De Novo Engineering of a Human Cystathionine-γ-Lyase for Systemic L-Methionine Depletion Cancer Therapy

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Ⓢ Supporting Information

ABSTRACT: It has been known for nearly a half century that human tumors, including those derived from the nervous system such as glioblastomas, medulloblastoma, and neuroblastomas are much more sensitive than normal tissues to L-methionine (L-Met) starvation. More recently, systemic L-Met depletion by administration of *Pseudomonas putida* methionine-γ-lyase (MGL) could effectively inhibit human tumors xenografted in mice. However, bacterial-derived MGLs are unstable in serum ($t_{1/2} = 1.9 \pm 0.2$ h) and highly immunogenic in primates. Since the human genome does not encode a human MGL enzyme, we created *de novo* a methionine degrading enzyme by reengineering the structurally homologous pyridoxal phosphate-dependent human enzyme cystathionine-γ-lyase (hCGL). hCGL degrades L-cystathionine but displays no promiscuous activity toward L-Met. Rational design and scanning saturation mutagenesis led to the generation of a variant containing three amino acid substitutions (hCGL-NLV) that degraded L-Met with a k_{cat}/K_M of 5.6×10^2 M^{-1} s^{-1} and displayed a serum deactivation $t_{1/2} = 78 \pm 5$ h (non-PEGylated). *In vitro*, the cytotoxicity of hCGL-NLV toward 14 neuroblastoma cell lines was essentially indistinguishable from that of the *P. putida* MGL. Intravenous administration of PEGylated hCGL-NLV in mice reduced serum L-Met from 123 μM to <5 μM for over 30 h. Importantly, treatment of neuroblastoma mouse xenografts with PEGylated hCGL-NLV resulted in near complete cessation of tumor growth. Since the mode of action of hCGL-NLV does not require breaching the blood-brain barrier, this enzyme may have potential application for sensitive tumors that arise from or metastasize to the central nervous system.
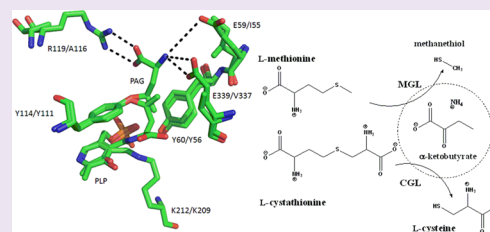
L-Methionine (L-Met) depletion has long been studied as a potential treatment for cancer, as many malignant human cell lines and tumors have a substantially higher requirement for L-Met than normal cells and tissues.[1-5] Depletion of any essential amino will disrupt protein synthesis, but L-Met is also required for polyamine synthesis, a known contributor to tumorigenesis;[6-8] furthermore, it is the major methylation source for DNA and other molecules.[9] L-Methionine-dependent tumor cell lines display an abnormally high demand for that amino acid and, additionally, can show defects in the expression of methionine synthase that recycles L-homocysteine to L-Met and of methylthioadenosine phosphorylase (MTAP) which cleaves methylthioadenosine to 5-methylthioribose-1-phosphate that is further metabolized to L-Met.[5,10-17] Most nonmalignant cells can grow on homocysteine/homocystine, whereas malignant cells must scavenge L-Met directly from their extracellular environment. When L-Met levels decrease from the normal human serum concentration of ~30 μM [18] to a threshold around ~5 μM, these tumors are unable to survive.[19] *In vivo*, a significant decrease in serum L-Met can be achieved by the systemic application of methionine-γ-lyase from *Pseudomonas putida* (pMGL) that degrades L-Met to α-ketobutyrate, methane thiol, and NH$_3$ resulting in drastic retardation of tumor growth in a variety of animal models and has been

shown to have synergistic effects in combination with chemotherapeutic agents such as 5-fluorouracil and vincristine.[20-27] However, pMGL is rapidly inactivated *in vitro* (this work) and *in vivo*[28,29] and has proven to be highly immunogenic in primate models.[20]

