# Serine Deprivation Enhances Antineoplastic Activity of Biguanides

Simon-Pierre Gravel<sup>1,2</sup>, Laura Hulea<sup>3,4,5</sup>, Nader Toban<sup>3,5</sup>, Elena Birman<sup>5</sup>, Marie-José Blouin<sup>5</sup>, Mahvash Zakikhani<sup>5</sup>, Yunhua Zhao<sup>5</sup>, Ivan Topisirovic<sup>2,3,4,5</sup>, Julie St-Pierre<sup>1,2</sup>, and Michael Pollak<sup>3,4,5,6</sup>

# Abstract

Metformin, a biguanide widely used in the treatment of type II diabetes, clearly exhibits antineoplastic activity in experimental models and has been reported to reduce cancer incidence in diabetics. There are ongoing clinical trials to evaluate its antitumor properties, which may relate to its fundamental activity as an inhibitor of oxidative phosphorylation. Here, we show that serine withdrawal increases the antineoplastic effects of phenformin (a potent biguanide structurally related to metformin). Serine synthesis was not inhibited by biguanides. Instead, metabolic studies indicated a requirement for serine to allow cells to compensate for biguanide-induced decrease in oxidative phosphorylation by upregulating glycolysis. Furthermore, serine deprivation modified the impact of metformin on the relative abundance of metabolites within the citric acid cycle. In mice, a serine-deficient diet reduced serine levels in tumors and significantly enhanced the tumor growth-inhibitory actions of biguanide treatment. Our results define a dietary manipulation that can enhance the efficacy of biguanides as antineoplastic agents that target cancer cell energy metabolism.

#### Introduction

There is evidence that metformin, like other biguanides, inhibits complex I of the mitochondrial electron transport chain (1, 2). In the liver, this causes energetic stress, resulting in inhibition of hepatic gluconeogenesis and reduction in the hyperglycaemia and hyperinsulemia associated with type II diabetes (3, 4). Pharmacoepidemiologic studies have generated the hypothesis that there are novel indications for metformin in oncology by associating its use for diabetes treatment with reductions in cancer risk and improvements in cancer prognosis (reviewed in refs. 5, 6). These findings led to investigation of metformin in preclinical cancer models, most of which demonstrate clear antineoplastic effects that are attributable to direct action on neoplastic cells and/or indirect effects resulting from alterations in the hormonal milieu (2, 5–7). However, the methodology used in some of the pharmacoepidemiologic studies is controversial (8), and most laboratory models involve drug exposure levels considerably higher than those used in diabetes treatment (9). Thus, it is uncertain whether conventional antidiabetic doses of metformin as a single agent have useful antineoplastic activity, and this possibility is being examined in ongoing clinical trials. However, in view of evidence that biguanides may have multiple mechanisms of action (for example, refs. 10–14) and that sensitivity to biguanides varies with tumor characteristics (15–17), nutrient conditions (17, 18), and other factors (19, 20), it is important to define metabolic conditions that influence the action of biguanides.

Cancer cells are metabolically adapted to meet the bioenergetics and biosynthetic demands of proliferation. Dysregulated metabolism in cancer cells presents potential therapeutic targets (21). Enzymatic imbalance in the metabolism of serine in cancer has been shown almost two decades ago (22), and was confirmed by more recent studies highlighting increased expression of serine synthesis pathway enzymes in breast cancer and melanoma (23, 24). In addition, serine starvation causes decreased proliferation and survival (25, 26) and induces metabolic reprogramming by inhibiting glycolysis (25-27). As cancer cells treated with biguanides have a particularly stringent reliance on glycolysis to compensate for the inhibition of mitochondrial ATP production and reduced glucose carbon flux to the citric acid cycle, we investigated the influence of serine deprivation on the antiproliferative effects of metformin and phenformin.

<sup>&</sup>lt;sup>1</sup>Goodman Cancer Research Centre, McGill University, Montreal, Quebec, Canada. <sup>2</sup>Department of Biochemistry, McGill University, Montreal, Quebec, Canada. <sup>3</sup>Division of Experimental Medicine, McGill University, Montreal, Quebec, Canada. <sup>4</sup>Department of Oncology, McGill University, Montreal, Quebec, Canada. <sup>5</sup>Segal Cancer Center, Lady Davis Institute for Medical Research, Jewish General Hospital, Montreal, Quebec, Canada. <sup>6</sup>Division of Cancer Prevention, McGill University, Montreal, Quebec, Canada.

S.-P. Gravel, L. Hulea, and N. Toban contributed equally to this article.

**Corresponding Authors:** Michael Pollak, Division of Experimental Medicine, McGill University, Montreal, QC H3T 1E2, Canada. Phone: 514-340-8222, ext. 4139; Fax: 514-340-8600; E-mail: michael.pollak@mcgill.ca; Julie St-Pierre, Goodman Cancer Research Centre, McGill University, Montreal, QC, Canada, julie.st-pierre@mcgill.ca; and Ivan Topisirovic, Segal Cancer Center, Lady Davis Institute for Medical Research, Jewish General Hospital, Montreal, QC, Canada, ivan.topisirovic@mcgill.ca

# **Materials and Methods**

# Cell lines, tissue culture, viral infections, and lentiviral shRNA silencing and proliferation assays

H1299, A549, MCF10A, and PrEC were purchased from and authenticated by the ATCC using identifiable short tandem repeat loci. MC38 cell line was generously provided by Dr. Pnina Brodt (McGill University, Montreal, OC, Canada) and authenticated by her laboratory. H1299 with AMPK $\alpha$ 1/2 stable knockdown and control cells were a generous gift from Dr. Russell Jones (McGill University) and authenticated by his laboratory (28). All the cell lines were used within 6 months of resuscitation. H1299, A549, and MC38 cell lines were cultured in RPMI-1640 with 10% (v/v) FBS (Wisent) and gentamycin. MCF10A were cultured in media supplemented with 10 µg/mL insulin, 20 ng/mL EGF, 100 ng/mL cholera toxin, and 0.5 µg/mL hydrocortisone; DMEM/F12 5% FBS media were use for regular cell culture and RPMI 5% dialyzed FBS media without serine were used for serine deprivation experiments. Primary prostate epithelial cells (PrEC) were cultured in media supplemented with the Prostate Epithelial Cell Growth Kit (ATCC); prostate epithelial cell basal medium (ATCC) was used for regular culture, and RPMI media without serine were used for serine deprivation experiments.

