

# Effects in Cancer Cells of the Recombinant L-Methionine Gamma-Lyase from *Brevibacterium aurantiacum*. Encapsulation in Human Erythrocytes for Sustained L-Methionine Elimination<sup>§</sup>

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Received January 12, 2019; accepted April 1, 2019

## ABSTRACT

Methionine deprivation induces growth arrest and death of cancer cells. To eliminate L-methionine we produced, purified, and characterized the recombinant pyridoxal 5'-phosphate (PLP)-dependent L-methionine  $\gamma$ -lyase (MGL) BL929 from the cheese-ripening *Brevibacterium aurantiacum*. Transformation of an *Escherichia coli* strain with the gene BL929 from *B. aurantiacum* optimized for *E. coli* expression led to production of the MGL-BL929. Elimination of L-methionine and cytotoxicity in vitro were assessed, and methylation-sensitive epigenetics was explored for changes resulting from exposure of cancer cells to the enzyme. A bioreactor was built by encapsulation of the protein in human erythrocytes to achieve sustained elimination of L-methionine in extracellular fluids. Catalysis was limited to

$\alpha,\gamma$ -elimination of L-methionine and L-homocysteine. The enzyme had no activity on other sulfur-containing amino acids. Enzyme activity decreased in presence of serum albumin or plasma resulting from reduction of PLP availability. Elimination of L-methionine induced cytotoxicity on a vast panel of human cancer cell lines and spared normal cells. Exposure of colorectal carcinoma cells to the MGL-BL929 reduced methyl-CpG levels of hypermethylated gene promoters including that of CDKN2A, whose mRNA expression was increased, together with a decrease in global histone H3 dimethyl lysine 9. The MGL-erythrocyte bioreactor durably preserves enzyme activity in vitro and strongly eliminates L-methionine from medium.

## Introduction

Methionine deprivation in cancer cells induces growth arrest and cell death, whereas normal cells are much more resistant (Halpern et al., 1974; Hoffman and Erbe, 1976; Stern et al., 1984).

The present work was supported by the Institut du Cancer et d'Immunogénétique (ICIG), Hospital Paul-Brousse, 94800 Villejuif, France. D.M. and P.Bo. are coauthors of US patent 9,051,562 B2, June 9, 2015, *Polypeptides isolated from Brevibacterium aurantiacum and their use for the treatment of cancer*. Assignees: Institut National de la Santé et de la Recherche Médicale (INSERM); Institut National de la Recherche Agronomique (INRA); and Université Paris Sud-Paris XI.

<https://doi.org/10.1124/jpet.119.256537>.

<sup>§</sup> This article has supplemental material available at [jpet.aspetjournals.org](http://jpet.aspetjournals.org).

Depletion of L-methionine can be achieved by methionine  $\gamma$ -lyases (L-methionine- $\alpha$ -deamino- $\gamma$ -mercaptoethane-lyase; MGL), pyridoxal 5'-phosphate (PLP) enzymes that catalyze  $\alpha,\gamma$ -elimination of L-methionine resulting in production of methanethiol,  $\alpha$ -ketobutyrate, and ammonia. Various MGLs have been obtained by extraction or by recombination of genes from bacteria, protozoa, and plants (Dias and Weimer, 1998; Sun et al., 2003; Tokoro et al., 2003; Sato and Nozaki, 2009). The MGL from *Pseudomonas putida* (*P. putida*-MGL) exerts cytotoxicity in most tumor cell lines in vitro, and enhances the effect of 5-fluorouracil (FUra). Potentiation is accompanied by changes in folate pools (Machover et al., 2001) and by a decrease in DNA methylation (Machover et al., 2002). Enhancement of

**ABBREVIATIONS:** ANOVA, analysis of variance; DMEM, Dulbecco's modified Eagle's medium; HSA, human serum albumin; IC<sub>50</sub>, 50% inhibitory concentrations; IPTG, isopropyl  $\beta$ -D-1-thiogalactopyranoside; 5-meC, 5-methylcytosine; MGL, L-methionine- $\alpha$ -deamino- $\gamma$ -mercaptoethane-lyase; L-methionine  $\gamma$ -lyase; PBS, phosphate-buffered saline; PLP, pyridoxal 5'-phosphate; PM, pyridoxamine; PN, pyridoxine; PCR, polymerase chain reaction; SAM, S-adenosyl L-methionine.

FUra, cisplatin, and bis (2-chloroethyl)-nitrosourea cytotoxicity by *P. putida*-MGL was described in mice given repeated injections of *P. putida*-MGL (Yoshioka et al., 1998; Kokkinakis, 2006). However, attempts at reducing anaphylaxis of the native *P. putida*-MGL in primates through polyethylene glycol (PEG) conjugation resulted in substances with unstable activity, which slowed down its use in humans (Sun et al., 2003; Yang et al., 2004).

We hypothesized that immunogenicity of any MGL derived from parasitic and potentially pathogenic microorganisms would not allow systemic administration to humans in safe conditions (Sato and Nozaki, 2009). Moreover, excepting for the L-methionine  $\gamma$ -lyase from the plant *Arabidopsis thaliana* (Goyer et al., 2007), other MGLs as yet characterized, including that engineered from human cystathionine  $\gamma$ -lyase, eliminate L-cysteine, whose deficiency may cause undesirable effects (Hori et al., 1996; Kudou et al., 2008; Sato and Nozaki, 2009; Stone et al., 2012). The recombinant MGL-BL929 presented herein is derived from *Brevibacterium aurantiacum*, a microorganism abundantly present in food, this MGL having a possibility for human oral immune tolerance (Forquin et al., 2011; Steele et al., 2012). Furthermore, MGL-BL929 substrates are restricted to L-methionine and its precursor, L-homocysteine.

**Authorizations.** Murine experiments were performed under authorization no. 94-323 to INSERM, Hospital Paul-Brousse, and University Paris XI, Villejuif, France, issued from the Veterinary Services, Val-de-Marne, France. Human blood for encapsulation experiments was obtained from adult volunteers under protocol no. 6589 approved by the Ethics Committee of the University of Urbino Carlo Bo.

## Materials and Methods

### Source of the Recombinant L-Methionine $\gamma$ -Lyase BL929

The enzyme MGL-BL929 originates from *Brevibacterium aurantiacum* (strain ATCC 9175), a cheese-ripening microorganism characterized by release of typical cheese aroma, and orange pigment production. *Brevibacteriaceae* reach proportions greater than 5% of bacteria in a number of cheese varieties.

Studies of gene expression by transcriptome analysis in *B. aurantiacum* in presence of L-methionine showed upregulation of two adjacent genes, including a gene encoding for a methionine transporter (*metPS*) and the gene *BL929* (Forquin et al., 2011). Upregulation of *BL929* was accompanied by production of volatile compounds resulting from degradation of methionine. The sequence *BL929* was identified within the genome of *B. linens* BL2 described in the Integrated Microbial Genomes and Microbiomes system (IGM/M; <https://img.jgi.doe.gov/>) under Locus Tag BlinB01000978. The sequence *BL929* is composed of 1182 base pairs (Table 1, Sequence 1) corresponding to a translated protein of 393 residues (Table 1, Sequence 2). Protein sequence alignment (Clustal Omega; <https://www.ebi.ac.uk/Tools/msa/clustalo/>) showed identity rates ranging from 27.4% to 40.1% between the MGL-BL929 and other described methionine  $\gamma$ -lyases (e.g., percent identity was 27.4, 37.4, 37.2, 33.1, 33.7, 37.8, 36.7, 36.7, 38.2, 39.3, 37.5, 37.2, 40.1, and 38.5 between the MGL-BL929 and the MGLs from *Arabidopsis thaliana*, *Aeromonas hydrophila* spp., *Entamoeba histolytica* 1 and 2, *Pseudomonas putida*, *Trichomonas vaginalis* 1 and 2, *Citrobacter freundii*, *Porphyromonas gingivalis*, *Treponema denticola*, *Fusobacterium nucleatum*, *Clostridium novyi*, and *Clostridium sporogenes*, respectively).

### Production and Purification of MGL-BL929

The open reading frame (ORF) encoding MGL-BL929 was optimized to adapt *B. aurantiacum* codon usage to *Escherichia coli*

expression (Table 1, Sequence 3). The optimized sequence flanked with restriction sites for endonucleases NdeI and XbaI was synthesized and then subcloned in the *E. coli* pGTPc608 expression vector (pET9d derivative including PTac promoter, Lac operator, LacIq, multiple cloning sites, and T1T2 terminator; GTP Technology, Labège, France), according to GeneArt procedures (GeneArt, Regensburg, Germany). Plasmids (pGTPc608-BL929) were verified by digestion and sequencing (GeneArt).

A single colony of *E. coli* BL21 (DE3) production strain (Agilent Technologies, Santa Clara, CA) transformed with the pGTPc608-BL929 plasmid was grown in a 50-l Applikon bioreactor containing 35 liters of GYP medium (yeast extract 24 g/l, soy peptone 12 g/l,  $\text{KH}_2\text{PO}_4$  4.8 g/l,  $\text{K}_2\text{HPO}_4$  2.2 g/l, and glycerol 5 g/l) supplemented with PLP 0.02% (w/v). Expression was induced with IPTG when the  $\text{OD}_{600\text{nm}}$  of the culture reached 2. Concentrated feeding solution (30% w/v glycerol and 30% w/v yeast extract) was added with a remote-controlled pH flow-regulated pump. Cells were harvested when the culture reached  $\text{OD}_{600\text{nm}}$  of 40–50.

Cells were suspended in 20 mM Tris-HCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM PLP, pH 7.5, and lysed by high pressure homogenization. The MGL-BL929 was purified from cell extracts by chromatography in five steps consisting of one hydrophobic interaction chromatography and then two successive sequences of anion exchange chromatography followed by endotoxin removal. Briefly, cell extracts were clarified by centrifugation and then filtered through a 0.45- $\mu\text{m}$  low-level protein binding filter (Sartorius, Göttingen, Germany). Ammonium sulfate (AS) was added to the cell extracts to a final 0.75 M concentration. After centrifugation at 4°C, and then filtration through a 0.45- $\mu\text{m}$  low-level protein binding filter, cell extracts were injected in a column of Phenyl Sepharose (High Sub) 6 Fast Flow resin (GE Healthcare Europe, Vélizy, France). The column was equilibrated with 20 mM Tris-HCl, 0.75 M AS, 0.1 mM PLP, pH 7.5, and proteins were eluted by stepwise decrease in AS concentration. Elution fractions were pooled, concentrated using an ultra-filtration cassette with a 10 kDa cut-off (Pall, Port Washington, NY), and then diafiltered versus seven volumes of 20 mM Bis-tris, 0.1 mM PLP, pH 6.0. Elution fractions were passed through a column of DEAE Sepharose Fast Flow resin (GE Healthcare), equilibrated in 20 mM Bis-tris, 0.1 mM PLP, pH 6.0. Proteins were eluted by increasing NaCl concentration from 0 to 1 M. Elution fractions were concentrated using an ultra-filtration cassette, and then diafiltered versus seven volumes of 40 mM sodium phosphate buffer and 20  $\mu\text{M}$  PLP, pH 7.5. Elution fractions were filtered with a 0.2- $\mu\text{m}$  low-level protein binding filter (Corning, Union City, CA) and then endotoxins were removed using a High Capacity Endotoxin Removal column (HCER; Thermo Pierce). Sequential DEAE Sepharose and HCER chromatography was repeated once. Protein fractions were concentrated to 50–80 mg/ml. Enzyme concentration and purity were determined by Bradford assay, capillary electrophoresis, and SDS-PAGE. Endotoxin levels were assessed by Lonza (Testing Services). Purified MGL-BL929 in a solution of 40 mM sodium phosphate buffer, 100 mM NaCl, and 20  $\mu\text{M}$  PLP, pH 7.5, was stored at  $-80^\circ\text{C}$ .