To circumvent the significant limitations of bacterial MGLs for human therapy, we developed a human methionine-γ-lyase by protein engineering. Cystathionine-γ-lyase (CGL) is the last enzyme of the mammalian transsulfuration pathway for the conversion of L-methionine to L-cysteine.[30] The human CGL (hCGL) displays 61% amino acid similarity with the *P. putida* MGL and 62% similarity to the MGL from *Trichomonas*. These enzymes are structural homologues (Figure 1a, structural alignment of hCGL and MGL from *Trichomonas*) and belong to the γ-family of pyridoxal phosphate (PLP) dependent enzymes. Although MGL and CGL are related in their reaction chemistry (Figure 1b), CGL catalyzes the α,γ-elimination of L-cystathionine to L-cysteine, α-ketobutyrate, and NH$_3$ but shows no detectable catalytic activity with L-Met. Using rational design and scanning saturation mutagenesis, coupled with a novel high
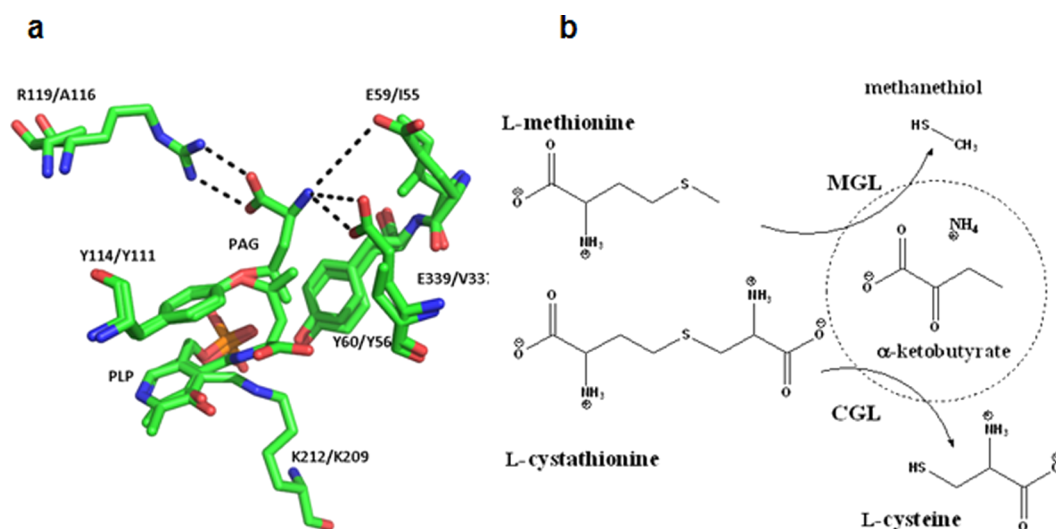
Figure 1. (a) Structural overlay of human CGL and MGL from Trichomonas vaginalis (PDB 3COG:1E5E) with the inhibitor propargylglycine (PAG) bound in two different orientations. (b) Reactions catalyzed by CGL and MGL.

throughput screen for methionine-γ-lyase activity, we engineered an hCGL variant containing 3 amino acid substitutions (hCGL-NLV) that displayed therapeutically significant rates of L-Met degradation. Biochemical analysis suggested possible mechanisms for the change in the selectivity of the enzyme from L-cystathionine to L-Met. hCGL-NLV was shown to be much more stable to deactivation in serum relative to the *P. putida* MGL and exhibited favorable *in vitro* cytotoxicity toward a large panel of human neuroblastoma cell lines. We show that administration of PEGylated hCGL-NLV into mouse xenografts bearing neuroblastoma tumors resulted in near complete cessation of tumor growth indicating that the engineered enzyme holds promise for cancer therapy.

Among enzyme therapies in cancer, asparaginase is one of the oldest and most successful. Its inclusion has led to a >90% cure rate for standard risk childhood ALL.[31,32] Currently, only bacterial enzymes derived from *E. coli* and *Erwinia chrysanthemi* (synonymous with Erwinia carotovora) are available. Pharmacokinetic studies have shown that asparaginase activity ≤0.4 U/mL provided insufficient deamination of ASN, whereas >0.4−0.7 U/mL was required for optimal (90%) ASN and glutamine deamination,[33] both important predictors of improved survival. Since bacterial asparaginase is highly immunogenic, antibody response in patients inactivates the enzyme, shortens its half-life, and induces anaphylaxis. Despite clinical success, asparaginase therapy remains limited by its immunogenicity.[34]

As hCGL-NLV is an engineered human protein, it is expected to be subject to immune tolerance, and moreover, computational predictions indicate that the 3 amino acid substitutions are unlikely to generate a T cell neo-epitope. While, as with any protein therapeutic candidate, it is impossible to rule out the possibility that hCGL-NLV could elicit antibody responses in some patients, the extensive clinical data with approved engineered antibodies[35,36] supports the notion that the introduction of a small, carefully selected number of amino acid substitutions into engineered human proteins does not generally result in an adverse immunogenicity profile.

RESULTS AND DISCUSSION

(See Supporting Information for additional Results of Construction of Synthetic Genes, Protein Expression and Purification, Detection limits of DTNB assay.)

In addition to their native substrates, both hCGL and pMGL exhibit appreciable activity toward L-cysteine and DL-homocysteine (Table 1). However, L-Met is not a substrate of hCGL,