All lentiviral shRNA vectors were retrieved from the arrayed Mission TRC genome-wide shRNA collections purchased from Sigma-Aldrich Corporation. Additional information about the shRNA vectors can be found at http://www.sigmaaldrich.com/life-science/functional-genomics-and-rnai/shrna/library-information.html or http://www.broad.mit.edu/genome\_bio/trc/rnai.html, using the TRCN number. The following lentiviral shRNA vector targeting mouse PHGDH was used: TRCN0000041627. The Non-Target shRNA Control (Sigma: SHC002) was used as negative control. Lentiviral supernatants were generated as described at http://www.broadinstitute.org/rnai/public/resources/protocols. Supernatants were applied on target cells with polybrene (6 µg/mL). Cells were reinfected the next day and, 2 days later, selected with puromycin for 72 hours (4 µg/mL; Sigma).

To assess proliferation, cells were seeded in culture plates and incubated for 24 hours to allow attachment. Subsequently, seeding media were replaced with treatment media and cells were grown for varying lengths of time, at the end of which they were detached by trypsinization and collected. Cells were stained with trypan blue and counted using an automated cell counter (Invitrogen), which assessed the number of total and viable cells. In each experiment, initial seeding density was chosen to avoid confluence in fastest growth condition at the intended time point.

For the bromodeoxyuridine (BrdUrd) incorporation assay (Cell Proliferation ELISA BrdUrd Kit from Roche), cells were seeded in 96-well plates (1,000 cells/well) and maintained as indicated in the Fig. 1 legend for 72 hours. The assay was performed as per the manufacturer's instruction. Absorbance at 450 nm (reference wavelength 690 nm) was measured using a FluoStar Optima microplate reader (BMG Labtech).

Viable cell count or BrdUrd incorporation values for the indicated samples were normalized to those obtained for vehicle-treated cells (control). Data are expressed as a percentage of inhibition relative to vehicle treated cells (control). Cell death was estimated by trypan blue staining and data are expressed as the ratio of trypan blue positive/total number of cells.

#### Animals

All protocols were approved by the McGill University Animal Care and Handling Committee. Male C57BL/6 mice were purchased from Charles River Laboratories (Saint-Constant, Québec, Canada) at 5 to 6 weeks of age. Animals were acclimatized for 1 week, after which, they were divided into two groups, one receiving a control diet and the other, a diet lacking serine and glycine (ser<sup>-</sup>/gly<sup>-</sup>; Purchased from TestDiet). The control diet (formulation #: 5CC7) consisted of sucrose (25.9%), corn starch (41.8%), corn oil (5.0%), and the following amino acids: glutamine (1.00%), asparagine (1.00%) arginine (0.83%), histidine (0.49%), isoleucine (0.80%) leucine (1.20%), lysine (1.12%), methionine (0.60%), cystine (0.40%), phenylalanine (0.80%), tyrosine (0.40%), threonine (0.78%), tryptophan (0.20%), valine (0.80%), alanine (1.00%), aspartic acid (1.00%), glutamic acid (1.00%), glycine (0.99%), proline (1.00%), serine (1.00%). The ser<sup>-</sup>/gly<sup>-</sup> diet (customized from 5CC7) was formulated identically; however, serine and glycine were omitted from the amino acid mixture. Animals were maintained on these diets for 2 weeks before the start of the allograft experiments to assess acceptance of new diets and weight gain indicative of adequate nutrition.

#### Allograft experiment

Animals were fed either control diet or serine and glycine deficient diet throughout the experiment. MC38 cells (5  $\times$  10<sup>5</sup> per animal) were implanted by s.c. injection into right flank (day 0). Mice from each diet group were further subdivided into treatment and vehicle groups (n = 8). On day 5, treatment groups began receiving twice-daily i.p. injections of 40 mg/kg phenformin, a biguanide with greater in vivo bioavailability than metformin, whereas control groups received twice-daily i.p. saline injections. In 2-day intervals and on the sacrifice day, mice were weighed and tumors were measured by electronic calipers. On day 15, animals were sacrificed and their tumors were excised, and flash-frozen in liquid nitrogen. For GC-MS (gas chromatography-mass spectroscopy) analyses, tumors were grounded in liquid nitrogen and 6 to 10 mg was weighted in tubes kept on dry ice. Each tumor was extracted independently three times with 80% (v/v) MeOH/milliQ water kept on dry ice. Suspensions were vortexed and sonicated for 10 to 20 minutes at 4°C (30 seconds ON and 30 seconds OFF, high setting, using Diagenode's Bioruptor), vortexed, and cleared by centrifugation (21,000  $\times$  g, 10 minutes/4°C). Supernatants were transferred to prechilled tubes and 800 ng of the internal standard myristic acid-D<sub>27</sub> diluted in pyridine was added to each sample. Samples were allowed to dry entirely in a Labconco CentriVap cold trap. GC-MS procedure (see GC-MS section) was carried out independently three times for the individual extraction.

# Plasma serine and glycine

Approximately 700  $\mu$ L of blood obtained by cardiac puncture from mice were transferred into BD Vacutainer Sodium Heparin collection tubes (BD Biosciences) and plasma was separated by centrifugation. Plasma samples were flash-frozen and stored at  $-80^{\circ}$ C. Plasma (2.5  $\mu$ L) thawed shortly on ice was diluted in 300  $\mu$ L 80% (v/v) MeOH/milliQ water kept on dry ice. Samples were vortexed, sonicated, and cleared by centrifugation (21,000  $\times$  g, 10 minutes/4°C). Supernatants were transferred to prechilled tubes and 800 ng of the internal standard myristic acid-D<sub>27</sub> diluted in pyridine was added to each sample. Samples were allowed to dry entirely in a Labconco CentriVap cold trap. The GC–MS procedure can be found in the GC–MS section.