### Characterization

Oligomerization was assayed by size exclusion chromatography using a Superdex 200 5/150 GL column (GE Healthcare). The ability of purified MGL-BL929 to catalyze elimination reactions was assessed with L-methionine, L-homocysteine, L-cysteine, L-cystathionine, S-adenosyl L-homocysteine, and D-methionine. Enzyme  $\geq 95\%$  pure was diluted in 100 mM potassium phosphate buffer, 0.01 mM PLP, pH 7.5, and 25 mM substrate. The mixture was incubated at 37°C for 5 minutes and sampled. The reaction was stopped by addition of trichloroacetic acid to a final concentration of 5%, and the  $\alpha$ -ketoacid formed was measured with 3-methyl-2-benzothiazolone hydrazone (MBTH) using spectrophotometry at 320 nm (Takakura et al., 2004). Kinetics for  $\alpha,\gamma$ -elimination catalysis was assessed with L-methionine and L-homocysteine. Studies of enzyme stability were done with the purified MGL-BL929 incubated

TABLE 1

DNA sequences of the L-methionine  $\gamma$ -lyase BL-929 and sequence of the protein

Sequence 1 DNA sequence from <i>Brevibacterium aurantiacum</i>	ATGACCTCACTGCACCCAGAAACGCTCATGGTCCACGGCGGAATGAAAGGCCTCACCGAG GCAGGAGTCCACGTACCGGCCATCGACCTCTCGACCACCAACCCAGTCAACGATGTCGCC ACCGGCGGTGACTCCTACGAATGGCTCGCCACCGGCCATACGCTCAAGGACGGCGACTCG GCCGTCTACCAAGCCCTCTGGCAGCCCGGTGTGCGACGCTTCGAGACCGCGCTGGCCGGG CTCGAACACGCTGAGGAAGCAGTCGCCCTTCGCCACGGGCATGGCCGCAATGACTGCCGCA CTTCTCGCGGCCGTGAGCGCAGGAACACCCACATCGTCGCGAGTGCCTCCCTCTATGGC GGAAGCGACCACCTCTCGAAACCGGACTGCTGGGGACACAGTACATGGGCAAAAGGAA GCCGACATCGCCTCGGCGATCCAAGATGACACCGGACTCGTCAATTGTCGAGACCCCGGCA AACCCAGCCTGGACCTTGTTGATCTCGACAGTGTGCTCTCAGCCGCGGCAACGTGCCT GTGCTGGTGGACAACACATTCTGCACACCTGTTCTCCAGCAGCCCATCTCCACGGAGCG GCCCTCGTACTGCACAGCGGACAAAATACCTCGGCGGTCTATGGCGATGCCATGGGCGGC ATCATCGCCACCAACGCGGACTGGGCGATGCGCCTGCGACAGGTCCGAGCCATCACAGGA GCCCTGCTCCACCCATGGGCGCGTATCTCTTCATCGGGGCTTGCGCACTCTGGCCGTG CGCATGCGCGCGGCTCAGACCACCGCGGTGAGCTCGCTGAGCGCCTGGACGCGCACCT GCCATCTCCGTCGTCCACTACCGGGACTGAAAGGCGCAAGCCACGCGGACTGCTCGGA CGCCAAATGTCCGGTGGTGGTGCATGATCGCGATGGAGCTCGCCGGTGGATTTCGACGCC GCCCGCAGCTTCGTGCAACACTGCAACCTCGTCTGTCACGCGGTGTCCCTGGGCGGCGCT GACACTCTCATCCAGCATCCGGCTCACTGACTACAGTCAAGTCCGCGCCACGCGGAAG CCCGCGGATGGTCTCATCCGACTCTCTGTGGGACTCGAACACGTGATGACCTGGCAGAC GATCTCATCGCTGCCCTCGACGCGAGTCGGGCGCGTGCCTGA
Sequence 2 Sequence of translated protein	MTSLHPETLMVHGGMKGLTEAGVHVPALDSTNPNVDVATGGDSYEWLATGHTLKDGD AVYQRLWQPGVARFETALAGLEHAEEAVAFATGMAAMTAALLAAVSAAGTTPHIVAVRPLYG GSDHLLTGLLGTVTWAKEADIASAIQDDTGLVIVETPANPSLDLVDLDSVSAAGNP VLVDNTFCTPVLQQPISHGAALVLHSATKYLGGHGDAMGGIATNADWAMRLRQVRAITG ALLHPMAYLLHRLRLAVMRMAAQTTAGELAERLDAHPAISVVHYPLKGGQDPRGLLG RQMSGGAMIAMELAGGFDAARSFVEHCNLLVHAVSLGGADTLIQHPASLTHRPVAATAK PGDGLIRLSVLEHVDLADDLIAALDASRAAA
Sequence 3 DNA sequence optimized for <i>E. coli</i> expression	ATGACCTCGCTGCATCCCGAGACGCTGATGGTTCATGGTGAATGAAGGGTCTGACCGAA GCTGGAGTTTCATGTCCTCCGGCAATTGATCTGAGCACCACCAATCCCGTCAATGATGTCGCA ACCGGTGGAGATTCTATGAGTGGCTGGCCACCGGTCATACGCTGAAAGATGGTGATAGC GCAGTTTATCAGCGTCTGTGGCAGCCTGGGGTGTCTGTTTGAACCGCTCTGGCAGGG CTGGAGCATGCTGAAGAGGCTGTTGCAATTGCAACGGGTATGGCAGCTATGACTGAGCT CTACTGGCTGCAGTTTCAGCTGGAACACCCACATTGTTGCTGTAAGACCTCTGTACGGT GGAAGCGACCATCTGCTGGAGACCGGACTGCTGGGAACACGCTTACCTGGGCTAAAGAG GCAGATATTGCAAGCGCTATTCAAGACGATACCGGACTGGTTATAGTTGAAACCCCGGT AATCCTTCACTGGATCTAGTCGACCTGGATTGCTGTTGTTGTCGACGAGCAAGTAATGTACCC GTCCTGGTGCATAATACTTTTGTACTCCCGTCTGTCAGCAGCCTATTTCCCATGGAGCT GCACTGGTCTGCTATCTGCTACTAAGTATCTGGGTGGGACCGGTGACGCAATGGGTGGT ATTATTGCAACCAATGCAGATTGGGCTATGCGTCTGAGACAGTTAGAGCAATTACCGGA GCACTGCTGCATCCTATGGGTGCTTACCTGCTACATCGGGGTCTCCGTACTCTGCGAGTA CGTATGCGTGCTGCTCAGACCACCGCAGGGGAACCTGGCTGAACGTCTGGATGCTCATCCC GCAATTTCCGTTGTTTATTATCCGGGACTGAAGGCTCAGGATCCCGTGGACTGCTGGGA CGTCAAATGTCCGGGGGGGGCGGATGATTGCTATGGAACCTGGCAGGGGGCTTTGATGCA GCACGTAGTTTTGTTGAGCATTGTAATCTGGTTGTTTCATGCTGTATCCCTGGGTGGTGTCT GATACTCTGATTAGCACCCTGGCTTCGCTGACTCATAGGCCGCTCGCGGCCACGGCGAAA CCTGGTGACGGGCTGATTAGACTGTCGGTAGGACTGGAGCATGTTGACGATCTGGCTGAT GACCTGATTGCTGCACTGGATGCTTCGCGGGCAGCTGCATAA

at 37°C in phosphate-buffered saline (PBS), PBS with human serum albumin (HSA) at 40 g/l, pure human plasma, and pure rat plasma, each of these containing four different concentrations of PLP up to 200  $\mu$ M (Fig. 1). Enzyme activity was measured at  $t_0$  and after 8 and 24 hours of incubation. Enzyme activity was assessed at 25°C in PBS. Experiments were done in quadruplicate. Western blots were performed in each condition at each time of incubation. Western blot analysis for estimation of apoenzyme levels under various conditions was done by SDS-PAGE probed with rabbit MGL-BL929 anti-serum (Eurogentec, Angers, France). The reaction was revealed with anti-rabbit IgG alkaline phosphatase conjugate, and visualized with Western Blue Substrate (Promega); sensitivity was 0.1  $\mu$ g/ml of protein.

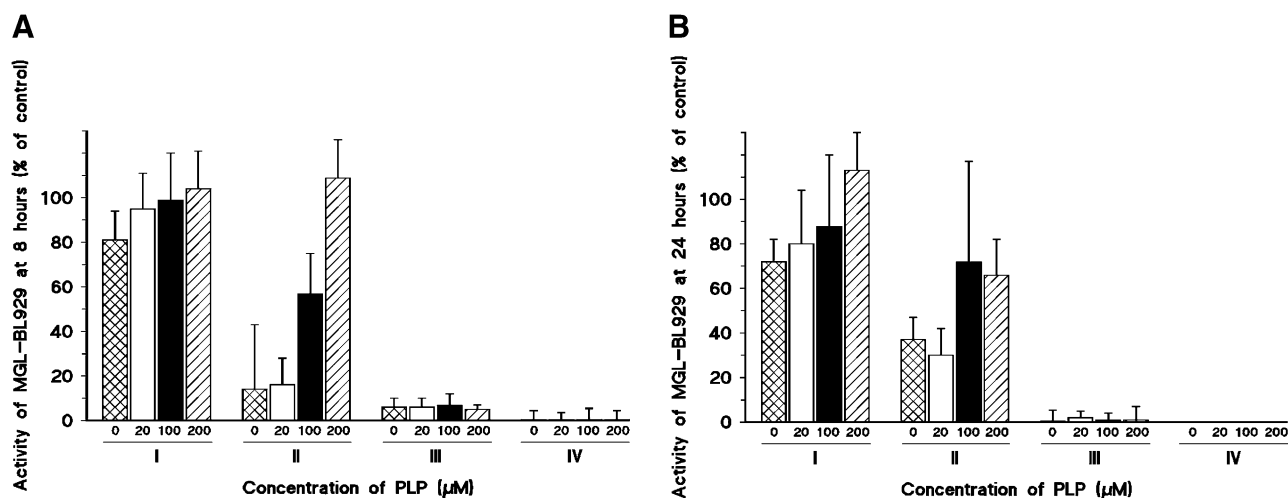
### Effects of MGL-BL929 in Cell Culture

L-Methionine concentration changes were assessed in supernatant of HT29, SKOV3, JURKAT, and REH human cancer cells exposed to MGL-BL929 in various concentrations (Fig. 2). Measurement was done by ultra-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS; Waters, Milford, MA) using L-methionine-(methyl)-D<sub>3</sub> as internal standard (Waterval et al., 2009). A panel of 35 human cancer cell lines originating from various cell types (American Type Culture Collection, Manassas, VA) was used to determine the

cytotoxicity spectrum of MGL-BL929 (Table 2). Cells were thawed from mycoplasma-free frozen stocks and were controlled for contamination. Cytotoxicity against noncancer cells was explored using primary 533 skin fibroblasts from a normal child, and fibroblasts from an individual with Congenital Disorder of Glycosylation II (CDG-II). Cells were grown in standard RPMI 1640 or Dulbecco's modified Eagle's medium (DMEM) culture media, supplemented with 10% fetal bovine serum and antibiotics at 37°C in an atmosphere with 5% CO<sub>2</sub>, and were exposed to the MGL-BL929 for 72 hours in 12-well cell plates. Viability was measured using flow cytometry. Living cells were defined by light double scatter, and counted in a BD Accuri C6 flow cytometer (BD Biosciences, Le-Pont-de-Claix, France). Each experiment was done in duplicate. Cell growth inhibition data were analyzed according to the median-effect principle for concentration-effect analysis. Dose-effect plots including 50% inhibitory concentrations (IC<sub>50</sub>) and 95% confidence intervals were obtained with the CalcuSyn v2 software (Biosoft, Cambridge, UK).

