of MEGC-PEG-rMETase on DEAE-Sepharose and Sephacryl

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S200 columns. Purified MEGC-PEG-rMETase was concentrated and sterilized by filtration and stored at -80°C .

PLP Infusion. Miniosmotic pumps with a reservoir volume of 200 μl , pumping rate of 1.0 $\mu\text{l/h}$ and duration 7 days (Model 2001) were purchased from Durect Corporation (Cupertino, CA). Five-hundred mg of PLP and 150 mg of sodium hydroxide were dissolved in 1 ml of distilled water (pH 7.4). Other concentrations of PLP were prepared by dilution. The PLP solution was drawn into a 3-ml syringe, which was attached to the blunt-tipped, 27-gauge filling tube of the minipump. For s.c. placement of the pump, a small incision is made in the skin between the scapulae. Using a hemostat, a small pocket is formed by spreading the s.c. connective tissues apart. The pump is inserted into the pocket with the flow moderator pointing away from the incision. The skin incision is closed with sutures.

Determination of Protein Content. Protein was measured colorimetrically using the Wako Protein Assay kit (Wako, Osaka, Japan) according to the instruction manual with slight modification (24).

Determination of PEGylation Degree of rMETase. The degree of modification of PEGylated rMETase was determined colorimetrically (25) and by matrix-assisted desorption ionization. For the colorimetric assay, various amounts of rMETase and MEGC-PEG-rMETase in 2 ml 0.1 M sodium phosphate buffer (pH 8.0) were mixed with 1 ml fluorescamine solution (0.3 mg/ml in acetone) and incubated for 5 min at room temperature. Samples were then assayed with a fluorescence spectrometer at 390 nm excitation and 475 nm emission. Results were plotted as fluorescence units *versus* concentration, with the slope of the line being determined by linear regression. The percentage of PEGylated primary amines was determined according to the following formula: $1 - (\text{slope PEGylated rMETase}/\text{slope naked rMETase}) \times 100$. Matrix-assisted desorption ionization analysis of naked and MEGC-PEG-rMETase was performed at the Scripps Research Institute using a PerSeptive Biosystems Voyager-Elite mass spectrometer (San Diego, CA).

Determination of MEGC-PEG-rMETase Apoenzyme. MEGC-PEG-rMETase apoenzyme was determined, in the presence of PLP, from α -ketobutyrate produced from L-methionine according to the method of Esaki and Soda (26) with slight modification. Fifty μl of sample, diluted in 100 mM potassium phosphate buffer (pH 8.0), containing 0.01% DTT, 1 mM EDTA_2Na , 10 μM PLP, and 0.05% Tween 80, were mixed with 1 ml substrate solution [100 mM potassium phosphate buffer (pH 8.0), containing 25 mM L-methionine and 10 μM PLP] in a glass test tube. The reaction mixture was vortexed immediately and incubated at 37°C without shaking for precisely 10 min. The reaction was stopped by adding 100 μl 50% trichloroacetic acid. The suspension was centrifuged at 13,000 rpm for 2 min. The supernatant (0.8 ml) was collected in a glass tube containing 1.6 ml of 1 M acetate buffer (pH 5.0). Then, 0.6 ml *N*-methyl-2-benzothiazolone hydrazone hydrochloride solution containing 0.1% 3-methyl-2-benzothiazolinone hydrazone hydrochloride monohydrate (Wako) was added to the tube, mixed well, and incubated at 50°C for 40 min. The absorbance of the reaction mixture was measured at 320 nm. The assay was carried out in triplicate. ΔE was calculated by subtracting the average absorbance of blanks from the average absorbance of the reaction mixture. The enzyme activity was calculated by the following formula: activity (units/ml) = $0.548 (1.07 + 2.2\Delta\text{E}) \Delta\text{E}$. One unit of enzyme is defined as the amount of enzyme which produced 1 μM α -ketobutyrate/minute at an infinite concentration of MET.

Determination of MEGC-PEG-rMETase Holoenzyme Activity. For determination of plasma MEGC-PEG-rMETase holoenzyme activity, no PLP was added in the substrate solution. All procedures were the same as for the determination of MEGC-PEG-rMETase apoenzyme, except that 5 μl of the sample in 45 μl distilled water were mixed with 1 ml substrate solution in the first reaction step. Because of the change of sample volume, the enzyme activities are calculated from the following formula: activity (units/ml) = $27.4 (1.07 + 2.2\Delta\text{E}) \Delta\text{E}$.