Table 1. Michaelis−Menten Kinetics^a

substrate	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (s^{-1} mM^{-1})
P. putida Methionine-γ-lyase			
L-methionine	20 ± 0.4	0.34 ± 0.03	59 ± 6
L-cystathionine	nd	nd	nd
L-cysteine	6.5 ± 0.2	1.1 ± 0.1	6 ± 0.7
DL-homocysteine	86 ± 3	2.9 ± 0.2	30 ± 3
Human Cystathionine-γ-lyase			
L-methionine	nd	nd	nd
L-cystathionine	3.7 ± 0.2	0.40 ± 0.07	9 ± 2
L-cysteine	0.15 ± 0.02	0.79 ± 0.30	0.2 ± 0.1
DL-homocysteine	0.35 ± 0.02	1.7 ± 0.30	0.21 ± 0.04
Human Cystathionine-γ-lyase-NLV			
L-methionine	7.9 ± 0.4 (≥10^{6b})	14 ± 1.5	0.56 ± 0.07
L-cystathionine	0.54 ± 0.02	0.83 ± 0.1	0.65 ± 0.07 (−14)
L-cysteine	2.7 ± 0.1	0.84 ± 0.1	3.2 ± 0.3 (16)
DL-homocysteine	2.6 ± 0.1	0.9 ± 0.1	2.8 ± 0.3 (13)

^{a}nd = not detected; () = fold change from hCGL. ^{b}Estimated from DTNB assay limits. All reactions were performed at 37 °C with the MBTH assay.

and likewise, pMGL has no activity with L-cystathionine as substrate, within the detection limit of our assay (see Supporting Information) (Table 1). Thus, hCGL does not exhibit promiscuous activity toward substrates with a methylthioethane side chain.[37] Structural overlays of hCGL and an MGL from *Trichomonas* revealed that, in hCGL, the side-chains of residues E59, R119, and E339 are important for the orientation of the cysteine terminus of its substrate, L-cystathionine, while in MGL, the corresponding positions are occupied by amino acids with hydrophobic side chains, namely, I55, A116, and V337 (Figure 1a). Positions 59, 119, and 339 in

hCGL were subjected to combinatorial pairwise saturation mutagenesis, and the resulting clones were screened for methionine-γ-lyase activity using a novel high throughput plate assay that relies on the colorimetric detection of the α-ketobutyrate product of the reaction using 3-methylbenzothiazolin-2-one hydrazone (MBTH).[38] Clones expressing mutagenized hCGL were grown in microtiter well plates to midlog phase; the cells were lysed chemically and incubated with 5 mM L-Met at pH 7.3 and 37 °C followed by MBTH, and the Abs_{320} was determined spectrophotometrically. Screening of >2000 colonies (2× theoretical diversity) from a saturation library (library A) at positions 119 and 339 gave 14 unique clones (Supporting Table S1, Library A) capable of producing α-ketobutyrate from L-Met. The active clones encoded hCGL with Pro or Val substitutions at pos. 339 with the latter resulting in higher product formation. The clone with the highest activity, hCGL(R119L, E339 V), was used as a template to randomize the E59/R119L codons. Screening of an additional 2000 clones led to the isolation of 31 clones exhibiting greater α-ketobutyrate formation than the hCGL-(R119L, E339 V) (Supporting Table S2, Library B). The respective enzymes were produced and purified by IMAC in small scale and then rank-ordered with respect to their relative second order rate constants (apparent k_{cat}/K_M when [S] is ≪ K_M) in a reaction with 0.1 mM L-Met and continuous monitoring of methane thiol formation with 5,5′-dithiobis-2-nitrobenzoic acid (DTNB, Supporting Table S3). The highest apparent k_{cat}/K_M was obtained with hCGL (E59N, R119L, and E339 V) designated hCGL-NLV. Steady-state Michaelis–Menten parameters for pMGL, hCGL, and hCGL-NLV with L-Met, L-cystathionine, L-cysteine, or DL-homocysteine were determined in 100 mM phosphate buffer, pH 7.3 at 37 °C (Table 1). hCGL-NLV displayed significant methionine-γ-lyase activity as well as increased activity toward L-Cys and DL-homocysteine. However, this enzyme had approximately 15-fold lower k_{cat}/K_M for L-cystathionine, the physiological substrate of hCGL, suggesting that its methionine-γ-lyase activity is not simply a consequence of broader substrate specificity, a rather common outcome in enzyme engineering. For comparison, the kinetics of hCGL-E339 V, hCGL-E339P, hCGL-R119Q-E339 V, hCGL-E339 V-R119L, and hCGL-E59A-R119S- E339 V (hCGL-ASV) for the degradation of L-Met and L-cystathionine were also determined (Supporting Table S4). Consistent with the results of the screening assay, hCGL-NLV displayed better kinetics than either the single or the double mutant enzymes above.