### Assessment of Methylation-Related Epigenetic Changes in Human Colorectal Cancer Cells Exposed to MGL-BL929

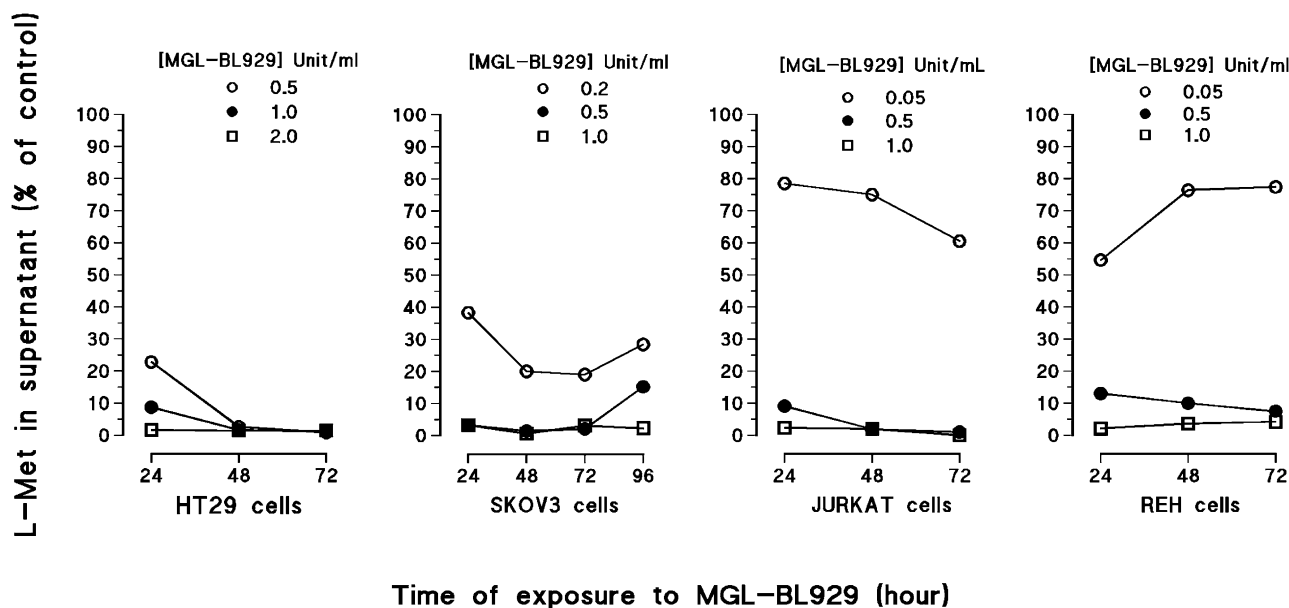
**Quantitation of 5-Methylcytosine in DNA from Gene Promoters.** Changes in 5-methylcytosine (5-meC) levels within CpG dinucleotide



**Fig. 1.** Enzymatic activity of native MGL-BL929 incubated for 8 hours (A) and 24 hours (B) was assayed at 37°C under four different conditions. Conditions were (I) phosphate-buffered saline; (II) PBS supplemented with human serum albumin at 40 g/l; (III) pure human plasma; and (IV) pure rat plasma. Measurement of enzyme activity was performed without additional PLP and under three PLP concentrations (i.e., 20, 100, and 200 μM). Bars are the mean of experiments done in quadruplicate  $\pm$  S.E. Analysis of variance found significant differences between incubation conditions at 8 hours ( $***P = 2.2 \times 10^{-16}$ ) and at 24 hours ( $***P = 2.75 \times 10^{-7}$ ), and between PLP concentrations at 8 hours ( $***P = 8.8 \times 10^{-5}$ ).

fragments from gene promoters containing 5-mC baseline levels greater than 70% were quantitatively assessed in HT29 cells exposed to MGL-BL929 at 1 U/ml and at 2 U/ml for 24, 48, and 72 hours. The MassARRAY System (Agena Bioscience, Hamburg, Germany) was used for quantitative methylation analysis of bisulfite-treated genomic DNA from 50 selected CpG sites from promoter sequences of 39 genes described as having hyper-methylated promoter regions in colon cancer cell lines (Ehrich et al., 2008). Genomic DNA was extracted from cells using a QIAamp DNA mini kit (Qiagen, Courtaboeuf, France). Concentration and purity of DNA were determined by absorbance using a NanoVue spectrophotometer. Sodium bisulfite DNA modification was performed using EZ DNA Methylation Kit (Zymo Research, Orange, CA). The 50 amplicons and polymerase chain reaction (PCR) primers were designed from

<http://agenacx.com/epidesigner/> (Supplemental Table S1). The amplification regions covered CpG islands of promoters of target genes overlapping with the DNA sequences corresponding to 5' untranslated regions (5' UTR). The target regions of the sodium bisulfite-treated genomic DNA were amplified by PCR using designed primers. Each forward primer contained a 10-nucleotide tag (5'-AGGAAGAGAG-3') to balance the PCR, and each reverse primer contained a T7 promoter tag and sequences insert (5'-CAGTAATACGACTCACTATAGGGAGAAGGCT-3') for in vitro transcription. Amplification comprised an initial incubation at 94°C for 4 minutes, 45 cycles of denaturation at 95°C for 20 seconds, annealing at 62°C for 30 seconds, extension at 72°C for 1 minute, and final incubation at 72°C for 3 minutes. Unincorporated dNTPs were dephosphorylated with premix for in vitro transcription including shrimp alkaline phosphatase (Agena Bioscience).



**Fig. 2.** L-Methionine concentration changes in supernatant of HT29, SKOV3, JURKAT, and REH cells growing in standard cell culture media (i.e., RPMI 1640, or DMEM) supplemented with 10% fetal bovine serum, exposed to various concentrations of MGL-BL929 for 24, 48, 72, and 96 hours. L-Methionine (L-Met) elimination rates were the ratios of the concentrations measured in supernatant of cells exposed to MGL-BL929 over those found in cells unexposed to the enzyme at same time to take into account the consumption of L-methionine by growing cancer cells. RPMI 1640 and DMEM media contain L-methionine at 100 and 200 μM, respectively.

TABLE 2  
Cytotoxicity of MGL-BL929 in human cells

Cancer Cell Line	Tumor Type	IC <sub>50</sub> of MGL-BL929 in U/ml (95% CI)
HCT116	Colorectal carcinoma	0.18 (0.13–0.25)
HT29	Colorectal carcinoma	0.56 (0.49–0.64)
LoVo	Colorectal carcinoma	1.32 (1.22–1.42)
SW480 <sup>a</sup>	Colorectal carcinoma	>3.5
SW620 <sup>a</sup>	Colorectal carcinoma	>3.5
HeLa	Cervix adenocarcinoma	0.76 (0.46–1.26)
A431	Vulvar epidermoid carcinoma	0.84 (0.58–1.20)
HUH7	Hepatocellular carcinoma	1.84 (1.77–1.93)
MAHLAVU	Hepatocellular carcinoma	1.78 (1.67–1.89)
HepG2	Hepatoblastoma	0.78 (0.61–0.99)
HOON	Pre-B-cell acute lymphocytic leukemia	0.29 (0.19–0.46)
REH	Pre-B cell acute lymphocytic leukemia	0.05 (0.02–0.14)
JURKAT	T-Cell acute lymphocytic leukemia	0.16 (0.07–0.37)
K562	CML blast crisis	0.20 (0.13–0.30)
HEL	Erythroleukemia	0.15 (0.08–0.27)
HL-60	Acute promyelocytic leukemia	0.08 (0.04–0.16)
KG-1	Acute myelocytic leukemia	0.48 (0.29–0.79)
BJAB	Burkitt's lymphoma	0.35 (0.31–0.40)
RAJI	Burkitt's lymphoma	0.18 (0.12–0.28)
U-2 OS	Osteosarcoma	0.59 (0.45–0.76)
HT-1080	Fibrosarcoma	0.64 (0.57–0.72)
PC-3	Prostate adenocarcinoma	0.80 (0.77–0.84)
OVCAR3	Ovarian adenocarcinoma	0.74 (0.53–1.05)
SKOV3	Ovarian adenocarcinoma	1.26 (1.06–1.51)
AsPC1	Pancreas adenocarcinoma	0.17 (0.09–0.35)
MCF7	Mammary adenocarcinoma	0.58 (0.23–1.43)
MDA-MB231	Mammary adenocarcinoma	0.61 (0.58–0.65)
SKBR3	Mammary adenocarcinoma	1.87 (1.41–2.48)
A375	Melanoma	0.39 (0.31–0.48)
SKMEL28	Melanoma	1.31 (1.04–1.65)
RCC7	Renal cell carcinoma	0.94 (0.74–1.19)
A-549	Lung adenocarcinoma	1.17 (0.95–1.45)
H1993	Lung adenocarcinoma	2.14 (1.89–2.43)
IMR32	Neuroblastoma	0.24 (0.10–0.54)
HTB-14	Glioma	1.56 (1.08–2.26)
Non-cancer cell	Cell type	IC <sub>50</sub> of MGL-BL929 in U/ml
533 <sup>b</sup>	Skin fibroblast	4.7
CDG-II <sup>c</sup>	Skin fibroblast	>10

<sup>a</sup>SW480 and SW620 colorectal cancer cells originate respectively from the primary tumor and a metastatic site from a single individual.

<sup>b</sup>Primary 533 skin fibroblasts originated from a normal child.

<sup>c</sup>CDG-II cells were from an individual with congenital disorder of glycosylation II. Cells were counted after 72-h exposure to MGL-BL929. Dose-effect plots were obtained with the CalcuSyn v2 software.

The reaction mixture was incubated at 37°C for 40 minutes before phosphatase heat inactivation. PCR product was then subjected to in vitro transcription with RNase-A cleavage for the T-reverse reaction. Samples were spotted onto a 384-pad Spectro-CHIP using MassARRAY NanoDispenser, and spectra were acquired with MassARRAY compact MALDI-TOF mass spectrometer (Agena Bioscience). Methylation calls were analyzed with EpiTYPER software (Agena Bioscience) to quantitate results for each CpG site. Results were expressed in percent methyl-CpG change between cells unexposed to the enzyme and cells exposed to each concentration of MGL-BL929 at same times of growth. Statistical comparisons of the difference between percent methylation in paired samples were done with two-sided Wilcoxon rank-sum test. One-way ANOVA was used to study differences in methylation rates according to time of cell culture in unexposed cells, and to time of enzyme exposure in treated cells. Statistical analysis was done with the R software, v3.4.4.

**Quantitation of mRNA Gene Expression by Real-Time Reverse Transcription-PCR.** Changes in mRNA expression of four genes by real-time reverse transcription-PCR were quantitatively assessed in HT29 and in HCT116 colon cancer cells exposed to MGL-BL929 at 1 U/ml, and at 2 U/ml for 48 and 72 hours. We selected three genes whose promoter CpGs were partially demethylated under MGL-BL929 in HT29 cells (CDKN2A, ATP10A, and MyoD) as presented below, and

one gene (DNM1L) that was not assessed for changes of 5-meC levels because its promoter was not reported to be hyper-methylated in colorectal cancer cells (Ehrich et al., 2008). The cyclin-dependent kinase inhibitor 2A (CDKN2A) gene encodes proteins that mainly regulate the retinoblastoma-susceptible gene product (pRb) and p53 pathways that play critical roles in the cell cycle and apoptosis (Li et al., 2011). Downregulation is frequently involved in oncogenic processes (Herman et al., 1995; Hinshelwood et al., 2009; Li et al., 2011). The MyoD gene encodes for a myogenic factor that is expressed in skeletal muscle lineage and is repressed in other tissues (Buckingham and Rigby, 2014). The ATP10A encodes a protein of the P-type cation transport ATPase family, and the DNM1L gene product belongs to the dynamin family of large GTPases. Messenger RNA of target genes (CDKN2A, MyoD, ATP10A, and DNM1L) and of endogenous reference genes (S18 rRNA and  $\beta$ -Actin) from HT29 and HCT116 cells was used for cDNA synthesis. Amounts of cDNA from target and reference genes obtained by PCR amplification under each experimental condition were determined from standard curves constructed with cDNA from the hepatocellular carcinoma MAHLAVU cells. Total RNA was extracted using a Qiagen kit (Qiagen). Complementary DNA was synthesized using QuantiTect Reverse Transcription reagents including DNase treatment (Qiagen) mixed with RNA. Cycling conditions were 42°C for 30 minutes, followed by denaturation at

95°C for 3 minutes. PCR amplification was performed on the LightCycler 480 instrument (Roche Applied Science, Mannheim, Germany), and data were analyzed with the LightCycler 480 Software, v1.5. PCR was performed with QuantiFast Sybr-Green PCR Master Mix reagents (Qiagen) for CDKN2A, and S18, and with QuantiFast Probe PCR Master Mix reagents for MYOD1, ATP10A, DNMT1L,  $\beta$ -Actin, and S18 for mRNA quantitation. One microliter of reverse transcription product was used for each round of PCR. One microliter of pure and diluted (1/10th; 1/100th; 1/1000th) cDNA from MAHLAVU cells was used for standard curves. Enzyme was activated by incubation at 95°C for 5 minutes. Each of the 50 PCR cycles consisted of a denaturation step at 95°C for 30 seconds, and then hybridization of probes and primers and DNA synthesis at 60°C for 90 seconds. Experiments were done in duplicate. Quantitative data of both S18 rRNA and  $\beta$ -Actin mRNA obtained under each condition were used as endogenous housekeeping references for normalization. The amount of target was divided by that of reference gene to obtain a normalized ratio value. Relative expression level (as n-fold change) was obtained by dividing normalized ratio values obtained with cells exposed to the MGL-BL929 under each condition by the normalized ratio in control cells unexposed to the enzyme at same time point (Bieche et al., 1998).