Plasma MET Determination. The MET level in the plasma was measured by precolumn derivatization, followed by high-performance liquid chromatography separation (27). Briefly, 10 μl plasma sample or MET standard were precipitated with 30 μl acetonitrile, followed by centrifugation at 10,000 rpm for 5 min. Ten μl of the supernatant were mixed with 5 μl of a fluoralddehyde derivative reagent, *o*-phthalaldehyde, for 1 min at room temperature, followed by addition of 150 μl 0.1 M sodium acetate (pH 7.0). Twenty μl of the reaction mixture were loaded on a reversed-phase Supelcosil LC-18DB column (25 \times 4.8-cm, particle size 5 μm ; Supelco, Bellefonte, PA). The amino

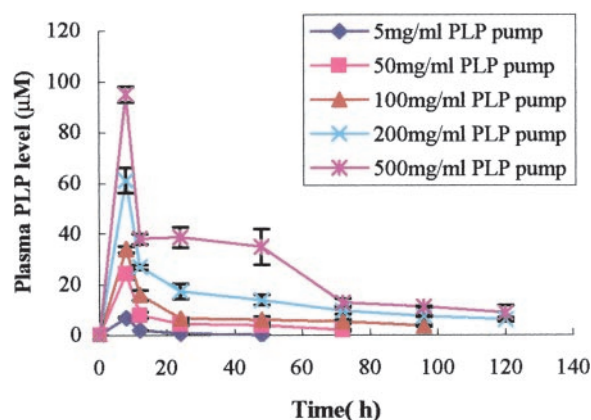


Fig. 1. Dose-dependent efficacy of PLP on plasma PLP concentration. Osmotic pumps filled with PLP ranging from 5 to 500 mg/ml were implanted s.c. 12 h before MEGC-PEG-rMETase was i.v. injected in mice. Blood samples were collected at different time points and measured for plasma PLP concentration as described in "Materials and Methods."

acid derivatives were separated by using a gradient elution of 40–60% solution B (methanol) in solution A [tetrahydrofuran/methanol/0.1 M sodium acetate (pH 7.2); 5/95/900] at a flow rate of 1.5 ml/min. A fluorescence spectrophotometer was used for detection with excitation at 350 nm and emission at 450 nm. The plasma methionine was identified by the retention time of a MET standard solution and quantitated according to a MET standard curve.

Determination of Plasma PLP. PLP in plasma was determined based on the conversion of pyridoxal 5'-phosphate to 4-pyridoxic acid 5'-phosphate by cyanide in alkaline medium, followed by a high pressure liquid chromatographic separation (28). All work was performed in a semi-darkened laboratory with low actinic lighting to avoid light-catalyzed destruction of the derivatives. Briefly, the diluted plasma sample and PLP standard solutions were mixed with an equal volume of trichloroacetic acid (100 g/liter) and incubated at 50°C in the dark for 5 min for removal of protein. After centrifugation at 10,000 rpm for 5 min, the supernates were taken and transferred to new vials. To 100 μl of the resulting supernatant, 35.5 μl dipotassium hydrogen phosphate (3.3 mM) were added, followed by 10 μl sodium cyanide (0.04 mM). The reaction mixture was kept at 50°C for 25 min in the dark, then 12.5 μl orthophosphoric acid (2.86 mM) were added. Fifty μl of the supernatant were loaded on a Waters Symmetry Shield RP8, 5 μm , 4.6 \times 250 mm column (Blackmooer Lane, Watford, United Kingdom). The column was eluted with water containing 75 mM semicarbazide and 50 mM potassium dihydrogen phosphate (pH 2.85) at a flow rate of 1.5 ml/min. A fluorescence spectrophotometer was used for detection with excitation at 325 nm and emission at 418 nm. The PLP peak was identified by the retention time of a PLP standard. The concentration of plasma PLP was calculated using a calibration curve.

Treatment of Mice with MEGC-PEG-rMETase. BALB-C NCR nude mice ages 4 weeks (20–25g) were used for the study with five mice/group. All animal studies were conducted in accordance with the principles and procedures outlined in the NIH National Research Council's "Guide for the Care and Use of Laboratory Animals"⁵ under assurance number A3873-1. Osmotic pumps filled with 250 μl PLP at different concentrations ranging from 5 to 500 mg/ml were implanted s.c. as described above. Twelve h after pump implantation, MEGC-PEG-rMETase at a dose of 4000 or 8000 units/kg was i.v. injected via the tail vein into each mouse, respectively. Blood (200 μl) was collected from the retroorbital plexus of each animal using heparinized capillary tubes. Blood was collected before injection and 1, 8, 12, 24, 48, 72, and 96 h after injection. The plasma was separated and stored in small aliquots at -80°C . MEGC-PEG-rMETase activity, methionine concentration and PLP level in the plasma were measured as described above.