Of importance to therapeutic applications, (non-PEGylated) hCGL-NLV displayed substantially greater stability in pooled human serum at 37 °C relative to the *P. putida* MGL with $t_{1/2}$ deactivation of 78 ± 5 h in contrast to 1.9 ± 0.2 h for the bacterial enzyme (Supporting Figure S1). Although hCGL-NLV has activity *in vitro* with other plasma metabolites (e.g., L-cystathionine, L-Cys, and L-homocysteine (L-Hcys)), it is not expected that this will greatly affect their homeostasis *in vivo*. Plasma L-cystathionine is excreted in the urine at a rate of 38.5 μM/day and maintained in the serum at concentration of 0.24 ± 0.06 μM[39] at which concentrations of hCGL-NLV would have the negligible turnover rate of 1.5×10^{-4} s^{-1} on its degradation. In human plasma, L-Cys and L-homocysteine exist either as the free amino acids, the (mixed) disulfide form, or bound to serum proteins at concentrations around 170 μM (54% free) and 6 μM (31% free), respectively.[40] Consistent with the existence of a protected fraction of L-Cys and L-Hcys,

mice injected with pMGL were shown to have a 90% reduction in L-Met but only a 46% reduction in L-Hcys and no effect on L-Cys levels.[41] hCGL-NLV is 2- and 10-fold less active toward L-Cys and L-homocysteine, respectively, relative to pMGL, and therefore, its effect on these metabolites would be expected to be significantly lower.

L-Met Inhibition of hCGL. It was surprising that we could not detect any L-Met hydrolysis with hCGL at any concentration despite its smaller size compared to L-cystathionine. DL-Homocysteine is also smaller than L-cystathionine, yet it is degraded by hCGL, though with a 40-fold lower k_{cat}/K_M. We examined the hypothesis that DL-homocysteine is a substrate of hCGL, whereas L-Met is not due to leaving group effects. Degradation of homocysteine leads to H_2S (pK_a = 7),[42] whereas L-Met degradation leads to methanethiol (pK_a = 10.3 [42]). The possibility that L-Met binds to the active site but the γ-lyase activity is disfavored by the high pK_a of methanethiol could be ruled out since we found that even 20 mM L-Met did not inhibit L-cystathionine degradation by hCGL. This observation raised the possibility that the relatively hydrophobic L-Met molecule is unable to partition into the hydrophilic active site of hCGL.

Hydropathy Studies. The log P values of the active-site residues unique to hCGL were summed (Σ log P) to yield a value = −19.7 (hydrophilic), while the Σ log P value of the pMGL unique active-site is −2.87 (hydrophobic). These values correlate well with the log P values of their respective substrates (L-cystathionine = −5.82; L-Met = −2.19). Consistent with this hypothesis, comparison of the active-site hydropathy values (Σ log P) of hCGL, hCGL-E339 V, hCGL-E339P, hCGL-E339Q-R119L, hCGL-E339 V-R119L, hCGL-ASV, and hCGL-NLV with the log k_{cat}/K_M values (log 10 value of k_{cat} divided by K_M values found from fits to the Michaelis–Menten equation) for L-Met and L-cystathionine hydrolysis revealed a positive linear relationship for the turnover of L-Met and a negative linear relationship for the turnover of L-cystathionine (Figure 2). This appears to hold true for DL-homocysteine and L-Cys as well those that have intermediate hydropathy values, log P = −2.58 and −2.79, respectively. DL-Homocysteine and L-Cys are substrates that can partition into the hCGL active-site, and
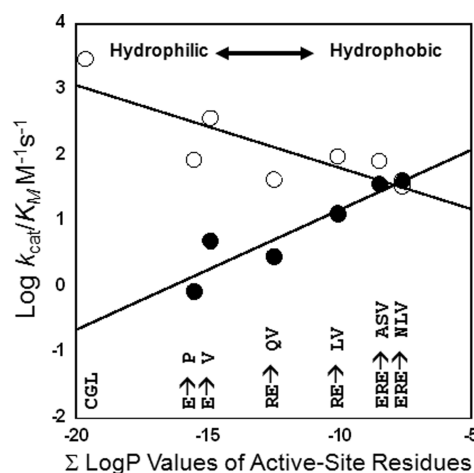
Figure 2. Plot of the sum of the calculated log P values of residues in the active sites of hCGL, hCGL-E339P, hCGL-E339 V, hCGL-R119L-E339Q, hCGL-R119L-E339 V, hCGL-ASV, and hCGL-NLV versus their log k_{cat}/K_M values for the hydrolysis of L-Met (●) and L-Cystathionine (○).