**Expression of the Protein p16<sup>INK4A</sup> in Cancer Cells.** Expression of the protein p16<sup>INK4A</sup>, a product of the CDKN2A gene, was assessed by Western blot in the HT29 and HCT116 colon carcinoma, and HeLa cervix adenocarcinoma cells exposed to the MGL-BL929 at 1 U/ml for 48 hours, and in cells unexposed to the enzyme. We chose to test the effect of the MGL-BL929 on p16 translation only at 1 U/ml for 48 hours with the aim of avoiding any possible deleterious effect of excessive L-methionine elimination on protein synthesis under high concentration of enzyme and longer times of exposure. p16<sup>INK4A</sup> primarily functions in cell-cycle control as a negative regulator of the pRb-E2F pathway; it specifically inhibits at the G1 to S transition the cyclin-dependent kinases 4 (CDK4)- and 6 (CDK6)-mediated phosphorylation of pRb (Li et al., 2011). After trypsinization, cells were lysed in Laemmli buffer, sonicated on ice, and then centrifuged at 4°C. Protein samples were transferred to a Low-Fluorescence PVDF Transfer Membrane (GE Healthcare) at 40 V overnight. After blocking for 90 minutes in Tris buffer saline, 5% BSA, and 0.1% Tween 20, blots were probed with rabbit antibodies against p16<sup>INK4A</sup> from two sources (anti-CDKN2A/p16INK4a 108349; Abcam, Cambridge, UK; and anti-p16INK4a 1D7D2; ThermoFisher Scientific, Illkirch, France), and against actin. Membranes were imaged with the LI-COR Odyssey Infrared Imaging System (LI-COR Biosciences, Bad Homburg, Germany), and band intensities were quantified with the LI-COR Odyssey software. Comparisons were performed between cells unexposed to the MGL-BL929 and those exposed to the enzyme at same time points. Two separate experiments were done.

**Quantitation of Dimethylated Histone H3-Lysine Levels by Western Blot.** To explore the influence of L-methionine deprivation on histone methylation, we quantitated global histone H3-dimethyl lysine levels by Western blot in HT29 and HCT116 cells exposed to the MGL-BL929 at 1 U/ml, and at 2 U/ml for 48 and 72 hours. Cells were lysed in Laemmli buffer, sonicated on ice, and then centrifuged at 4°C. Protein samples were transferred to a Low-Fluorescence PVDF Transfer Membrane and then probed with antibodies against histone H3 dimethylated on different lysines (H3K4me2, H3K9me2, H3K27me2, H3K36me2, H3K79me2), and against total histone H3 (Methyl-Histone H3 Ab Sampler Kit 9847S; Cell Signaling Technology, Leiden, The Netherlands). Membranes were imaged with the LI-COR Odyssey Infrared Imaging System. Band intensities were quantified with the LI-COR Odyssey software and compared with that in control cells unexposed to MGL-BL929 at same time point.

**Assessment of Plasma L-Methionine Depletion and Enzyme Levels in Mice Given Parenteral Native MGL-BL929.** Measurement of L-methionine levels (Waterval et al., 2009) and Western blots were done in plasma after a single injection of enzyme in groups of three mice per dose of enzyme and time point. Female C57Bl/6 mice

aged 6 weeks were given MGL-BL929 i.p. at 50 or 200 U per mouse. Female Balb/C mice aged 6 weeks were given the enzyme i.v. at 50 U per mouse. Blood was drawn 1, 3, 6, and 24 hours after injection.

**Construction of a Heterologous Bioreactor by Encapsulation of the MGL-BL929 in Human Erythrocytes.** With the aim at preserving MGL function for long periods of time in vivo, we constructed a heterologous bioreactor by encapsulation of the MGL-BL929 in human erythrocytes. These cells internalize L-methionine and have the capacity to synthesize PLP, the cofactor required to preserve the intracellular activity of the PLP-dependent L-methionine  $\gamma$ -lyase (Zempleni and Kübler, 1994; Machover et al., 2018). Carrier erythrocyte lifespan does not differ from or is only slightly shorter than that of intact cells (Bax et al., 1999), a characteristic that allows maintenance of therapeutic blood levels of loaded cells for prolonged periods of time. Moreover, carrier erythrocytes prevent loaded proteins from being released into the extracellular environment and are progressively degraded in the organism by the mononuclear phagocytic system without producing toxic substances. Human blood was obtained from healthy adults and collected in heparin. Recombinant MGL-BL929 was loaded into erythrocytes by sequential hypotonic dialysis, isotonic resealing, and reannealing as previously described (Magnani et al., 1988), with slight modifications. Erythrocytes were first washed twice at 4°C in solution A (154 mM NaCl, 10 mM HEPES-NaOH, 5 mM glucose, and 200  $\mu$ M PLP; pH 7.4). Cells were then suspended at 50% erythrocyte volume fraction in encapsulation solution consisting of solution A supplemented with various concentrations of MGL-BL929 ranging from 11 to 270 U/ml. Hypotonic hemolysis was performed by dialysis of 1 ml of cell suspension in a cellulose tube (cutoff 14 kDa) versus 50 ml of 60-mOsm hypotonic solution (Fiske 210 micro sample Osmometer, Norwood, MA) consisting in 10 mM NaHCO<sub>3</sub>, 10 mM NaH<sub>2</sub>PO<sub>4</sub>, 20 mM glucose, 2 mM ATP, 3 mM glutathione, and 200  $\mu$ M PLP; pH 7.4, for 90 minutes at 4°C. At the end of dialysis, osmolality of cell suspensions was approximately 90 mOsm. Erythrocyte resealing and reannealing were accomplished by incubating 10 volumes of cell suspension in one volume of hypertonic solution of 100 mM inosine, 100 mM sodium pyruvate, 33 mM NaH<sub>2</sub>PO<sub>4</sub>, 1.66 M KCl, 190 mM NaCl, 10 mM glucose, 30 mM ATP, 4 mM MgCl<sub>2</sub>, and 200  $\mu$ M PLP; pH 7.4 (approximately 390 mOsm) for 25 minutes at 37°C. Finally, after three gentle washes in solution A at 4°C, cells were suspended in this solution and used for experiments. Enzymatic activity (unit of MGL per milliliter of packed cells) was measured in loaded cells obtained immediately after encapsulation and at various times after loading (Takakura et al., 2004). Morphologic parameters of loaded erythrocytes were assessed at various times after the loading procedure. These included optical morphologic study, and measurement of red cell parameters (Hematology Analyzer Coulter Ac T 5diff; Beckman Coulter, Miami, FL). Estimation of anaerobic glycolysis by measurement of lactate production (micromoles per hour per milliliter of packed cells; GEM Premier 3000 Analyzer; Instrumentation Laboratory, Bedford, MA) was carried out immediately after the encapsulation procedure. Western blot analysis (sensitivity, 0.1  $\mu$ g/ml of protein, i.e., approximately 0.6 mU/ml) was performed in cell supernatant of encapsulated cells at various times of incubation to control for release of free MGL-BL929 from loaded cells. Unloaded erythrocyte suspensions (i.e., cells subjected to encapsulation procedure without enzyme) and untreated cells were used as control in all experiments. Elimination of L-methionine was assessed in supernatant of erythrocytes loaded with MGL-BL929 (at 11, 54, or 270 U/ml in encapsulation solution) incubated at 0.5% cell volume fraction for up to 96 or 120 hours in media consisting of PBS (50 mM NaH<sub>2</sub>PO<sub>4</sub>/Na<sub>2</sub>HPO<sub>4</sub>, 90 mM NaCl; pH 7.4), 5 mM glucose, PLP (0.1 or 200  $\mu$ M), and L-methionine (100 or 1000  $\mu$ M) at 37°C in an atmosphere containing 5% CO<sub>2</sub>. Experiments in medium containing vitamin B6 as pyridoxine (PN) or pyridoxamine (PM) at 200  $\mu$ M instead of PLP were performed to test MGL function of the bioreactor under exposure to unphosphorylated B6 vitamins (Di Salvo et al., 2011). Potency and duration of L-methionine elimination was assessed in medium supplemented with L-methionine

once at  $t_0$ , twice at  $t_0$ , and after 24 hours of incubation, and three times at  $t_0$  and after 24 and 48 hours of incubation. At planned intervals up to 96 or 120 hours, 2-ml aliquots of cell suspension were used for measurement of L-methionine in supernatant (Waterval et al., 2009).

## Results

**Production and Characterization of the Recombinant MGL-BL929.** Purification of MGL-BL929 was achieved to homogeneity from a 50-l scale cell pellet. Protein more than 95% pure containing small levels of endotoxin (49.4 endotoxin U/ml) was concentrated to 79.6 mg/ml. Molecular mass is 46.5 kDa and the hydrodynamic volume assessed by size exclusion chromatography corresponds to a 171.5 kDa globular protein, which suggests that MGL-BL929 is a monodisperse homotetrameric protein. Catalytic activity is limited to  $\alpha, \gamma$ -elimination of L-methionine and L-homocysteine (Table 3). Mean  $K_M$  for L-methionine was  $6.83 \pm 2.45$  mM and specific activity was  $5.77 \pm 1.4$   $\mu\text{mol mn}^{-1} \text{mg}^{-1}$ . The enzyme had no detectable catalytic activity toward L-cysteine, L-cystathionine, S-adenosyl-L-homocysteine and D-methionine. The MGL-BL929 was stable at 25°C for >24 hours without additional PLP. At 37°C, the enzyme lost 20% of its initial activity after 24 hours in PBS in the presence of  $\leq 2$   $\mu\text{M}$  of PLP, but activity remained stable with PLP at >20  $\mu\text{M}$  (Fig. 1). Activity of MGL-BL929 decreased in HSA in the absence of PLP, and augmented with increasing concentrations of cofactor at 8 hours of incubation. In pure plasma, the enzyme lost activity by  $\geq 90\%$  after 8 hours of incubation that could not be preserved by PLP up to 200  $\mu\text{M}$ . Loss of activity was not accompanied by decrease of apoenzyme as estimated by Western blot.