## Immunoblots

For protein analysis, cells were seeded and cultured in complete medium for 24 hours to obtain 30% confluence. Culture medium was replaced with indicated treatment media, and cells were cultured for various lengths of time, after which they were washed with ice-cold PBS and lysed in lysis buffer containing 10 mmol/L Tris-HCl (pH 7.3), 150 mmol/L NaCl, 1% sodium deoxycholate, 1% Triton X, 1 mmol/L EDTA, 10 mmol/L  $\beta$ -glycerophosphate, and 0.5 mmol/L NaF, supplemented with protease inhibitor pills (Roche). The crude lysates were centrifuged at 13.000 rpm for 15 minutes at 4°C and the resulting soluble fractions were isolated. Protein concentration was assessed using a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific). Loading buffer was added to cleared lysates, which were subsequently boiled for 5 minutes. Samples were loaded into SDS-polyacrylamide gels, resolved by electrophoresis, transferred onto nitrocellulose membranes, and probed using the indicated antibodies. Protein bands were visualized with the ChemiDoc XRS<sup>+</sup> System (Bio-Rad) using SuperSignal West Femto Chemiluminescent Substrate (Thermo Fisher Scientific) or by using enhanced chemiluminescence (GE Healthcare). Antibodies for PHGDH were obtained from Novus Biologicals and Sigma (HPA021241) whereas those against phospho-S240/244 RPS6 (rpS6), phospho-T389 RPS6KB1 (p70 S6K), phospho-T37/46 4E-BP1, phospho-T172 PRKAA1 (AMPKa), phospho-S79 ACACA (ACC), RPS6, p70 S6K, 4E-BP1, AMPK, ACC, and ACTB ( $\beta$ -actin) were obtained from Cell Signaling Technology.

#### Glucose consumption and lactate production assays

Cells were seeded and cultured in complete medium for 24 hours to obtain 30% density. Medium was replaced with indicated treatment media, and cells were cultured for 24 hours. Subsequently, supernatant samples were collected. Cells were also collected, lysed, and assayed for protein content. Measurement of glucose concentration in samples was done as previously described (29). Total consumption was calculated by subtracting results from baseline glucose concentration, measured in samples from media incubated in identical conditions, without cells. Lactate production was quantified using a commercial lactate assay kit (BioVision). Both glucose consumption and lactate production were normalized to protein content as described previously (30). The magnitude of

changes in lactate production and glucose uptake over the 24-hour incubation was much larger than the changes in cell number or in protein content. Cells were collected, lysed, and protein content was determined using a BCA protein assay kit by multiplying protein concentration by total volume of lysate. Molar concentrations of metabolites (in mmol/L) were multiplied by total media volume per well (2 mL) and data expressed as nmol/µg of protein.

#### Stable isotope tracing experiments

MC38 cells grown up to 40% confluence in 35-mm plates were subjected to  ${}^{13}C_6$ -glucose (CML-1396; Cambridge Isotope Laboratories, Inc.) or <sup>13</sup>C<sub>5</sub>-glutamine (CML-1822; Cambridge Isotope Laboratories, Inc.) pulse labeling along with metformin treatment, serine deprivation, or pyruvate supplementation for 24 hours (citrate isotopomer analyses), or pulsed for 6 hours in the presence of  ${}^{13}C_6$ -glucose after the 24 hours incubations (serine isotopomer analyses). Basal medium used was RPMI-1640 lacking glucose, glutamine, glutamate, serine, and glycine (Wisent) and supplemented with 2% (v/v) dialyzed FBS (Wisent). <sup>13</sup>C<sub>6</sub>-glucose and <sup>13</sup>C<sub>5</sub>-glutamine were used at half the final concentration, with unlabeled counterparts kept at 5.55 mmol/L glucose and 2 mmol/L glutamine (citrate isotopomer analyses) or at final 5.55 mmol/L for  ${}^{13}C_6$ -glucose (serine isotopomer analyses). When used as supplements, serine was added at 30 µg/mL and pyruvate was added at 1 mmol/L. Following incubations, cells were washed once with 2 mL 0.9% (w/v) NaCl/4°C on ice, then 300 µL 80%(v/v) MeOH kept on dry ice was added to each well. Cells were removed from plates and transferred to prechilled tubes. MeOH quenching and harvest were repeated one or two times to ensure complete recovery. Suspensions were sonicated for 10 to 20 minutes at 4°C (30 seconds ON and 30 seconds OFF, high setting, using Diagenode's Bioruptor), vortexed, and cleared by centrifugation (21,000  $\times$  g, 10 minutes/4°C). Supernatants were transferred to prechilled tubes and 800 ng of the internal standard myristic acid-D<sub>27</sub> diluted in pyridine was added to each sample. Samples were allowed to dry entirely in a Labconco CentriVap cold trap.

## GC-MS

The GC–MS procedure was the same for plasma samples, tumor extracts, and cell extracts. Pyridine ( $30 \mu$ L) containing 10 mg/mL methoxyamine hydrochloride (Sigma) was added to dried samples. Samples were vortexed and sonicated, cleared by centrifugation, and supernatants were heated at 70°C for 30 minutes in GC–MS injection vials. Samples were further incubated for 1 hour after the addition of 70-µL N-Methyl-N-tertbutyldimethylsilyltrifluoroacetamide (MTBSTFA; Sigma). One µL was used per sample for GC–MS analysis. GC–MS installations and softwares were all from Agilent. GC–MS methods are as previously described (31).