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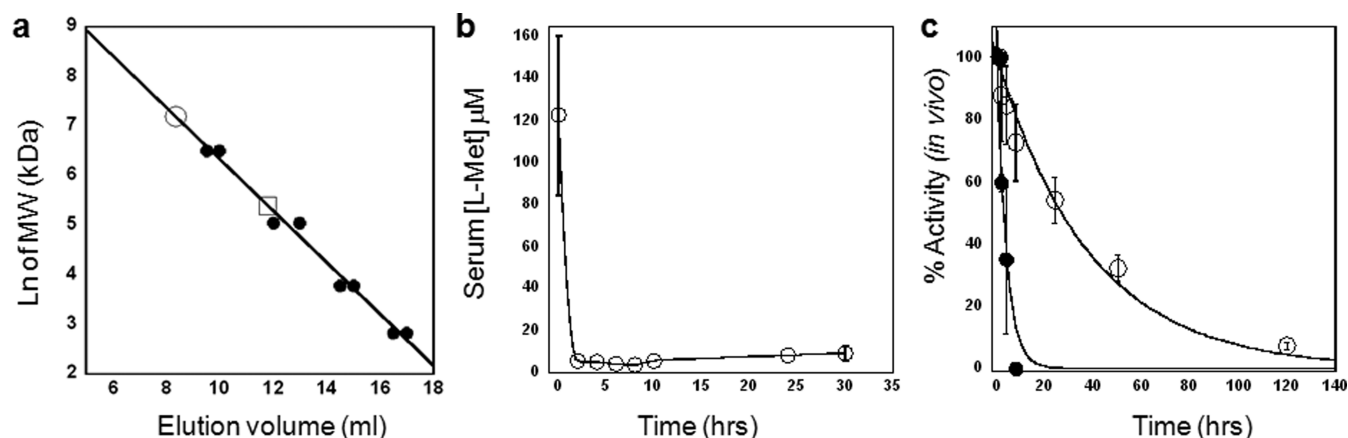
Figure 3. (a) Analytical size exclusion chromatography of MW standards (●), hCGL-NLV (□), and PEGylated hCGL-NLV (○). (b) Pharmacodynamics of PEG-hCGL-NLV in balb/c mice maintained on a Met(−)Hcys(−)Chl(−) diet. Mice ($n = 5$) were treated with 200 U PEG-hCGL-NLV administered by tail-vein injection and blood methionine level (○) was monitored by HPLC. (c) *In vivo* activity of PEG-hCGL-NLV (○) and unPEGylated hCGL-NLV (●) in serum as a function of time following tail-vein injection of 100U PEG-hCGL-NLV in athymic mice ($n = 5$).

hCGL-NLV with a more hydrophobic active site ($\Sigma \log P = -7.64$) increases the k_{cat}/K_M for these substrates 13- and 6-fold, respectively. It should be noted that, in previous studies, when E339 of hCGL was mutated to either Lys, Ala, or Tyr, the k_{cat}/K_M for L-Cys increased by 2-, 3-, and 7-fold, respectively, results that are also consistent with the effect of active site hydrophobicity as a key determinant of CGL substrate specificity.[43]

Immunogenicity Assessment. To assess the likelihood that the three-point mutations (E59N-R119L-E339 V) in hCGL could generate a novel T cell epitope (i.e., generate a peptide fragment with affinity to MHC II), we compared the computationally predicted MHC(II) binding scores (consensus method) for peptides containing each mutated residue relative to the corresponding wt sequence for 8 of the most common HLA alleles that collectively cover nearly 95% of the human population.[44] The consensus percentile rank (CPR) score ratios indicated no significant change in predicted MHCII binding suggesting that the E59N-R119L-E339 V mutations are unlikely to introduce neo-epitopes (Supporting Table S5).

Pharmacological Optimization, *in Vitro* Cytotoxicity, and *in Vivo* Half Life of PEG-hCGL-NLV. To investigate the therapeutic efficacy of hCGL-NLV *in vivo*, the enzyme was PEGylated by conjugation to methoxy PEG succinimidyl carboxymethyl ester, MW 5000 Da at an 80:1 molar ratio (80 PEGs per subunit). Initial tests using PEG:enzyme ratios of 10:1, 20:1, 40:1, and 80:1 showed the greatest homogeneity at an 80:1 ratio (Supporting Figure S2). PEGylation has been extensively exploited to increase the hydrodynamic radius of proteins, preventing renal filtration and, in turn, markedly increasing circulation persistence; note that PEGylation significantly increased the circulation $t_{1/2}$ of the structurally homologous pMGL in mice and primates.[20] To prevent inactivation during PEGylation, it was essential to preincubate the enzyme with 10 mM pyridoxal phosphate (PLP), to avoid conjugation of K212 to the PEG succinimidyl ester. Analytical size exclusion chromatography of the resulting PEG-hCGL-NLV gave a single homogeneous peak with an apparent MW of 1300 KDa per tetramer, a 6-fold increase from the unmodified tetramer (Figure 3a). PEG-hCGL-NLV displayed L-Met degradation kinetics identical to those of the unmodified hCGL-NLV with $k_{cat} = 7.8 \pm 0.4$ s^{-1}, $K_M = 13.5 \pm 1.5$ mM, and