**The Native MGL-BL929 Eliminates L-Methionine in Cell Culture, and Exerts Strong Cytotoxicity in Human Cancer Cell Lines In Vitro.** L-Methionine was eliminated from supernatant of all four cell lines studied, and the effect was augmented with increasing concentrations of enzyme (Fig. 2). The MGL-BL929 induced cytotoxicity against 33 of the 35 cancer cell lines tested in the panel (Table 2). In the 33 cancer cell lines that were sensitive to the MGL-BL929, cytotoxicity appeared at  $\leq 0.5$  U/ml and increased with increasing concentrations of enzyme; in these cells,  $\text{IC}_{50}$  values ranged from 0.05 to 2.14 U/ml. Resistance to methionine deprivation was only found in SW480 and SW620 colorectal cancer cells that originate respectively from the primary tumor and a metastatic site from the same tumor. Exposure of human skin fibroblasts CDGII and 533 to the enzyme affected their growth at concentrations much greater than

that required for cytotoxicity of cancer cells.  $\text{IC}_{50}$  was 4.7 U/ml in CDG-II cells, and it was not reached at 10 U/ml in 533 cells.

### Methylation-Related Epigenetic Changes Occurred in Human Colorectal Cancer Cells Exposed to MGL-BL929.

Of 50 CpG sites from 39 selected gene promoters in HT29 cells, variable numbers of fragments comprising a single CpG or groups of 2–5 contiguous CpGs were amplified (Fig. 3; Supplemental Fig. S1; and Table 4). Exposure of cells to the MGL-BL929 decreased 5-methyl-CpG levels of hyper-methylated gene promoters. Differences in 5-methyl-CpG rates between paired samples from cells unexposed and exposed to enzyme were not significant after 24 hours and became highly significant after 48 and 72 hours of exposure to both concentrations of enzyme studied (Table 4). No significant difference was found with one-way analysis of variance (ANOVA) in methylation rates between control cells sampled at different times of growth that were not exposed to the enzyme and were used for comparisons with cells exposed to MGL-BL929 at 1 U/ml ( $P = 0.99$ ) and at 2 U/ml ( $P = 0.17$ ). Significant differences in methylation rates according to time of enzyme exposure were found by one-way ANOVA in cells exposed to MGL-BL929 at 1 U/ml ( $***P = 2 \times 10^{-10}$ ) and 2 U/ml ( $***P = 5 \times 10^{-5}$ ), which indicate that 5-meC promoter DNA levels decrease with increasing time of exposure to the enzyme. With the exception of MyoD, whose transcripts were not detected in HT29 and HCT116 cells, transcripts of CDKN2A, ATP10A, and DNMT1 were amplified in both colorectal carcinoma cells unexposed to the MGL-BL929. Exposure of these cell lines to the enzyme at 1 U/ml and 2 U/ml for 48 and 72 hours resulted in marked increase in CDKN2A mRNA expression of variable degree, from approximately 10- to more than  $10^5$ -fold of that in unexposed cells at same time points (Fig. 4). No change in mRNA level from that found in unexposed cells was observed for ATP10A, whose promoter had been partially demethylated in HT29 cells exposed to MGL-BL929 (Fig. 3; Supplemental Fig. S1), and for DNMT1. No appreciable differences in relative gene expression were observed when normalized ratios were obtained either with the 18S rRNA gene (Fig. 4) or the  $\beta$ -Actin gene (data not shown). We analyzed in the HT29 cell line the individual data of the three genes whose promoter methylation levels under exposure to the MGL-BL929 were assessed, together with mRNA expression (i.e., CDKN2A, MyoD, and ATP10A). From this analysis, we did not observe appreciable differences in mean levels of baseline CpG methylation of individual gene promoters or in mean rates of demethylation under L-methionine depletion at the two concentrations of enzyme (1 and 2 U/ml) and times of exposure (48 and 72 hours)

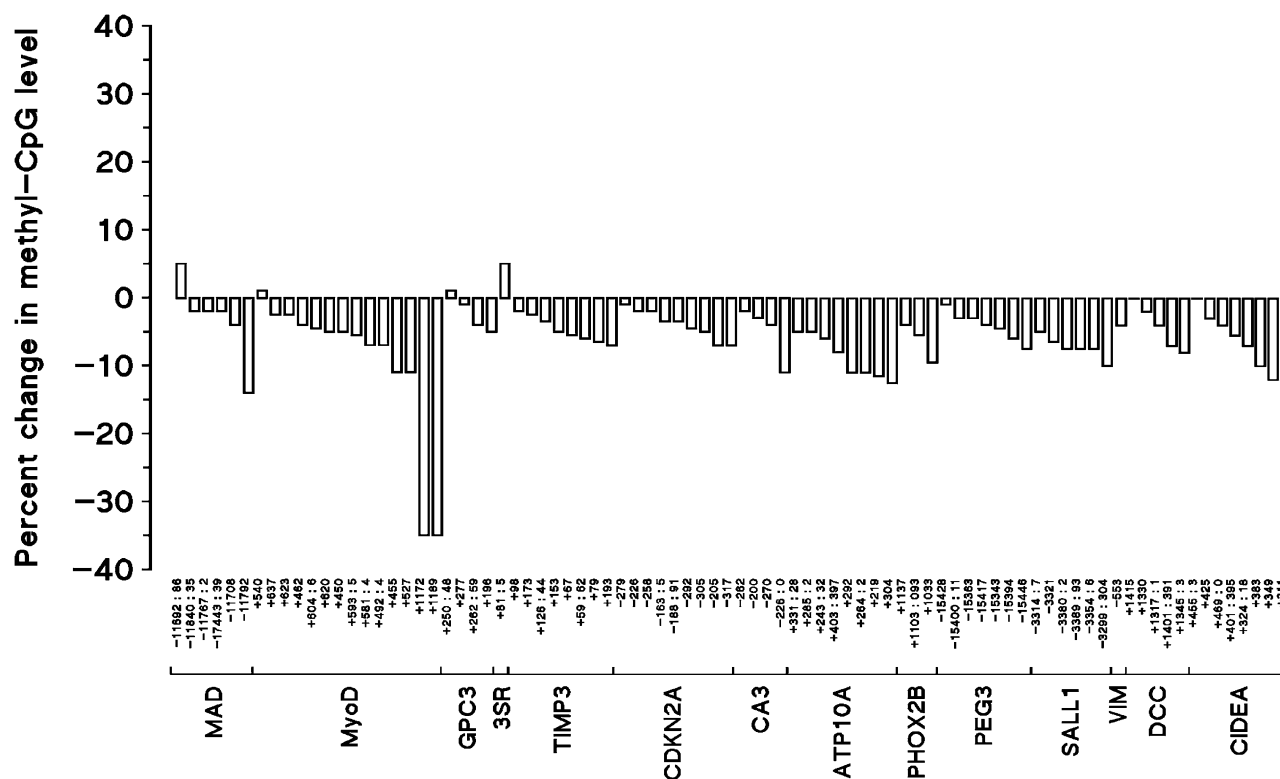
TABLE 3

Enzyme kinetics of MGL-BL929 with sulfur-containing amino acids and substituted amino acids

Values are mean  $\pm$  SE from two to four separate experiments with each amino acid or substituted amino acid. One unit of enzyme is defined as the amount that catalyzes formation of 1  $\mu\text{mol}$  of  $\alpha$ -ketobutyrate per minute from L-methionine.

Amino Acid and Substituted Amino Acid	$K_M$	$k_{\text{cat}}$	Specific Activity
	mM	$\text{s}^{-1}$	$\mu\text{mol mn}^{-1} \text{mg}^{-1}$
L-Methionine	$6.83 \pm 2.45$	$15.51 \pm 3.23$	$5.77 \pm 1.4$
L-Homocysteine	$0.94 \pm 0.8$	$72.44 \pm 12.38$	$27.7 \pm 5.7$
L-Cysteine	—	—	Nd
L-Cystathionine	—	—	Nd
S-Adenosyl-L-homocysteine	—	—	Nd
D-Methionine	—	—	Nd

Nd, no detectable activity.



**Fig. 3.** Mean methyl-CpG percent change from control of gene promoters in colorectal cancer HT29 cells exposed to MGL-BL929 at 2 U/ml for 72 hours. Methylation rates in fragments with a single CpG or groups of 2–5 contiguous CpGs were measured in 84 DNA fragments comprising 131 CpG dinucleotides whose baseline methyl-CpG levels in control cells were greater than 70% (mean percent methyl-CpG, 88.67). Bars represent the difference in mean methyl-CpG level of fragments in cells exposed to the MGL-BL929 and that in control cells unexposed to the enzyme. Numbers under bars indicate positions from the first nucleotide of coding DNA sequences to each single 5-meC or groups of 5-meCs within CpG dinucleotides of gene promoters. The difference between percent methylation in paired samples was statistically significant (two-sided Wilcoxon rank-sum test,  $***P = 6.5 \times 10^{-5}$ ). Methyl CpG change in cells exposed to MGL-BL929 at 1 U/ml for 72 hours is shown in Supplemental Fig. S1.

that could explain the changes observed only in CDKN2A mRNA expression. Statistical comparisons of mRNA expression levels in HT29 cells using the Student's *t* test found that CDKN2A expression decreased with increasing concentration of MGL-BL929 from 1 to 2 U/ml at 48 hours ( $*P = 0.02$ ), and at 72 hours of exposure ( $*P < 0.02$ ), as well as with increasing

time of exposure to the enzyme from 48 to 72 hours at 1 U/ml ( $*P < 0.02$ ), and at 2 U/ml ( $*P < 0.02$ ). In HCT116 cells, statistical difference was only found for a decrease in CDKN2A mRNA expression with increasing concentration of MGL-BL929 from 1 to 2 U/ml in cells exposed for 72 hours to the enzyme ( $*P < 0.05$ ). The effect of the MGL-BL929 on levels

**TABLE 4**  
Changes in gene promoter CpG methylation in HT29 cells exposed to MGL-BL929

MGL-BL929	Mean Percent 5-Methyl-CpG and Mean Difference from Control at <sup>a,b</sup>			
	24 h		48 h	
1 U/ml <sup>b,c,e</sup>	Enz <sub>control</sub>	89.1	Enz <sub>control</sub>	89.21
	Enz <sub>1U/ml</sub>	89.68	Enz <sub>1U/ml</sub>	86.35
	Δ Enz <sub>control:Enz<sub>1U/ml</sub></sub>	0.58	Δ Enz <sub>control:Enz<sub>1U/ml</sub></sub>	-2.86
	<i>P</i>	0.52 (NS)	<i>***P</i>	$5.7 \times 10^{-3}$
2 U/ml <sup>b,d,e</sup>	Enz <sub>control</sub>	89.16	Enz <sub>control</sub>	91.22
	Enz <sub>2U/ml</sub>	88.54	Enz <sub>2U/ml</sub>	85.7
	Δ Enz <sub>control:Enz<sub>2U/ml</sub></sub>	-0.61	Δ Enz <sub>control:Enz<sub>2U/ml</sub></sub>	-5.52
	<i>P</i>	0.63 (NS)	<i>***P</i>	$4.1 \times 10^{-6}$
				Enz <sub>control</sub> 88.64
				Enz <sub>1U/ml</sub> 81.84
				Δ Enz <sub>control:Enz<sub>1U/ml</sub></sub> -6.8
				<i>***P</i> = $1.3 \times 10^{-9}$
				Enz <sub>control</sub> 88.67
				Enz <sub>2U/ml</sub> 82.9
				Δ Enz <sub>control:Enz<sub>2U/ml</sub></sub> -5.77
				<i>***P</i> = $6.5 \times 10^{-5}$

<sup>a</sup>Methyl-CpG level under each experimental condition is the mean percent methylation of fragments from selected gene promoters comprising a single CpG or groups of two to five contiguous CpGs.