#### Mass isotopomer distribution analysis

Ion integration was done with the Agilent Chemstation software. Integrations of all m+i ions, where m is the M-57 fragment of TBDMS derivatives and *i*, the number of possible <sup>13</sup>C for this fragment, were transferred to a spreadsheet



Figure 1. Serine deprivation enhances the antiproliferative effects of metformin *in vitro*. H1299 (A) and A549 (B) cells were treated for 72 hours with vehicle (PBS) or metformin (2.5 mmol/L) in media with or without 30 mg/L serine. Viable cells were counted; data, shown as mean  $\pm$  SEM (*n* = 3); \*, *P* < 0.001. Results are representative of at least three independent experiments. C, cell lysates from H1299 and A549 cells treated as shown for 24 hours were immunoblotted using the indicated antibodies. (*Continued on the following page*.)

together with the integration of the internal standard myristic acid-D<sub>27</sub>. A correction matrix was generated for each metabolite using an in-house algorithm adapted from ref. 32. Integration values for a given metabolite were multiplied by the corresponding correction matrix to remove the abundances of naturally occurring isotopes that mask the labeling provided by exogenous <sup>13</sup>C-substrates. Values obtained for a given metabolite correspond to proportional isotopomer enrichment, for example, the proportion of a labeled fraction within the pool of total ions. To assess relative amounts of labeled ions, values of the proportional labeling for a given metabolite were multiplied by the abundance of this metabolite previously divided by the integration of the internal standard and further divided by cell number. Values obtained are presented as normalized ion amounts, in which the proportions of labeled ions are adjusted to the amount of the metabolite analyzed. For GC-MS data corrected on cell number, cells counts were obtained on three biological replicates using a TC10 counting device (Bio-Rad).

## **Statistical analyses**

All experiments were independently performed at least three times unless otherwise specified. When biological replicates are shown, they are taken from a single experiment that was representative of multiple independent experiments. Student t tests were used for comparisons of individual treatments versus control. Two-way ANOVA with Bonferroni posttests was used for multiple comparisons. Statistical tests were performed with Microsoft Excel, GraphPad Prism, or GraphPad InStat.

#### Results

# Cell proliferation is influenced by interactions between serine availability, phosphoglycerate dehydrogenase expression, and metformin exposure

Either directly or through conversion to glycine, serine allows for the replenishment of one-carbon units, which play an important role in DNA methylation, maintenance of redox balance, and biosynthesis of nucleotides, phospholipids, and other amino acids (33). Consequently, serine has been shown to be indispensable for the growth and proliferation of certain cancer cell lines, which must obtain it through uptake or through de novo biosynthesis from glucose. Thus, strategies combining serine deprivation and disruption of serine biosynthesis are antiproliferative in vitro (23, 24, 34). We investigated the impact of serine availability on the proliferative capacity of the human cancer cell lines H1299 and A549. Serine withdrawal exerted a substantial inhibitory effect on the proliferation of A549 cells, whereas it inhibited more weakly the proliferation of H1299 cells (Fig. 1A and B). This led us to hypothesize that these cell lines differ in their serine metabolism. As expected, relative to H1299 cells, A549 cells show reduced expression of PHGDH, the enzyme catalyzing the first step of the *de novo* serine synthesis pathway (Fig. 1C). To confirm that robust expression of PHGDH is key to sustaining proliferation in the absence of serine, we used MC38 colon cancer cells, previously demonstrated to be metformin-sensitive *in vivo* (11). We depleted MC38 cells of PHGDH using shRNA (>90% depletion as compared with control; Fig. 2A). Serine deprivation had a drastic effect on cell proliferation in PHGDH-depleted cells (Fig. 2B).

Cancer cells must coordinate the diversion of glucose metabolism into biosynthesis pathways to meet the macromolecular synthesis requirements for proliferation (21). Given the importance of serine for many biosynthetic pathways, its intracellular level may be an important indicator of cellular biosynthesis needs. In addition, serine deprivation has been shown to reduce the rate of glycolysis (25-27), which we hypothesized would increase sensitivity to the metabolic stress induced by biguanides. Therefore, we treated serine-deprived MC38 cells with metformin. In agreement with our hypothesis, serine withdrawal increased the antiproliferative effectiveness of otherwise suboptimal doses of metformin (1-2.5 mmol/L) in MC38 cells (Fig. 1D). At 2.5 mmol/L, metformin did not greatly hinder proliferation of H1299 (Fig. 1A) or MC38 (Fig. 1E and F) cells in serine-replete conditions. This contrasts with results obtained with A549 cells (Fig. 1B) that are known to be sensitive to biguanides through a separate mechanism related to a lack of STK11 (LKB1) expression (15). Under serine deprivation, metformin caused a marked inhibition of proliferation in both H1299 and MC38 cells that was not seen with either serine deprivation alone or metformin treatment alone (Fig. 1A, E, and F, respectively). Proliferation curves for MC38 under serine deprivation combined with 2.5 mmol/L metformin revealed that arrest occurs within the first 24 hours of treatment and that neither serine deprivation nor the administration of 2.5 mmol/L metformin in the presence of serine affected the proliferation rate of MC38 cells (Fig. 1H). The observed decrease in cell number caused by metformin and serine withdrawal relative to control is primarily due to decreased proliferation (Fig. 1F), whereas cell survival was only modestly decreased under these conditions (Fig. 1G).