$t_{1/2} = 39 \pm 2$ h in pooled human serum (Supporting Figure S3). The specific activity of PEG-hCGL-NLV was 10 U/mg protein at 37 °C and pH 7.3 and had an endotoxin level of ≤12.5 EU/mg as assessed by Limulus Amebocyte Lysate assay. (The endotoxin received by the mice was ≫2 orders of magnitude lower than doses showing no observed effect in mice.[45,46]) The cytotoxicity of PEG-hCGL-NLV towards 14 neuroblastoma (NB) lines established to be L-Met-dependent[25] was compared to that of pMGL (the myelocytomatosis viral related oncogene, neuroblastoma derived (MYCN) amplification[47] status, anaplastic lymphoma kinase (ALK) mutation[48] status, α-thalassemia/mental-retardation-syndrome-X-linked (ATRX) mutation[49] status, and IC$_{50}$ values are summarized in Supporting Table S6). While PEG-hCGL-NLV has a lower k_{cat}/K_M than pMGL for L-Met, its much higher stability in serum and in growth media led to nearly identical IC$_{50}$ values with an average of 0.091 ± 0.043 U and 0.086 ± 0.037 U, respectively. In Balb/c mice ($n = 5$) maintained on a L-Met(−) homocysteine(−)choline(−) {Met(−)Hcys(−)Chl(−)} diet, tail-vein administration of 200 U PEG-hCGL-NLV, led to a decrease in the serum L-Met concentration from 124 ± 37 μM (pretreatment) to 3.9 ± 0.7 μM at 8 h postinjection. Maintaining the animals on a Met(−)Hcys(−)Chl(−) diet during treatment is necessary due to the high metabolic rate of mice, which leads to rapid replenishment of the serum L-Met pool following depletion.[19,25] (It is not yet known if L-Met depletion therapy in humans will necessitate that the patients are placed on L-Met-deficient diet. The serum L-Met replenishment rate in man is estimated to be roughly 2 μM/h (est. from ref 24) compared to ∼4 μM/h in mice (est. from ref 50). An L-Met deficient diet would likely augment a treatment regimen but may not be necessary with an enzyme displaying a good PK/PD profile.) In mice treated with PEG-hCGL-NLV, the serum L-Met was maintained at a therapeutically relevant level for at least 30 h (Figure 3b). This is on par with various formulations of PEGylated-pMGL, which have been reported to maintain serum L-Met levels in mice at <5 μM for 8 h in one study[50] to 48 h in an earlier report.[28] The PEG-hCGL-NLV activity in the blood of the injected mice decreased with a $t_{1/2}$ of 30 ± 3 h (Figure 3c), much longer than non-PEGylated hCGL-NLV ($t_{1/2} = 1.8 \pm 0.7$ h).

Therapy of Established NB Xenografts. Athymic mice bearing LAN-1 neuroblastoma tumor xenografts were treated with 100 Units PEG-hCGL-NLV, administered via tail-vein injection once daily for 3 times a week for 4 weeks. Met(−)Hcys(−)Chl(−) diet was fed to the animals on the days of PEG-hCGL-NLV administration. Xenografted control mice were either fed a constant normal diet or a Met(−)-Hcys(−)Chl(−) diet 3 times a week for 4 weeks using an identical schedule as in the treatment group. Twenty-four hours after the third PEG-hCGL-NLV injection during the last (4th) week of treatment, plasma methionine concentration was 6 ± 2 μM among PEG-hCGL-NLV-treated mice fed Met(−)-Hcys(−)Chl(−) diet, in contrast to 124 ± 37 μM before treatment ($n = 10$). Mice fed Met(−)Hcys(−)Chl(−) diet exhibited a 10−15% reversible weight loss (Supporting Figure S4). While control mice on Met(−)Hcys(−)Chl(−) diet only had no significant reduction in tumor growth, the administration of PEG-hCGL-NLV completely inhibited the growth of the LAN-1 tumors during the treatment period ($p < 0.01$, Figure 4). Even three weeks after the last round of treatment
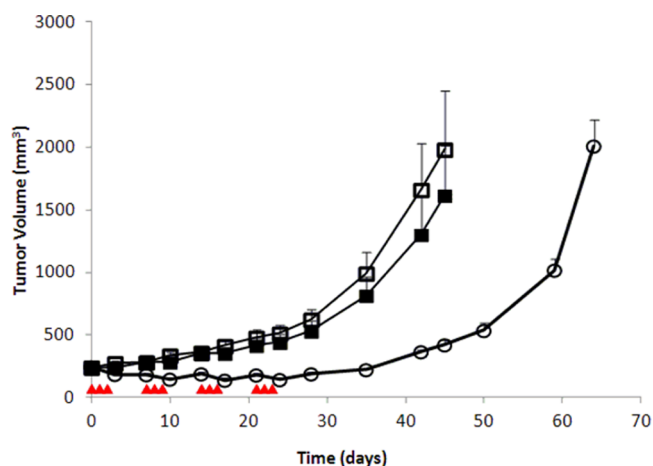
Figure 4. Evaluation of PEG-hCGL-NLV in athymic mice bearing LAN-1 xenografts. (□) Control animal group maintained on normal diet ($N = 10$); (■) animals maintained on Met(−)Hcys(−)Chl(−) diet ($N = 10$); (○) animals treated with 100 U PEG-hCGL-NLV in combination with Met(−)Hcys(−)Chl(−) mouse feed ($N = 10$). Treatment days are designated by (▲). Tumor growth rate was expressed as mean ± SEM (standard error of the mean) for each group. *, $p < 0.01$ for the PEG-hCGL-NLV treated group relative to the two untreated groups.

with PEG-hCGL-NLV, rebound tumor volume was small (0.5 fold increase), whereas in the control groups, tumor growth increased 3- and 4-fold in mice maintained on Met(−)-Hcys(−)Chl(−) or on normal diet, respectively.