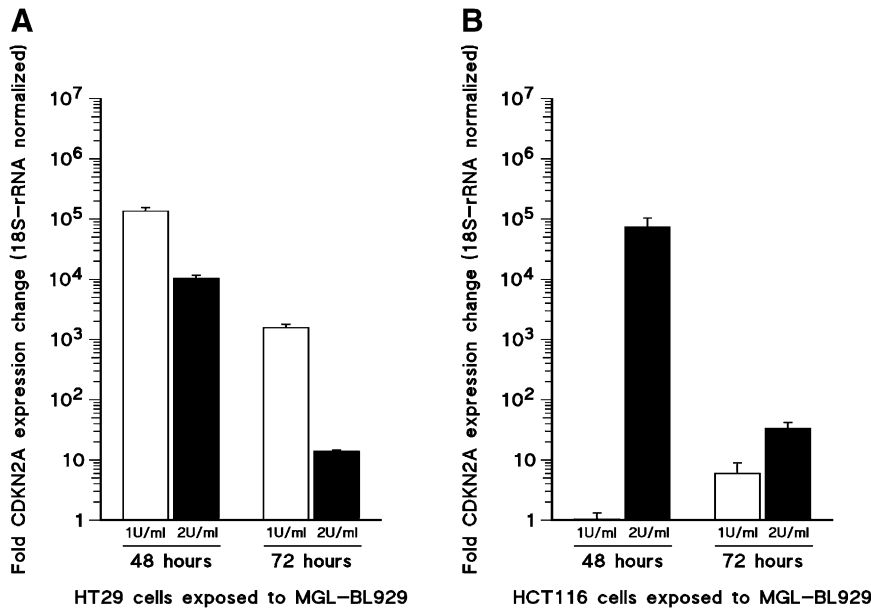
<sup>b</sup>Changes in 5-methylcytosine (5-meC) levels within CpG dinucleotide in fragments from selected gene promoters containing 5-meC baseline levels greater than 70% were studied in cells exposed to MGL-BL929 at 1 U/ml, and at 2 U/ml for 24, 48, and 72 h. Results were expressed in percent methyl-CpG change between control cells unexposed to the enzyme and cells exposed to each concentration of MGL-BL929 at same times of growth. Mean percent methyl-CpG  $\pm$  S.E. of gene promoter fragments from untreated cells was  $89.25 \pm 7.9$ .

<sup>c</sup>For cells exposed to MGL-BL929 at 1 U/ml, 142 fragments (including 230 CpGs), 141 fragments (including 228 CpGs), and 142 fragments (including 229 CpGs) were analyzed after 24, 48, and 72 h of exposure, respectively.

<sup>d</sup>For cells exposed to MGL-BL929 at 2 U/ml, 91 fragments (including 146 CpGs), 81 fragments (including 129 CpGs), and 84 fragments (including 131 CpGs) were analyzed after 24, 48, and 72 h of exposure, respectively.

<sup>e</sup>Statistical comparisons of the difference between percent methylation in paired samples were done with two-sided Wilcoxon rank-sum test. One-way ANOVA found significant differences in methylation rates according to time of enzyme exposure in cells exposed to MGL-BL929 at 1 U/ml ( $***P = 2 \times 10^{-10}$ ) and 2 U/ml ( $***P = 5 \times 10^{-5}$ ).





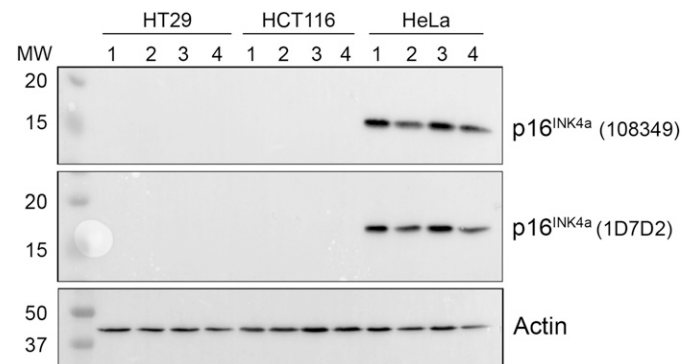
**Fig. 4.** CDKN2A gene expression changes by quantitative RT-PCR in HT29 (A) and in HCT116 (B) colorectal carcinoma cell lines exposed to MGL-BL929 at 1 and 2 U/ml for 48 and 72 hours. CDKN2A mRNA expression was normalized using 18S-rRNA as reference gene. The amount of cDNA from CDKN2A gene was divided by that of 18S-rRNA gene under each condition to obtain a normalized ratio value. Relative expression level was obtained by dividing normalized ratio values obtained with cells exposed to the MGL-BL929 under each condition by the normalized ratio in control cells unexposed to the enzyme at same time point. Bars indicate N-fold change  $\pm$  S.E.

of CDKN2A mRNA expression (Fig. 4) was greater at the lowest concentration of enzyme (i.e., 1 U/ml), as it also was on DNA fragments of gene promoters successfully amplified whose number decreased with increasing concentration of the enzyme (Table 4). These observations could result from profound L-methionine depletion under exposure to MGL-BL929 at 2 U/ml below limits allowing precise analysis of changes resulting from L-methionine deprivation in cancer cells. The protein p16<sup>INK4A</sup> was not detected by Western blot using two different antibodies in HT29 and HCT116 cells unexposed to the MGL-BL929 (Fig. 5). Expression of the protein neither occurred in cells exposed to the enzyme despite great increase of CDKN2A mRNA found in this condition. In contrast, HeLa cells strongly expressed p16<sup>INK4A</sup>, with no appreciable change in band intensities according to enzyme exposure. Decrease in overall H3K9me2 band intensities from control ranging from 77% to 89% (mean decrease, 83%) occurred in both HT29 and HCT116 cells exposed to MGL-BL929 at 1 or 2 U/ml, except for a single smaller decrease by 38% in HT29 cells exposed to the enzyme at 1 U/ml for 72 hours (Fig. 6). Relative band intensity changes by  $\pm 15\%$  in total histone H3 and in histone H3-dimethyl lysine levels H3K4me2, H3K27me2, H3K36me2, and H3K79me2 levels did not occur.

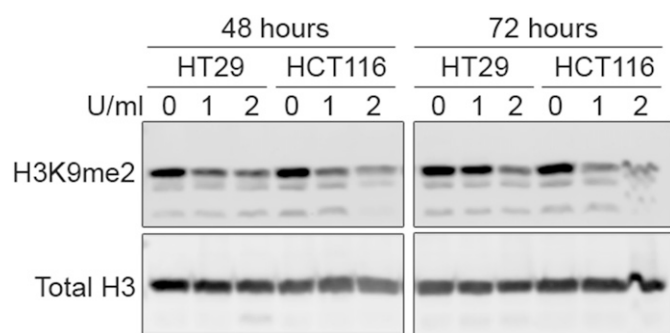
**Methionine Elimination in Mouse Plasma In Vivo with the Native Enzyme Is Effective for Short Periods of Time.** Purified MGL-BL929 at 200 U i.p. reduced L-methionine to undetectable levels 1 hour after injection, but recovery to 60% from baseline occurred in 3 hours. At 50 U i.p. or i.v., methionine concentration decreased to 60% from baseline 1 hour after injection, and the decrease lasted for 3 hours or less. Loss of effectiveness was not accompanied by appreciable changes in Western blot band intensities for apoenzyme in plasma after 24 hours from injection.

**Human Erythrocytes Encapsulated with MGL-BL929 Retain Enzyme Activity for Long Periods of Time In Vitro, and Form a Bioreactor with Sustained MGL Function That Strongly Eliminates L-Methionine.** Morphologic and functional parameters were assessed in carrier cells loaded with the enzyme and in unloaded cells incubated

for 96 hours in 50 mM NaH<sub>2</sub>PO<sub>4</sub>/Na<sub>2</sub>HPO<sub>4</sub>, 90 mM NaCl, 5 mM glucose, 200  $\mu$ M PLP, and 100  $\mu$ M L-methionine; pH 7.4 at 37°C in an atmosphere containing 5% CO<sub>2</sub>. No change in cell number, total amount of hemoglobin (Hb), mean cell Hb, and mean cell Hb concentration was observed during the entire period of time. Unchanged Hb content together with absence of extracellular MGL-BL929 by Western blot in supernatant indicates that encapsulated cells do not release intracellular macromolecules. After 96 hours of incubation, both loaded and unloaded cells showed an increase in mean cell volume and red blood cell distribution width by approximately 23%, and 13%, respectively, and a number of erythrocytes acquired echinocyte-like morphology. Lactate production was not modified by the process; it was 4.25, 3.75, and 4.15  $\mu$ mol/h per milliliter of packed erythrocytes in untreated cells, in unloaded cells, and in cells loaded with MGL-BL929,



**Fig. 5.** Western blots of p16<sup>INK4A</sup> in HT29, HCT116 colon carcinoma, and in HeLa cervix adenocarcinoma cells exposed to MGL-BL929 at 1 U/ml during 48 hours. Figure represents two separate experiments: *Experiment 1*: Lanes 1 and 3 represent blots from cells unexposed and exposed to the enzyme, respectively; and *Experiment 2*: Lanes 2 and 4 represent blots from cells unexposed and exposed to the enzyme, respectively. The protein p16<sup>INK4A</sup> was not detected in colon carcinoma cell lines but was expressed in HeLa cells. No appreciable change in band intensities was observed according to experimental conditions in each experiment. Antibodies against p16<sup>INK4A</sup> were 108349 (Abcam), and 1D7D2 (ThermoFisher).

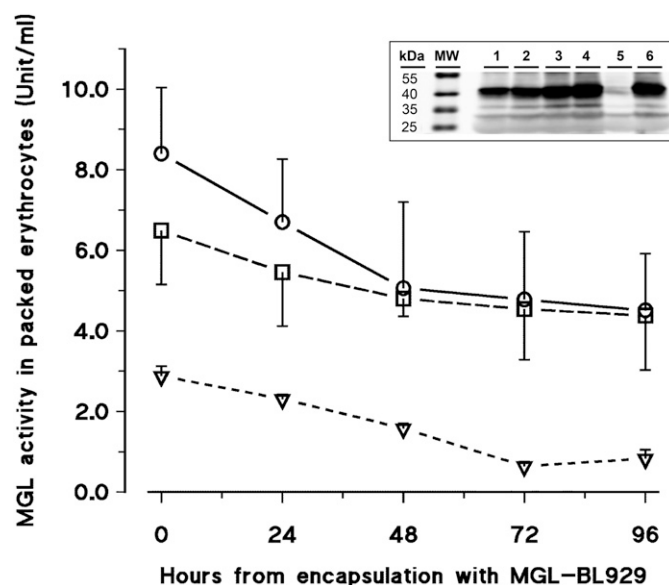


**Fig. 6.** Western blots of global histone H3 dimethyl lysine 9 (H3K9me2) and of total histone H3 in HT29 and HCT116 cells exposed to MGL-BL929 at 1 or 2 U/ml for 48 and 72 hours. Decrease in band intensities of global H3K9me2 ranging from 77% to 89% (mean decrease, 83%) from that in control occurred in HT29 and HCT116 cells exposed to MGL-BL929 at 1 and 2 U/ml, except for a single smaller decrease by 38% in HT29 cells exposed to the enzyme at 1 U/ml for 72 hours. Relative band intensity changes by  $\pm 15\%$  were not observed in total histone H3, nor in H3K4me2, H3K27me2, H3K36me2, and H3K79me2.