Importantly, although serine withdrawal or metformin treatment reduced proliferation of nontransformed, immortalized MCF10A cells, the combination did not significantly further suppress proliferation (Fig. 1E, right side). This is in direct contrast with the results obtained with transformed MC38 cells (Fig. 1E, left side). Moreover, serine deprivation did not bolster the antiproliferative effects of metformin on PrECs (Fig. 1I). These findings provide evidence that serine withdrawal potentiates the antiproliferative effects of metformin

<sup>(</sup>*Continued.*) D, metformin, at concentrations between 0.5 and 5 mmol/L, substantially inhibited proliferation in MC38 cells cultured for 72 hours without serine. Data, mean  $\pm$  SEM (n = 3; \*, P < 0.01). E–G, MC38 cells and MCF10A cells were treated as indicated for 72 hours. Proliferation was assessed by cell count (E) or BrdUrd incorporation (F) and cell death by trypan blue exclusion (G). Data, mean $\pm$  SEM (n = 3; \*, P < 0.05). H, the combination of serine deprivation and metformin (2.5 mmol/L) results in proliferation arrest within 24 hours of exposure in MC38 cells. Cells were cultured as shown and were counted at the indicated times. Data, mean  $\pm$  SEM (n = 3) \*, P < 0.05 compared with control. I, PrECs were treated with metformin in the absence of serine for 48 hours; viable cells were counted. Effect of serine deprivation was compared with data obtained for H1299, MC38, and MCF10A cells in equivalent conditions. Data, mean  $\pm$  SEM (n = 3; \*, P < 0.05).



**Figure 2.** PHGDH knockdown exacerbates the sensitivity to serine deprivation in MC38 cells, but metformin does not inhibit serine biosynthesis. A, immunoblot analysis of cell lysates from MC38 cells stably expressing control or PHGDH shRNA, treated as indicated for 16 hours. B, MC38 control or PHGDH-depleted cells were treated as indicated for 72 hours and viable cells were counted. For each cell line (PHGDH shRNA or control shRNA), values were normalized to the vehicle control-treated cells (set to 100%). Data, mean  $\pm$  SEM (n = 4);\*, P < 0.01. C, schematic depicting the glucose carbon flow into serine biosynthesis. Unlabeled carbons (<sup>12</sup>C) are shown in white and <sup>13</sup>C in black. 3-PG: 3-phosphoglycerate. D, stable isotope tracer analysis of serine in MC38 cells incubated with <sup>13</sup>C<sub>6</sub>-glucose for 6 hours following the indicated 24 hour treatments. Each bar integrates serine isotopomer amounts and shows the relative metabolite levels per cell. Data, mean  $\pm$  SEM (n = 3); \*, P < 0.05 (sum of glucose-derived isotopomers); #, P < 0.05 (m+0). E, immunoblot analysis of MC38 cell lysates treated as indicated for 96 hours.

on transformed cells to a greater extent than nontransformed cells, which is likely a consequence of reduced capacity of transformed cells to adapt to energetic stress.

Next, we investigated the effects of the attenuation of *de novo* serine biosynthesis on metformin-induced inhibition of proliferation in MC38 cells (Fig. 2A). In the presence of serine, PHGDH depletion did not significantly potentiate the antiproliferative effects of 2.5 mmol/L metformin as compared with control. Inhibition of proliferation induced by a combination of PHGDH depletion and metformin (~10% inhibition relative to metformin treated, control shRNA infected cells) was lower in magnitude compared with the combination of metformin and serine deprivation. This is likely due to the compensatory increase in serine

uptake (see below) and/or the ability of remaining PHGDH to support low levels of serine biosynthesis. Of note, combined serine deprivation and PHGDH depletion induced dramatic inhibition of proliferation (more than 95% of the control), which rendered determination of the combined effects of serine withdrawal, PHGDH depletion, and metformin treatment unfeasible (Fig 2B). Collectively, these findings suggest that intracellular serine levels influence antineoplastic activity of biguanides.

# Dietary restriction of serine and glycine increases *in vivo* effectiveness of phenformin as an antineoplastic agent

It has been shown that a diet deficient in serine and glycine significantly reduces the serum levels of these amino acids in



**Figure 3.** Serine deprivation enhances the antiproliferative effects of phenformin *in vivo*. A, serine- and glycine-free diet sensitizes tumors to an otherwise ineffective dose of phenformin. Mice (C57BL/6 male) were fed either control diet containing all amino acids or isocaloric diet lacking serine and glycine. After 2 weeks on the respective diets, MC38 cells were injected i.p. (day 0). On day 5, mice were injected i.p. twice daily with saline or 40 mg/kg phenformin for 10 days (n = 8 for each diet/drug combination). Animals were sacrificed on day 15. Results are representative of two independent experiments. Data, mean values of tumor volume  $\pm$  SEM (\*, P < 0.01). B, plasma serine and glycine concentrations in mice described in A were glycine levels in tumors of mice described in A. Each data point represents the average metabolite abundance determined by three independent extractions and GC-MS runs per tumor. Data, mean  $\pm$  SEM (6-7 animals/group); \*, P < 0.05.

mice (25). We sought to extend our in vitro findings by creating a similar diet-induced serine and glycine deficiency in C57BL/6 mice bearing MC38 allografts and treating them with the more bioavailable biguanide phenformin (9). MC38 tumors grew rapidly regardless of the presence or absence of serine and glycine in the diet. Phenformin, dosed at 40 mg/ kg by i.p. injection twice daily did not impair tumor growth in mice fed the control diet. However, we observed a significant reduction in the growth rate and in the final size of the tumors in mice on the diet deficient in serine and glycine combined with phenformin treatment (Fig. 3A). All groups tolerated the combinations of diets and treatments administered, and constant weight gain was seen in all groups. To better understand the link between dietary amino acids and tumor growth, plasma and tumor serine and glycine levels were determined by GC-MS. Plasma of mice on the serine/glycine-deficient diet showed reduced concentration of these amino acids (Fig. 3B). Tumor serine and glycine levels were also reduced in the serine/glycinedeprived groups at the conclusion of the experiment (Fig. 3C). This observation provides pioneering evidence that dietary amino acid intake manipulation can potentiate the antineoplastic activity of biguanides in vivo.