RESULTS AND DISCUSSION

Characterization of MEGC-PEG-rMETase. rMETase was PEGylated at a ratio of MEGC-PEG to rMETase of 60:1, which resulted

⁵ Internet address: <http://oacu.od.nih.gov/regs/guide/guidex.htm>.

Table 1 Dose-dependent efficacy of PLP on plasma half-life of MEGC-PEG-rMETase

Concentration of PLP in pump	Apoenzyme half-life	Holoenzyme half-life
0	18 h	1.5 h
5 mg/ml	18 h	2.5 h
50 mg/ml	16 h	3 h
200 mg/ml	15 h	6 h
500 mg/ml	14 h	7 h

NOTE. Osmotic pumps containing PLP ranging from 5 to 500 mg/ml were implanted s.c. MEGC-PEG-rMETase at 4000 units/kg was i.v. injected in each mouse 12 h after pump implantation. Blood samples were collected at different time points and measured for MEGC-PEG-rMETase apoenzyme and holoenzyme enzyme activity as described in "Materials and Methods."

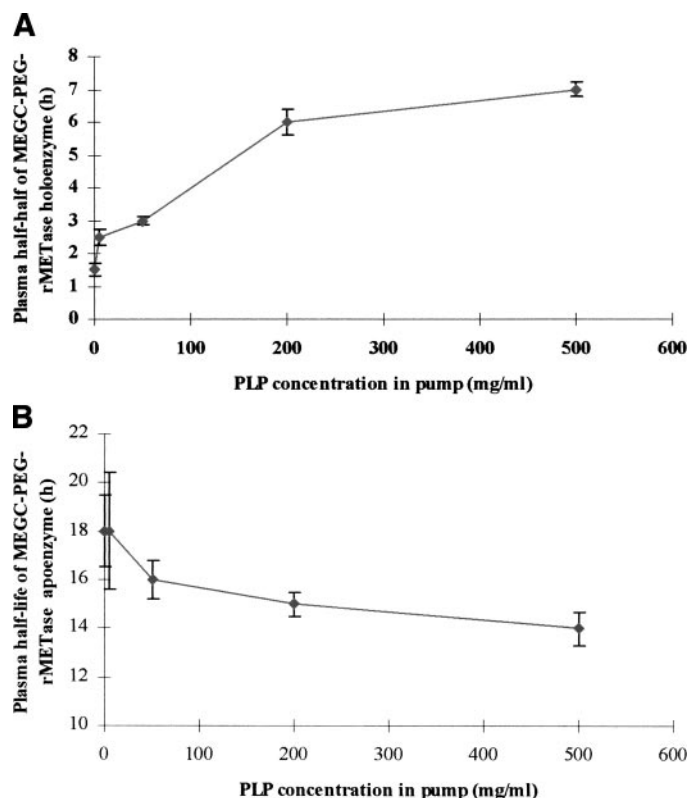


Fig. 2. Dose-dependent efficacy of PLP on plasma half-life of MEGC-PEG-rMETase holoenzyme (A) and apoenzyme (B) half-life. Osmotic pumps filled with PLP ranging from 5 to 500 mg/ml were implanted s.c. MEGC-PEG-rMETase at a dose of 4000 units/kg was i.v. injected into each mouse 12 h after pump implantation. Blood samples were collected at different time points and measured for PEG-rMETase holoenzyme (A) and apoenzyme (B) activity as described in "Materials and Methods."

in MEGC-PEG-rMETase molecules with a range of three to seven PEG chains/monomer of rMETase, specific enzyme activity, 44 units/mg (23).

Dose Response of PLP Infusion on Plasma PLP Levels. The activity of rMETase depends on stable binding of PLP to the protein to form the holoenzyme (29). We have previously shown that PLP readily dissociates from MEGC-PEG-rMETase *in vivo* (23). To increase the time of maintenance of the holoenzyme form of PEG-rMETase and MET-depletion time *in vivo*, PLP was continuously infused to MEGC-PEG-rMETase-treated mice using implanted miniosmotic pumps containing various concentrations of PLP. Plasma PLP levels were found to increase in a dose-dependent manner (Fig. 1). Peak plasma PLP concentrations in mice implanted with 5, 50, 100, 200, and 500 mg/ml PLP-containing pumps resulted in 7, 24, 34, 60, and 95 μM plasma concentrations, respectively, compared with a baseline of 0.3 μM . Pumps with 200 mg/ml PLP could sustain plasma PLP at $\sim 20 \mu\text{M}$ for 24 h and 10 μM for 72 h. Pumps with 500 mg/ml

PLP could sustain plasma PLP levels at $\sim 40 \mu\text{M}$ for 48 h and 10 μM for 96 h (Fig. 1).