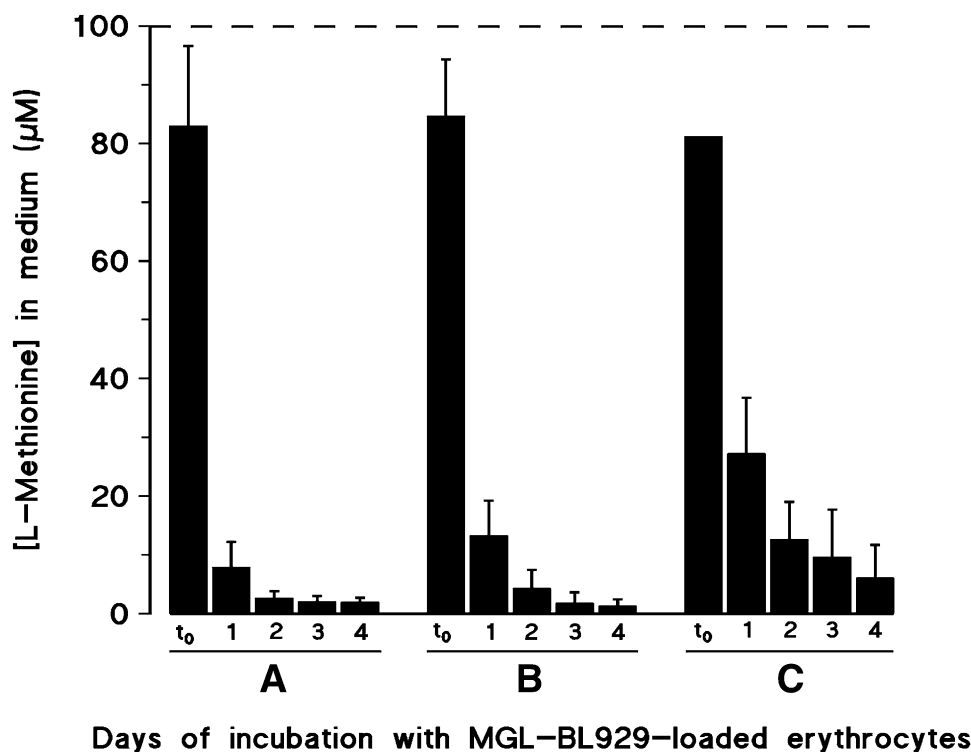
respectively. Enzyme load in erythrocytes augmented gradually with increasing concentration of enzyme in encapsulation solution as estimated by Western blot band intensities (Fig. 7). However, L-methionine  $\gamma$ -lyase activity within loaded erythrocytes at  $t_0$  (in units per milliliter of packed cells) augmented with increasing concentrations of enzyme in encapsulation solution only from 11 to 54 U/ml but did not increase further, or minimally, at 270 U/ml (Fig. 7). Activity decreased partially in the long term; after 96 hours of incubation, residual MGL activity was 54%, 67%, and 29% of values at  $t_0$  in cells loaded with 270, 54, and 11 U/ml of enzyme in encapsulation solution, respectively. From comparisons between enzyme activity and Western blot band intensities in erythrocytes at  $t_0$  (Fig. 7), no apparent correlation was observed between activity and protein load obtained with MGL-BL929 above 54 U/ml in encapsulation solution. Further studies are needed to explain this discrepancy.

Human erythrocytes loaded with the MGL-BL929 incubated at 0.5% erythrocyte volume fraction in medium with 50 mM  $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ , 90 mM NaCl, 5 mM glucose, and 100  $\mu\text{M}$  L-methionine supplemented with PLP at 200  $\mu\text{M}$  strongly eliminated L-methionine from supernatant (Fig. 8). Unloaded control cells had no effect. L-Methionine elimination rates augmented as enzyme load increased in erythrocytes encapsulated with 11 and 54 U/ml. However, rates were similar with erythrocytes loaded with 270 U/ml and with those loaded with 54 U/ml; these two formulations of the bioreactor eliminated L-methionine from supernatant almost completely at 48 hours, with only minimal differences in elimination rates at 24 hours of incubation (Fig. 8). Erythrocytes loaded with 11 U/ml eliminated L-methionine at lower rates than did cells with higher concentrations of the enzyme; under this condition, residual concentrations of L-methionine in supernatant after 96 hours of incubation were approximately 10% of initial levels (Fig. 8). These findings are in accordance with data of MGL activity obtained within erythrocytes (Fig. 7). Initial L-methionine elimination rates were more marked from incubation medium containing PLP at 200  $\mu\text{M}$  than those from medium containing PLP at 0.1  $\mu\text{M}$  (Fig. 9). However, gradual elimination of L-methionine was produced by MGL-BL929-loaded cells incubated in medium

with the lowest concentration of PLP; in this condition, depletion was completed after 120 hours. Similar figures were found in experiments with cells incubated in medium containing pyridoxine (PN) or pyridoxamine (PM) instead of PLP (Fig. 9). Potency and duration of L-methionine elimination by enzyme-loaded erythrocytes at 0.5% erythrocyte volume fraction was assessed from medium with 50 mM  $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ , 90 mM NaCl, 5 mM glucose, and 200  $\mu\text{M}$  PLP, supplemented with high concentration L-methionine (1 mM) once (at  $t_0$ ), twice (at  $t_0$  and after 24 hours), and three times (at  $t_0$ , after 24 hours, and after 48 hours) from the start of the experiment (i.e., media containing cumulative amounts of L-methionine of 1, 2, or 3 mM) (Fig. 10). Elimination of these great amounts of L-methionine occurred rapidly and persisted during the 96 hours of the experiment as demonstrated in supernatant supplemented twice or three times with L-methionine. Erythrocytes loaded with MGL at 270 U/ml in encapsulation solution eliminated high concentrations L-methionine slightly faster than did cells loaded with 54 U/ml, but residual methionine was less than 0.5% of control by 48 hours from the start of the experiment in erythrocytes loaded with each of these two concentrations of enzyme (data not shown). These findings are comparable to those with smaller concentrations of L-methionine in medium (Fig. 8). Elimination of L-methionine from extracellular medium resulted from the ability of the reactor to internalize and then catabolize L-methionine, since enzyme was not released from cells into medium. Highly sensitive Western blot analysis of supernatants could not



**Fig. 7.** L-Methionine  $\gamma$ -lyase activity in human erythrocytes encapsulated with MGL-BL929. Cells were incubated at 0.5% erythrocyte volume fraction in 50 mM  $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ , 90 mM NaCl, 5 mM glucose, 200  $\mu\text{M}$  PLP, and 100  $\mu\text{M}$  L-methionine; pH 7.4 at 37°C in an atmosphere containing 5%  $\text{CO}_2$ . Enzyme activity was assayed at  $t_0$ , and then every 24 hours for 96 hours. Erythrocytes were loaded with encapsulation solution containing the enzyme at 270 U/ml (circles), 54 U/ml (squares), or 11 U/ml (triangles). Values (in units per milliliter of packed cells) represent mean activity  $\pm$ SE. The inset represents estimation of MGL-BL929 load in erythrocytes by Western blot at  $t_0$  in cells subjected to encapsulation in the presence of various concentrations of the enzyme; concentrations of MGL-BL929 in encapsulation solution (U/ml) were: 45 (lane 1), 90 (lane 2), 180 (lane 3), and 270 (lanes 4 and 6). Lane 5 was obtained from unloaded (UL) cells (i.e., cells that were subjected to the loading procedure without the enzyme).



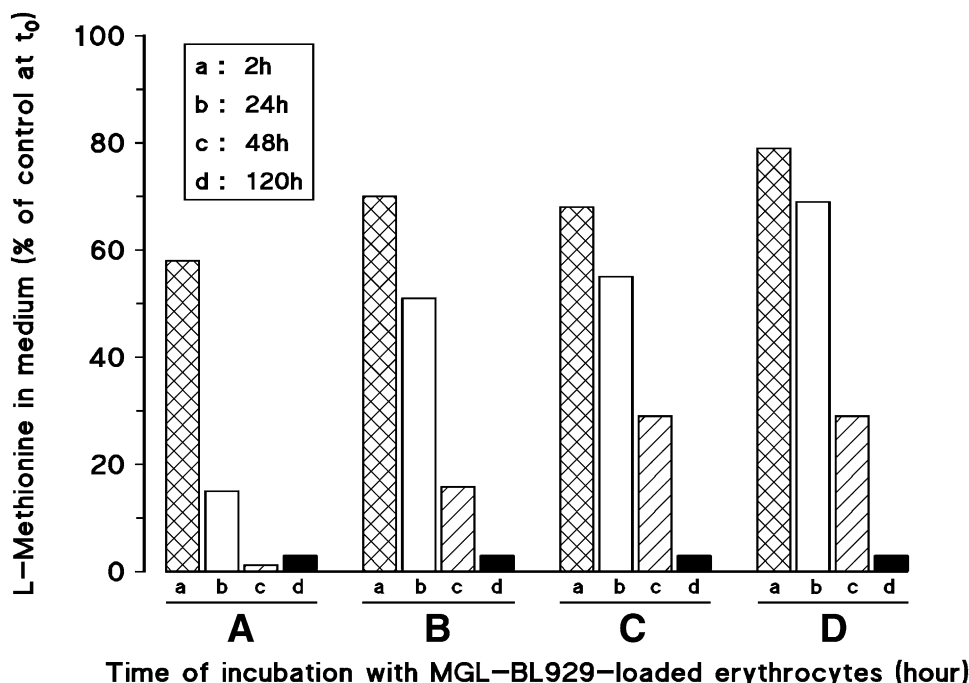
**Fig. 8.** L-Methionine elimination from medium by human erythrocytes loaded with various amounts of MGL-BL929. Concentrations of enzyme in encapsulation solution were 270 U/ml (A), 54 U/ml (B), or 11 U/ml (C). Elimination of L-methionine was assessed in supernatant of MGL-BL929-loaded erythrocytes incubated at 0.5% cell volume fraction in 50 mM  $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ , 90 mM NaCl, 5 mM glucose, 200  $\mu\text{M}$  PLP, and 100  $\mu\text{M}$  L-methionine; pH 7.4 at 37°C in an atmosphere containing 5%  $\text{CO}_2$ . Measurements of L-methionine were done at  $t_0$ , and then every 24 hours for 4 days. Bars indicate mean values of  $\geq 2$  experiments  $\pm$  S.E.

detect free extracellular enzyme, with the exception of traces found in only rare instances of long incubation times, which are accompanied by morphologic alteration of erythrocytes.

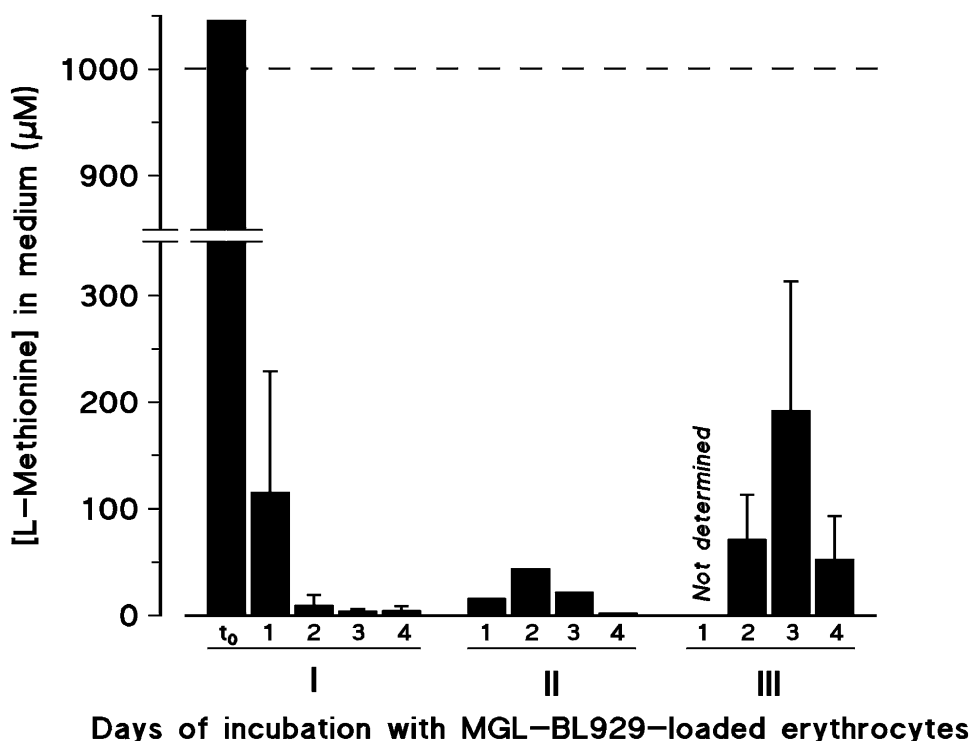
### Discussion

The MGL-BL929 exhibits the conserved homotetrameric structure found in all other methionine  $\gamma$ -lyases that belong to the  $\gamma$ -family of PLP-dependent enzymes (Sun et al., 2003;

Yang et al., 2004; Kudou et al., 2007, 2008; Sato and Nozaki, 2009). Protein sequence alignment showed identity rates ranging from 27.4% to 40.1% between the MGL-BL929 and other known MGLs that originate from various bacterial, protozoan, and plant sources. The MGL-BL929 is different from that encoded by the putative sequence from *Brevibacterium linens* that was previously described (Amarita et al., 2004). Using the latter sequence, we obtained a recombinant protein in *E. coli* that does not possess MGL activity, and



**Fig. 9.** Elimination of L-methionine in supernatant of MGL-BL929-loaded human erythrocytes incubated at 0.5% cell volume fraction at 37°C in an atmosphere containing 5%  $\text{CO}_2$  in media containing various B6 vitamers. Media consisted of 50 mM  $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ , 90 mM NaCl, 5 mM glucose, and 100  $\mu\text{M}$  L-methionine; pH 7.4, supplemented with vitamin B6 in the form of (A) PLP at 200  $\mu\text{M}$ , (B) PLP at 0.1  $\mu\text{M}$ , (C) pyridoxine (PN) at 200  $\mu\text{M}$ , or (D) pyridoxamine (PM) at 200  $\mu\text{M}$ . Erythrocytes were loaded with MGL-BL929 at 54 U/ml in encapsulation solution. Measurements were done at  $t_0$ , and then at various times up to 120 hours (inset) from loading. Concentrations of L-methionine after 120 hours of incubation (d columns) were  $\leq 3$   $\mu\text{M}$  in all conditions.