# Metformin does not inhibit serine biosynthesis

Given that high expression of the serine biosynthesis enzyme PHGDH limits the impact of serine deprivation on cell proliferation (Fig. 2B), we sought to determine whether the enhanced effects of metformin or phenformin under serine deprivation were due to inhibition of serine biosynthesis. To evaluate the flux of glucose into serine under metformin and/or serine deprivation, we performed stable isotope tracer analysis using  ${}^{13}C_6$ -glucose (Fig. 2C).  ${}^{13}C_6$ -glucose is converted to the glycolytic intermediate 3-phosphoglycerate (3-PG)m+3, which is further transformed into serine m+3 through the sequential reactions catalyzed by PHGDH, phosphoserine aminotransferase and 3-phosphoserine phosphatase. Serine is reversibly converted into glycine m+2, Thus, total serine isotopomers derived from  ${}^{13}C_6$ -glucose will contain m+1, m+2, and m+3 enrichments. Metformin did not inhibit  $^{13}C_6$ -glucose contribution to the serine pool, whereas serine deprivation drastically reduced it (Fig. 2D). Metformin increased unlabeled serine (m+0) relative to control, and this effect was ablated by concomitant serine withdrawal, suggesting that metformin increases the uptake of exogenous serine. Metformin did not affect expression of PHGDH, which indicates that the effects of metformin on intracellular serine levels are not mediated by modulating expression of this enzyme (Fig. 2E). Collectively, these data show that the decrease in proliferation upon metformin exposure in serine-deprived MC38 cells cannot be explained by a decrease in de novo serine biosynthesis.

# Serine deficiency enhances the activity of biguanides in AMPK-independent manner

AMPK activation due to energetic stress leads to downregulation of proliferation and other energy-consuming processes, thereby favoring cellular survival (35). Antiproliferative effects of biguanides are in part mediated by AMPK-dependent inactivation of mTORC1 (10, 36), whereas changes in



Figure 4. Under serine deprivation, metformin decreases S6K and rpS6 phosphorylation. H1299 (A and C) or MC38 (B) cells were treated as indicated for 24 hours (A and B) or for the shown time periods (C). Immunoblots were performed using indicated antibodies.  $\beta$ -Actin served as a loading control. Ser<sup>-</sup>, serine-free media; Ser<sup>+</sup>, media with 30 mg/L serine. Blots, representative of three independent experiments.

intracellular serine levels have been shown to reduce the activity of mTORC1 in cell lines (27). Therefore, we explored the possibility that the enhancement of metformin-induced inhibition of proliferation by serine deprivation is mediated by the AMPK and/or mTORC1 pathways. Serine withdrawal only marginally reduced the phosphorylation of mTOR effectors S6 kinase 1 (S6K1) and its downstream substrate ribosomal protein S6 (rpS6) in H1299 and MC38 as compared with control, whereas 2.5 mmol/L metformin had no effect (Fig. 4A and B). In stark contrast, treatment with 2.5 mmol/L metformin combined with serine deprivation resulted in further suppression of mTORC1 as judged by a strong reduction in S6K1 and rpS6 phosphorylation (Fig. 4A-C). The data show that the potentiation of the antiproliferative effects of metformin by serine withdrawal is paralleled by decreased mTORC1 signaling.

We next determined whether the effects of combination of serine withdrawal and metformin on mTORC1 are mediated by AMPK. To this end, we activated AMPK pharmacologically using AICAR, while varying serine availability in MC38 cells. Although 25 µmol/L AICAR had no effect on AMPK activation or ACC phosphorylation, both AMPK and ACC phosphorylation were stimulated with 50 µmol/L AICAR to levels comparable with those observed in 2.5 mmol/L metformin-treated cells, and this was paralleled by modest inhibition of mTORC1 as judged by slightly decreased phosphorylation of rpS6 (Fig. 5A, compare lanes 1 vs. 3 and 1 vs. 7). Moreover, the antiproliferative effects of 50 µmol/L AICAR and 2.5 mmol/L metformin were comparable. Surprisingly, although serine deprivation increased the inhibitory effects of both 50 µmol/L AICAR and 2.5 mmol/L metformin on mTORC1 signaling (Fig. 5A; compare lanes 3 and 4 and 7 and 8), serine withdrawal potentiated the antiproliferative effects of metformin but not AICAR (Fig. 5B). These results suggest that the potentiation of the effects of metformin by serine withdrawal is not mediated by AMPK or mTORC1.

To further corroborate these findings, we measured the effects of metformin and serine withdrawal on the proliferation of H1299 cells depleted of AMPK $\alpha$ 1/2 by shRNA (Fig. 5C and D). These experiments revealed that similarly to AICAR, AMPK $\alpha$ 1/2 subunit depletion does not affect the inhibition of proliferation by metformin in cells depleted of serine. Taken together, these findings show that serine withdrawal bolsters the antiproliferative effects of biguanides independently of AMPK. This suggests that serine withdrawal and metformin exert their combinatory antiproliferative effects by directly perturbing the metabolism of cancer cells.

# Serine deprivation reduces the upregulation of glycolysis and shifts in the relative abundance of citric acid cycle metabolites induced by metformin

In the setting of energy stress due to inhibition of oxidative phosphorylation by biguanides, changes in metabolic fluxes occur. Primarily, oxygen consumption and glucose oxidation are decreased, whereas glycolytic flux to lactate is upregulated (18, 20). Serine acts as a critical modulator of glycolysis (26). We therefore sought to explore the effects of serine deprivation and metformin treatment on glucose metabolism. We treated MC38 and H1299 cells with metformin for 24 hours and measured glucose consumption and lactate secretion. As expected, we observed substantial increases in glucose consumption and lactate secretion, indicative of upregulated glycolysis (Fig. 6A and B). Compared with cells in the serine-replete control condition, cells under serine deprivation exhibited a reduced rate of glycolysis (Fig. 6A and B). Furthermore, serine deprivation effectively inhibited the metformininduced increase in glucose consumption, lactate secretion, and the lactate:pyruvate ratio (Fig. 6A, B, and E). These results