■ SUMMARY

The differential susceptibility of malignant and nonmalignant cells to L-Met starvation provides a useful therapeutic window for cancer therapy. Even partial depletion solely through L-Met omission in a parenteral nutrition (TPN) regimen has been shown to result in tumor reductions in gastric cancer in both the rat model and in humans.[51−53] However, dietary restriction alone only results in ~40% decrease in serum L-Met levels.[54] A much more significant reduction in the serum L-Met concentration, resulting in drastic inhibition of tumor growth in a number of animal models,[27,41,55,56] has been achieved by

the administration of MGLs. Humans do not encode an MGL, and consequently, bacterial enzymes have been investigated as agents for L-Met depletion therapy. In particular, the *P. putida* MGL was found to display the most favorable catalytic properties, having a low K_M and a relatively high k_{cat}.[57,58] Administration of pMGL was shown to drastically affect the growth of human neuroblastoma and lung, colon, and brain tumors in mouse xenografts maintained on L-Met restricted diet.[21,22,25,59] Unfortunately, both in mice and in the nonhuman primate model, pMGL was shown to be deactivated very rapidly with a half-life of approximately 2 h due to the loss of the PLP cofactor.[26] Nonetheless, pMGL given at a 90 mg/kg dose every 8 h for 2 weeks resulted in a steady-state depletion of plasma methionine to less than 2 μM in animals maintained on L-Met restricted diet. Only mild toxicities were observed and manifested with decreased appetite and slight weight loss. However, anti-MGL antibodies were detected after administration, and an additional round of treatment with pMGL resulted in anaphylactic shock and death in some animals.[26] Yang et al. sought to increase the pharmacokinetic half-life and reduce the immunogenicity of MGL by amine conjugation to polyethylene glycol (PEGylation). PEGylation of pMGL drastically improved circulatory persistence (≥36 fold) in primates and attenuated severe immunological responses (with anti-MGL antibody titers increasing upon repeated treatments); however, only a marginal improvement in pharmacodynamics was observed (serum L-Met was maintained at <5 μM for 12 h vs 8 h for the unPEGylated enzyme).[20,29] PEGylation of bacterial and even of mammalian proteins appears to delay but not eliminate immune responses as evident in clinical studies of PEGylated *M. arginii* arginine deiminase[60] or pegloticase.[61]

To augment the antineogenic potential of systemic L-Met depletion and overcome the disadvantages observed with pMGL, we engineered hCGL into a pharmacologically efficacious human methionine-γ-lyase. Although the overall structures of hCGL and pMGL align with a root-mean-square (rms) deviation of 0.8 Å, they only share a 61% amino acid sequence similarity. Many of the differences are seen in the degree of hydrophobicity in the active sites, which correlates with the relative hydrophobicities of their substrates. The hCGL-NLV is engineered from a native human enzyme, and thus, it is likely to be much less immunogenic than pMGL circumventing problems with adverse immune responses, including neutralizing antibodies and anaphylactic reactions observed in earlier studies. L-Met depletion may be particularly attractive for the treatment of brain tumors, a hypothesis we plan to test in future studies using orthotopic glioblastoma mouse models.[62] The paucity of chemotherapeutics that can target tumors from across the blood-brain barrier makes systemic L-Met depletion an appealing strategy for the treatment of tumors that arise from or metastasize to the central nervous system, both significant challenges in cancer treatment.

■ MATERIALS AND METHODS

(See Supporting Information for additional Materials and Methods of Construction of Synthetic Genes; Saturation Mutagenesis Libraries; Construction of hCGL-E339 V Variant; Protein Expression and Purification; Kinetic Assays; 96-Well Plate Screen; L-Met Inhibition of hCGL; Immunogenicity Calculations; WST8 Assay for NB Cell Proliferation; *in Vitro* Half-Lives; Serum Stability; Pharmacological

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Optimization of hCGL Variants; Methionine Depletion in Mouse Plasma; and Mouse Feed.)

Hydropathy Calculations. Using structural alignments of hCGL (3COG) and pMGL (2O7C) as a guide, we identified residues in the active site within 5−6 Å of a covalently bound inactivator, propargylglycine. We found that F58, L62, C116, A119, and V339 were unique to the pMGL active-site, whereas in hCGL, these residues correspond to E59, S63, G116, R119, and E339, respectively. By convention, the relative hydropathy of a solute is described by the logarithm of its octanol−water partition coefficient (log P). Using ChemAxon software, we calculated the log P values of each of the aforementioned residues with their α amino and carboxy termini in the amide form.[63] (We used this method because the log P values are in good agreement with available experimental values and allowed us to estimate log P values for compounds without known reference values like L-cystathionine.) We also calculated the active site log P values for three hCGL variants, hCGL-E339 V, hCGL-R119L -E339 V, and hCGL-E59N-R119L-E339 V (hCGL-NLV). These log P values were summed for each enzyme to calculate an overall hydropathy value for each active site and plotted against the log k_{cat}/K_M values for each enzymes ability to hydrolyze L-Met and L-cystathionine (this is similar to an analysis of lipase performed by Hirata et al.[64]). We also calculated the log P values for the substrates L-Met, L-homocysteine, L-Cys, and L-cystathionine.