**Fig. 10.** Elimination of high-concentration L-methionine from medium by MGL-BL929-encapsulated human erythrocytes. Cells were loaded with the enzyme at 270 U/ml in encapsulation solution. Elimination of L-methionine was assessed in supernatant of MGL-BL929-loaded erythrocytes incubated at 0.5% cell volume fraction at 37°C in an atmosphere containing 5% CO<sub>2</sub> in media consisting of 50 mM NaH<sub>2</sub>PO<sub>4</sub>/Na<sub>2</sub>HPO<sub>4</sub>, 90 mM NaCl, 5 mM glucose, and 200 μM PLP; pH 7.4, supplemented with 1 mM L-methionine once at t<sub>0</sub> (I), twice at t<sub>0</sub> and t<sub>24 h</sub> (II), and three times at t<sub>0</sub>, t<sub>24 h</sub>, and t<sub>48 h</sub> (III). Measurements were done at t<sub>0</sub> and then every 24 hours from loading up to 96 hours. Comparisons of residual L-methionine from days 1 to 4 by analysis of variance did not find any significant difference between experimental conditions I, II, and III ( $P = 0.12$ ), and between days of incubation ( $P = 0.36$ ). Bars represent mean values from experiments done in duplicate  $\pm$  S.E.

whose function remains unknown (unpublished data). Our failure to produce an MGL with this sequence is not in agreement with the reported recombinant enzyme that was obtained with the same gene in *Lactococcus lactis* (Hanniffy et al., 2009).

Substrate specificity of the MGL-BL929 restricted to L-methionine and its immediate precursor L-homocysteine has not been described for other characterized recombinant MGLs whose catalytic activity extend to various sulfur-containing amino acids or substituted amino acids (Hori et al., 1996; Tokoro et al., 2003; Kudou et al., 2008; Sato and Nozaki, 2009; Stone et al., 2012). In particular, excepting for the methionine  $\gamma$ -lyase from *Arabidopsis thaliana* (Goyer et al., 2007), other MGLs catalyze  $\alpha,\beta$ -elimination of L-cysteine. The vast cytotoxic activity spectrum of MGL-BL929, comprising 33 of 35 human cancer cell lines of different origin and cell type, demonstrates that enzymatic methionine-specific deprivation induces growth inhibition and cell death in most cancer cells. However, sensitivity to L-methionine depletion, whose degree depends on concentration of MGL-BL929 (Fig. 2), varies among cancer cell lines, resulting in IC<sub>50</sub> values ranging from 0.05 to 2.14 U/ml (Table 2). It is noteworthy that amounts of MGL-BL929 producing high L-methionine elimination rates leading to concentrations close to the detection limit lead to variable degrees of cytotoxicity. The mechanisms underlying cancer cell growth inhibition with respect to degree of L-methionine availability have not yet been determined. Resistance to methionine deprivation was only found in two colorectal cancer cell lines that originated from two different tumor sites of the same primary. These rare exceptions indicate that methionine dependence may not be a constant metabolic feature in cancer as previously suggested (Stern et al., 1984), and that growth independence with respect to exogenous methionine supply can be retained at different stages of the neoplastic evolution. Instability of

the MGL-BL929 activity in vitro can be explained, in part, by the affinity of HSA for PLP, and by dephosphorylation of PLP by plasma alkaline phosphatases that may reduce cofactor availability (Bohney et al., 1992; di Salvo et al., 2011). The requirement for cofactor is probably owing to weak binding of the apoenzyme to PLP, a feature that may be exhibited by most MGLs (Sun et al., 2003; Yang et al., 2004). However, differences in binding affinities of apoenzyme for cofactor between these enzymes have been reported, e.g., dissociation constant ( $K_D$ ) values for binding to PLP were found to be 0.62 μM for the MGL from *Citrobacter freundii* and 14.5 μM for the MGL from *Clostridium novyi* (Kulikova et al., 2017).

Reduction of methylation levels of hyper-methylated gene promoters in HT29 carcinoma cells, as shown by a highly sensitive method for 5-meC quantitation, confirms prior results of global genomic DNA methylation analysis performed with the MGL from *P. putida* in CCRF-CEM lymphoma cells (Machover et al., 2002). Decrease in promoter 5-meC DNA levels with increasing time of exposure to the enzyme fits with the hypothesis of a mechanism resulting from progressive decrease in availability of S-adenosyl L-methionine (SAM), the cofactor of DNA methyltransferases, owing to L-methionine deficiency. Results suggest that partial demethylation of CDKN2A promoter contributed to the increase in mRNA expression level. However, genes needed for cell maintenance, including DNMT1L and ATP10A, as well as MyoD, a mesenchymal lineage specification gene that is repressed in epithelial cells, did not have their mRNA expression changed even though ATP10A and MyoD promoters were partially demethylated under exposure to MGL-BL929. L-Methionine deficiency-induced histone H3 dimethyl lysine level changes were restricted to global H3K9me2, whose levels were strikingly reduced. These findings of selective decrease in overall H3K9me2 accompanying demethylation of a large

number of highly hypermethylated gene promoters suggest the triggering of a process that is probably not explained solely by a decrease in SAM availability. Analysis of the epigenetic significance of these changes requires further experiments using chromatin immunoprecipitation (ChIP) to explore histone H3 dimethyl lysine distribution along specific genes and their relation with promoter methylation and transcription. Prior studies using this method showed that DNA methylation-dependent gene silencing is associated with a gain of H3K9me2 at specific gene regions (Kondo et al., 2003, 2004; Hinshelwood et al., 2009). Increased gene-associated H3K9me2 occurred with CDKN2A promoter hypermethylation and silencing in HCT116, RKO, and SW48 colorectal cancer cells (Kondo et al., 2003); and enrichment for H3K9me2 marks accompanying CDKN2A gene promoter hyper-methylation was found in primary human mammary cells progressing in vitro from normal to premalignant cells (Hinshelwood et al., 2009). Absence of the p16<sup>INK4A</sup> suggests the presence of a process preventing this protein's expression in HT29 and HCT116 cells grown in standard conditions as well as under exposure to MGL-BL929. This repressive yet unidentified mechanism is cell line-specific because p16<sup>INK4A</sup> was present in control HeLa cells. Transcription of altered CDKN2A mRNA and impairment of p16<sup>INK4A</sup> translation in cancer cells by several mechanisms have been reported [reviewed by Li et al. (2011)]. We hypothesize that cytotoxicity induced by L-methionine deficiency is related to mechanisms resulting from SAM deficiency. Within the context of gene expression complexity found in cancer cell lines, the present findings suggest that the cytotoxic effect of MGL-BL929 is attributable, in part, to changes in methylation-sensitive epigenetic abnormalities involved in oncogenesis, including DNA methylation.

As predicted by data from enzyme stability in vitro, duration of L-methionine depletion in mouse plasma after parenteral administration of MGL-BL929 was short, falling within time-range limits reported for the native MGL from *Pseudomonas putida* (Sun et al., 2003; Yang et al., 2004; Stone et al., 2012). Parenteral supply of PLP was found to overcome inactivation of the PEG-conjugated MGL from *P. putida* in mouse plasma (Sun et al., 2003; Yang et al., 2004), suggesting that high-dose PLP, as well as high doses of unphosphorylated B6 vitamers (e.g., PN or PM) given by parenteral route, should be studied with the aim of improving stability in plasma of the native MGL-BL929 whose inactivation is, at least in part, owing to insufficient cofactor availability. However, exogenous or synthesized PLP is probably cleared from plasma by serum albumin and alkaline phosphatases at rates that may prevent achievement of cofactor levels required to sustain activity of the MGL lying free in plasma for long periods of time (Bohney et al., 1992; Zemleni and Kübler, 1994; di Salvo et al., 2011). From these limitations to the use of the free native enzyme, we hypothesized that carrier erythrocytes could provide encapsulated MGL-BL929 the environment required for prolonged catalytic function. Furthermore, in this configuration, absence of  $\alpha,\beta$ -elimination of L-cysteine with the MGL-BL929 should have limited or no impact on glutathione synthesis, whose deficiency would shorten enzyme-loaded erythrocyte lifespan (Meister and Anderson, 1983). As reported with the MGL from *P. putida* (Gay et al., 2017), we successfully built a bioreactor with durable catalytic MGL function by encapsulation of the MGL-BL929 into human erythrocytes. Under present

experimental conditions, the intracellular erythrocyte environment durably preserves MGL activity. The intense and durable elimination of high concentrations of L-methionine from medium highlights the robustness of the bioreactor whose MGL function is retained in vitro. The variable rapidity of L-methionine elimination according to concentration of PLP in medium is probably the result of differences in amounts of cofactor uptake by MGL-loaded erythrocytes (Maeda et al., 1976). Furthermore, on the basis of the naturally occurring rapid decline of PLP concentration in red blood cells (Zemleni and Kübler, 1994; Machover et al., 2018), evidence of elimination of L-methionine by the bioreactor from medium containing a low concentration of PLP, as well as PN or PM instead of PLP, suggest that sustained MGL function within loaded erythrocytes in the longer term is probably relative to the newly synthesized intracellular cofactor (Anderson et al., 1971; di Salvo et al., 2011). These findings together with data from previously reported erythrocyte pharmacokinetics of vitamin B6 (Zemleni and Kübler, 1994; Machover et al., 2018) suggest the possibility of modulating the bioreactor's MGL activity in vivo by supplying cells with B6 vitamers.

Treatment of cancer with the powerful cytotoxic MGL-BL929 from *Brevibacterium aurantiacum* requires a pharmaceutical delivery system that preserves activity of the enzyme to achieve sustained L-methionine depletion in vivo. Development of a bioreactor with this capacity is in progress.

#### Acknowledgments

We thank Dr. Ibrahim Casal for support with animal experiments.

#### Authorship Contributions

*Participated in research design:* Machover, Goldschmidt, Saffroy, Hamelin, Rossi, Gaston-Mathé.

*Conducted experiments:* Machover, Hamelin, Saffroy, Mollicone, Rossi, Pierigè, Haghighi-Rad.

*Contributed new reagents or analytic tools:* Machover, Bonnarne, Rossi, Pierigè, Saffroy, Hamelin, Chadefaux-Vekemans, Desterke, Magnani, Mollicone, Haghighi-Rad, Dairou, Briozzo, Kopečný, Boucheix.

*Performed data analysis:* Machover, Saffroy, Hamelin, Goldschmidt, Rossi, Desterke, Mollicone, Haghighi-Rad, Chadefaux-Vekemans, Pierigè, Dairou.

*Wrote or contributed to the writing of the manuscript:* Machover, Goldschmidt, Rossi, Bonnarne, Briozzo, Desterke, Saffroy, Boucheix.

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