**Figure 5.** AMPK is dispensable for the effect of metformin under serine deprivation. A, MC38 cells were treated as indicated for 24 hours and the levels of indicated proteins were determined by immunobloting.  $\beta$ -Actin served as a loading control. B, MC38 cells were treated as indicated for 72 hours and viable cells were counted. C, loss of AMPK did not reduce the effect of metformin exposure with serine deprivation on viable cell count. AMPK $\alpha$ 1/2-depleted (shRNA) or control H1299 cells were treated with metformin in the absence of serine for 72 hours and viable cells were counted. D, immunoblot analysis of H1299 AMPK $\alpha$ 1/2- and control shRNA–expressing cells treated for 24 hours as indicated.  $\beta$ -Actin served as a loading control.

confirm that cells respond to metformin-induced energy stress by upregulating glycolysis, and demonstrate that serine deprivation is a potent inhibitor of this compensatory response. To further explore whether serine depletion potentiates the antiproliferative effects of biguanides by impeding the compensatory switch to glycolysis, we supplemented MC38 and H1299 cells with 1 mmol/L pyruvate. Given that in the presence of metformin, pyruvate entry into the citric acid cycle will be limited (15, 31), pyruvate supplementation will lead to the conversion of pyruvate into lactate by lactate dehydrogenase and the generation of NAD<sup>+</sup> that is required for the activity of the glycolytic pathway. Accordingly, pyruvate alleviated the inhibitory effects of the combination of metformin and serine withdrawal on glycolysis (Fig. 6B), which was paralleled by rescue of proliferation (Fig. 6C). Hence, the combined inhibitory effect of metformin and serine withdrawal on cell proliferation can be explained at least in part by abrogation of metformin-induced compensatory switch to glycolysis in cells deprived of serine.

In addition to upregulating glycolysis, metformin alters the citric acid cycle due to accumulation of NADH as a consequence of complex I inhibition, promoting the usage of



Figure 6. Metabolic alterations underlie enhanced antineoplastic action of metformin under serine deprivation. A, H1299 cells were treated for 24 hours as follows: Ser<sup>+</sup>, control; Ser<sup>-</sup>, serine-free; Met, metformin (2.5 mmol/L); Ser<sup>-</sup> Met, serine-free with metformin (2.5 mmol/L). Glucose consumption (white bars) and lactate production (black bars) were measured. Data, mean  $\pm$  SEM (n = 6); \*, P < 0.001. B, MC38 cells were treated as in A with or without pyruvate (1 mmol/L). Glucose consumption (white bars) and lactate production (white bars) and lactate production (black bars) were measured. Data, mean  $\pm$  SEM (n = 6); \*, P < 0.001. C, MC38 cells were treated for 72 hours with metformin (2.5 mmol/L) in the presence or absence of serine, in the presence or absence of 1 mmol/L pyruvate. (*Continued on the following page*.)

glutamine to support lipogenesis (37-40). Metformin was found to increase the  $\alpha$ -ketoglutarate/citrate ratio by reducing glycolytic input into the mitochondria (41). Therefore, we assessed lactate, citrate and  $\alpha$ -ketoglutarate levels in MC38 cells under serine deprivation and/or metformin treatment. Strikingly, metformin and serine deprivation had opposite effects on the concentration of these metabolites in cells, whereby serine withdrawal antagonized both the increase in lactate and  $\alpha$ -ketoglutarate, and the decrease in citrate induced by metformin (Fig. 6D). Importantly, pyruvate rescued the levels of all three metabolites (Fig. 6D). As expected, metformin-treated MC38 cells showed an increase in the  $\alpha$ -ketoglutarate/citrate ratio as compared with control (Fig. 6F). This effect was abolished by serine deprivation, and pyruvate partially restored the  $\alpha$ -ketoglutarate/citrate ratio in metformin and serum depleted cells.

Considering these findings, we further studied the effects of metformin and serum withdrawal on citric acid cycle activity by monitoring citrate isotopomers with stable isotope tracer analysis using <sup>13</sup>C<sub>6</sub>-glucose or <sup>13</sup>C<sub>5</sub>-glutamine. Serine deprivation increased m+2 citrate derived from <sup>13</sup>C-glucose (Fig. 6G) and m+4 citrate derived from <sup>13</sup>C<sub>5</sub>glutamine (Fig. 6H), thereby supporting the observation that serine deprivation increases citric acid cycle activity (25). In contrast, metformin dramatically reduced the flux of  ${}^{13}C_6$ -glucose into m+2 citrate (Fig. 6G), whereas the flux of  $^{13}C_5$ -glutamine into m+5 citrate was maintained (Fig. 6H). Importantly, serine deprivation alone led to an accumulation of <sup>13</sup>C-glutamine-derived m+5 citrate, which was further increased when serine deprivation was combined with metformin treatment (Fig. 6H). This finding, together with the serine withdrawal-induced decrease in the  $\alpha$ -ketoglutarate/citrate ratio (Fig. 6F), suggests that serine deprivation alters the metabolism of glutamine-derived citrate. Taken together, these findings suggest that serine withdrawal potentiates the antineoplastic effects of metformin at least in part by antagonizing compensatory metabolic pathways that are activated on exposure to biguanides.

#### Discussion

The hypothesis that the antidiabetic biguanide metformin may be "repurposed" for indications in oncology is receiving considerable attention, with more than 100 clinical trials involving metformin treatment for cancer presently underway (6). However, there are important gaps in knowledge with respect to the mechanisms of action. Although metformin influences levels of circulating hormones in a manner that may reduce proliferation of certain cancers, these changes are modest in magnitude (5), and it is not established clinically that they are sufficient to have a therapeutic effect. The possibility that biguanides act directly on cancer cells *in vivo* is supported by many preclinical models (5–7, 13, 15, 17). There is evidence that the direct antiproliferative actions of biguanides on cancer cells are a consequence of metabolic stress caused by inhibition of oxidative phosphorylation (1, 2).