Dose Response of PLP Infusion on MEGC-PEG-rMETase Holoenzyme Half-Life. MEGC-PEG-rMETase holoenzyme activity is measured *in vitro* without addition of PLP in the enzyme assay solution. PEG-rMETase holoenzyme activity was markedly dependent on the dose of PLP infused *in vivo* (Table 1, Fig. 2A). MEGC-PEG-rMETase holoenzyme half-life was 1.5 h without PLP infusion, 2.5 h with 5 mg/ml PLP pumps, and ranged to 7 h with 500 mg/ml PLP pumps (Table 1), a 4.5-fold increase compared with the PEG-rMETase half-life without PLP supplementation. A high degree of correlation of PLP concentration in

Table 2 Dose-dependent efficacy of PLP on time of plasma MET depletion by MEGC-PEG-rMETase

PLP in pumps	Time of MET depletion to $\leq 5 \mu\text{M}$	
	4000 units/kg	8000 units/kg
0	8 h	12 h
5 mg/ml	8 h	12 h
50 mg/ml	24 h	24 h
200 mg/ml	72 h	72 h
500 mg/ml	72 h	72 h

NOTE. Osmotic pumps containing PLP ranging from 5 to 500 mg/ml were implanted subcutaneously. MEGC-PEG-rMETase at 4000 or 8000 units/kg was i.v. injected in each mouse 12 h after pump implantation. Blood samples were collected at different time points and measured for plasma MET level as described in "Materials and Methods."

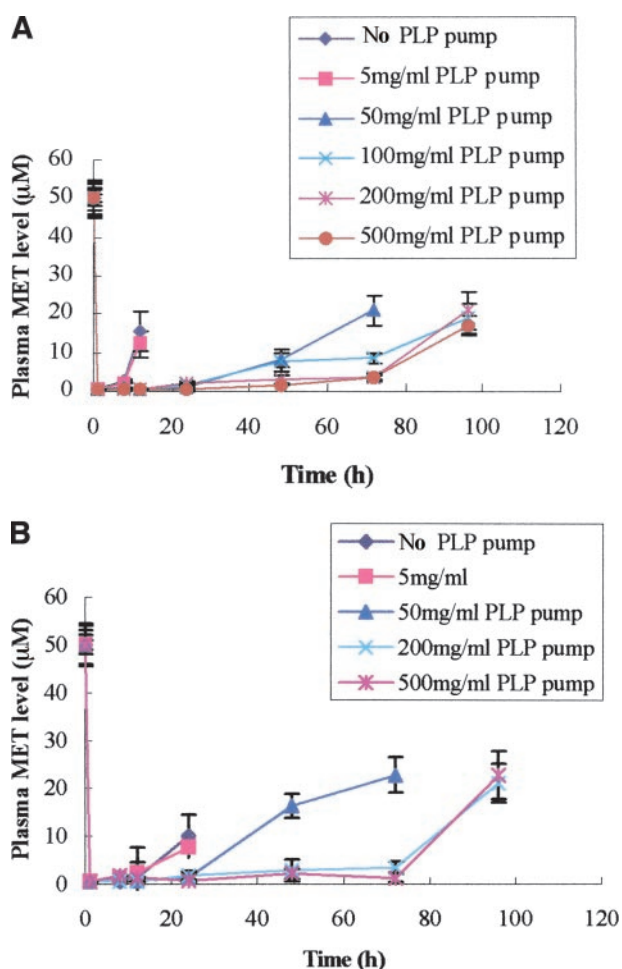


Fig. 3. Time and extent of plasma MET depletion by MEGC-PEG-rMETase at various doses of PLP infusion. Osmotic pumps filled with PLP ranging from 5 to 500 mg/ml were implanted s.c. PEG-rMETase at a dose of 4000 units/kg (A) or 8000 units/kg (B) was i.v. injected into each mouse 12 h after pump implantation. Blood samples were collected at different time points and measured for plasma MET level as described in "Materials and Methods."