Tumor Cell Lines. NB cell line LAN-1 was obtained from Dr. Robert Seeger (Children's Hospital of Los Angeles; Los Angeles, CA), and NB cell line NMB-7 was from Dr. Liao of McMaster University (Hamilton, ON, Canada). SK-N-BE(1)N, SK-N-BE(2)C, SK-N-BE(2)N, SK-N-BE(2)S, LAI-5S, SH-EP-1, 55N, and 66N were kindly provided by Dr. Robert Ross, Fordham University (New York, NY). NB cell lines SK-N-LD and SK-N-MM were established at Memorial Sloan-Kettering Cancer Center (MSKCC, New York, NY). IMR-32 and CHP-212 were purchased from American Type Culture Collection (Manassas, VA). Cells were cultured in RPMI with 10% fetal calf serum (F10) as previously described.[65]

In Vivo **Half-Lives.** hCGL-NLV and PEG-hCGL-NLV activity was assayed on sera following injection of 50 U of enzyme in nude mice. Serum samples were obtained at 1, 2, 4, 8, 24, 50, and 120 h after injection. Half-lives of enzymatic activity was calculated by SigmaPlot using the exponential decay equation ($y = ae^{-bx}$).

Therapy of Established NB Xenografts. All animal experiments were carried out according to an Institutional Animal Care and Use Committee (IACUC) approved protocol at Memorial Sloan-Kettering Cancer Center, following institutional guidelines for the proper and humane use of animals in research. Athymic female nude mice were purchased from the National Cancer Institute. Mice with 5−10 mm established tumors were randomly separated into groups of 10 mice each. In mice xenografted with LAN-1 neuroblastoma, 100 units of PEG-hCGL-NLV was given IV three times a week for 4 weeks. The 0.5−1 cm tumors are generally regarded as standard for tumor therapy studies since Institutional Animal Care and Use Committee (IACUC) requirement stipulates that mice with tumors that are >15% body weight (or 2 cm maximal tumor diameter) cause undue distress, and these animals have to be sacrificed. Tumor volume was calculated as $(a^2b)/2$ where a is the width of the tumor (small diameter) and b the length (large diameter), both in millimeters.[66] All of the tumors were growing at the time when the treatment was started, and all of the mice finally died of tumor when the treatment was stopped. There were no spontaneous regressions in this experiment and in this tumor model. Met(−)Hcys(−)Chl(−) diet was fed to the animals on the days of PEG-hCGL-NLV administration. Mice were bled 24 h after the third PEG-hCGL-NLV injection during the last week of treatment. The weight of mice was measured twice weekly and normalized to the weight at day 0 (start of experiment) and used as an index of toxicity. A 20% maximum weight loss was used as a guideline in designing PEG-hCGL-NLV dosing, and none of the mice required sacrificing because of toxicity. Tumor size was measured twice per week and expressed as mean ± SEM (standard error of the mean) for each group. Mice were sacrificed when their tumor sizes exceeded 20 mm in diameter.

■ ASSOCIATED CONTENT

ⓢ Supporting Information

Descriptions of the Constructed Synthetic genes, Protein Expression and Purification; Construction of Synthetic Genes; Saturation Mutagenesis Libraries; Construction of hCGL-E339V variant; Protein Expression and Purification; Kinetic Assays; 96-well plate screen; L-Met Inhibition of hCGL; Immunogenicity Calculations; WST8 Assay for NB Cell Proliferation; in vitro half-lives; Serum Stability; Pharmacological Optimization of hCGL Variants; Methionine Depletion in mouse plasma; Mouse Feed. Tables with data including relative activity of clones from Library A and B, Relative apparent k_{cat}/K_M of select clones from Library B, Kinetics (k_{cat}/K_M) of hCGL, and variants E339V, E339P, R119Q-E339V, R119L-E339V, and hCGL-NLV, Computational comparison of T-Cell epitopes, and Inhibition of in vitro proliferation of various neuroblastoma cell lines. Figures depicting stability of pMGL and hCGL-NLV incubated in pooled human serum, SDS-PAGE gel of purified hCGL-NLV and hCGL-NLV conjugated to PEG, stability of PEG-hCGL-NLV incubated in pooled human serum, and mouse toxicity as assessed by body mass. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): E.S. and G.G. are inventors on a patent application related to the work described here.

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