Prior work suggests that both host and tumor-related factors influence the antineoplastic activity of biguanides. For example, cancers with baseline impairment of oxidative phosphorylation due to mutations in genes encoding proteins in respiratory complex I are particularly sensitive to biguanides, whereas high glucose levels attenuate the antiproliferative effects of biguanides by facilitating high rates of glycolysis, which can relieve the energy stress caused by biguanide-induced reduction in oxidative phosphorylation (17, 18). We observed that sensitivity to biguanides in vitro is significantly increased under conditions of serine deprivation, and extended this observation to an in vivo model, in which a level of biguanide exposure that is well tolerated but insufficient to achieve antineoplastic activity under control conditions inhibited tumor growth when mice were fed a diet deficient in glycine and serine. This diet was well tolerated and was not by itself associated with significant in vivo tumor growth inhibition in the aggressive MC38 cancer cell model. In contrast, a recent report showed that dietary restriction of serine and glycine is sufficient to have an antiproliferative effect on the slower growing HCT116 cancer cell model (25).

Serine is directly involved in folate and methionine cycles, and is thus important for nucleotide biosynthesis, NADPH production, and reactive oxygen species clearance (42). We initially suspected a novel action of metformin as an inhibitor of serine biosynthesis to account for its enhanced activity when serine is removed from culture medium, but this hypothesis was not supported by our metabolic studies. Rather, our results show that cell survival in the presence of biguanide-induced inhibition of oxidative phosphorylation is associated with increased glycolysis, and that under serine deprivation this compensatory increase in glycolysis does not take place (Fig. 7). This demonstrates a new context in which the positive impact of serine on glycolysis (25–27, 33) is important.

<sup>(</sup>*Continued.*) Proliferation was determined by viable cell count and data are expressed as a percentage of growth inhibition as compared with control. Data, mean  $\pm$  SEM (\*, P < 0.01). D, intracellular lactate, citrate, and  $\alpha$ -ketoglutarate levels in MC38 cells treated for 24 hours as indicated were determined by GC–MS. Pyruvate addition (1 mmol/L) was simultaneous with other treatments. Data, means  $\pm$  SEM (n = 3 biological replicates). E, the intracellular lactate to pyruvate ratio in MC38 was determined by GC–MS. Data, mean  $\pm$  SEM (n = 3 independent experiments); \*, P < 0.05. F, the  $\alpha$ -ketoglutarate ( $\alpha$ -KG) to citrate ratio, indicator of glutamine-dependent reversal of citric acid cycle, in MC38 cells was determined by GC–MS. Pyruvate addition (1 mmol/L) was simultaneous to other indicated treatments. Data, mean  $\pm$  SEM (n = 3 biological replicates); \*, P < 0.05. F, the  $\alpha$ -ketoglutarate ( $\alpha$ -KG) to citrate ratio, indicator of glutamine-dependent reversal of citric acid cycle, in MC38 cells was determined by GC–MS. Pyruvate addition (1 mmol/L) was simultaneous to other indicated treatments. Data, mean  $\pm$  SEM (n = 3 biological replicates); \*, P < 0.05. G, stable isotope tracer analyses of citrate in MC38 cells treated as indicated and incubated with <sup>13</sup>C<sub>6</sub>-glucose for 24 hours. Each bar integrates principal isotopomer ion amounts and shows the relative metabolite present per cell. Data, mean  $\pm$  SEM (n = 3 biological replicates); \*, P < 0.05. H, stable isotope tracer analyses of citrate in MC38 cells treated as indicated with <sup>13</sup>C<sub>5</sub>-glutamine. Each bar integrates principal isotopomer ion amounts and shows the relative metabolite present per cell. m+5 reflects reverse citric acid cycle cycling through reductive carboxylation, whereas m+4 reflects forward citric acid cycle cycling. Data, mean  $\pm$  SEM (n = 3 biological replicates); \*, P < 0.05.



Figure 7. Schematic of the proposed mechanism by which serine deprivation interferes with biguanide-induced metabolic remodeling. Left, biguanide-induced reduction in oxidative phosphorylation leads to (i) a compensatory increase in glycolysis with augmentation of glucose uptake and lactate production and (ii) reductive carboxylation in the citric acid cycle (CAC). These effects on the citric acid cycle are associated with an increase in the  $\alpha$ -ketoglutarate ( $\alpha$ -KG) to citrate ratio. Right, serine withdrawal counteracts these compensatory metabolic responses to biguanides associated with enhanced antineoplastic activity.

Inhibition of cancer growth by manipulation of dietary amino acids has previously been proposed (43, 44), but leads to adverse effects of deficiency in essential amino acids. This contrasts with our approach, in which a well-tolerated dietary restriction of two nonessential amino acids sensitizes cancer cells to biguanide treatment. Substantial interindividual differences in circulating levels of glycine and serine exist between normal individuals (45), even in the absence of dietary interventions, and may influence the efficacy of biguanides as antineoplastic agents. Our findings indicate that characterizing and targeting compensatory metabolic pathways activated in response to biguanideinduced energy stress can identify strategies to improve the efficacy of these compounds as antineoplastic agents.

### **Authors' Contributions**

Conception and design: S.-P. Gravel, L. Hulea, N. Toban, M.-J. Blouin, M. Zakikhani, I. Topisrovic, J. St-Pierre, M. Pollak

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.-P. Gravel, L. Hulea, N. Toban, M. Zakikhani, I. Topisirovic, J. St-Pierre, M. Pollak Writing, review, and/or revision of the manuscript: S.-P. Gravel, L. Hulea, N. Toban, M.-J. Blouin, M. Zakikhani, I. Topisirovic, J. St-Pierre, M. Pollak Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): N. Toban, M.-J. Blouin, M. Pollak Study supervision: M. Zakikhani, I. Topisirovic, J. St-Pierre, M. Pollak

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Development of methodology: S.-P. Gravel, L. Hulea, N. Toban, E. Birman, M.-J. Blouin, M. Zakikhani, Y. Zhao, J. St-Pierre

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.-P. Gravel, L. Hulea, N. Toban, M.-J. Blouin

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