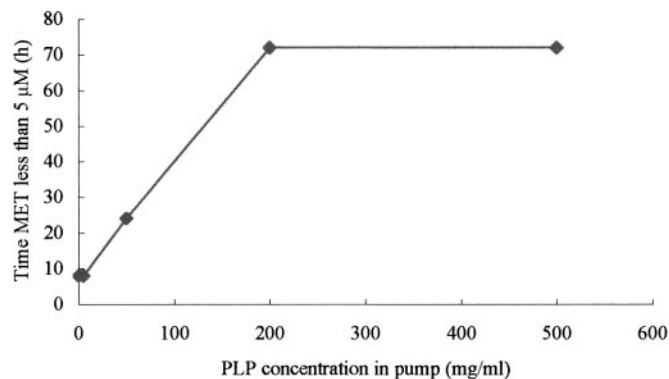


Fig. 4. Dose-dependent efficacy of PLP on time of plasma MET depletion by MEGC-PEG-rMETase. Osmotic pumps filled with PLP ranging from 5 to 500 mg/ml were implanted s.c. MEGC-PEG-rMETase at a dose of 4000 units/kg was i.v. injected in each mouse 12 h after pump implantation. Blood samples were collected at different time points and measured for plasma MET level as described in "Materials and Methods."

the osmotic pumps and MEGC-PEG-rMETase holoenzyme half-life was found up to 200 μM PLP ($r^2 = 0.96$; Table 1, Fig. 2A). In contrast, the half-life of PEG-rMETase apoenzyme, measured in the presence of PLP *in vitro*, was ~ 18 h in animals with or without PLP infusion (Table 1, Fig. 2B). Thus, MEGC-PEG-rMETase holoenzyme half-life was highly dependent on the dose of PLP in contrast to apoenzyme half-life (Fig. 2A).

Dose Response of PLP Infusion on Time of MET Depletion by PEG-rMETase. MEGC-PEG-rMETase holoenzyme half-life highly correlated with time of MET depletion, both of which depend on the dose of infused PLP (Table 2, Fig. 3, A and B). PLP-supplemented mice were injected with a single i.v. injection of 4000 or 8000 units/kg PEG-rMETase. Blood samples were collected at various time points and measured for plasma MET concentration. Four-thousand units/kg MEGC-PEG-rMETase administration in the mice infused with 5, 50, 200, and 500 mg/ml PLP-containing pumps resulted in plasma MET depletion to $\leq 5 \mu\text{M}$ for 8, 24, 72, and 72 h, respectively (Table 2, Fig. 3A), demonstrating a strong PLP dose dependence of MET depletion. The mice receiving 8000 units/kg PEG-rMETase and the same varying amounts of PLP showed a similar plasma MET depletion time course (Table 2, Fig. 3B). However, mice given 8000 units/kg MEGC-PEG-rMETase supplemented with 500 mg/ml PLP had no detectable levels of MET for 72 h (Fig. 3B). The efficacy of PLP to extend the time of MEGC-PEG-rMETase-mediated MET depletion to $\leq 5 \mu\text{M}$ was linearly dose dependent on PLP up to 200 mg/ml in the osmotic pump ($r^2 = 0.99$; Fig. 4). Five-hundred mg/ml PLP-containing pumps did not further extend the time of MET depletion.

This study indicated that PLP supplementation *in vivo* greatly extended the half-life of MEGC-PEG-rMETase holoenzyme, which in turn greatly extended the time of MET depletion by MEGC-PEG-rMETase in a dose-dependent manner. PLP extended the half-life of MEGC-PEG-rMETase holoenzyme ~ 4 – 5 -fold in a dose-dependent manner, which resulted in a ~ 10 -fold increase in time of plasma MET depletion to $\leq 5 \mu\text{M}$. PLP appeared to either associate with the MEGC-PEG-rMETase apoenzyme or prevent PLP dissociation from the holoenzyme in the plasma. Although PLP remains tightly bound to the enzyme *in vitro*, it readily dissociates from the enzyme *in vivo*. In a recent study with macaque monkeys, MEGC-PEG-rMETase had an extended apoenzyme half-life of 90–143 h,⁶ but holoenzyme half-life was only 1.5 h. The results of the present study in mice suggest that PLP would greatly extend holoenzyme half-life in primates as well as

humans. The combination of MEGC-PEG-rMETase treatment with PLP supplementation suggests an effective clinical strategy for long-term MET depletion to arrest cancer cell growth.

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