Serine deprivation potentiates anti-neoplastic effects of biguanides: Inducing and targeting a novel weakness in the regulation of cancer cell metabolism

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Abstract

Altered metabolism has recently re-emerged as a hallmark of cancer. New evidence from preclinical models and from epidemiological studies suggests that targeting cancer metabolism is possible and practical. A promising aspect is the near universality of certain defining characteristics of this altered metabolism. Notably cancer cells sustain elevated rates of aerobic glycolysis and express pyruvate kinase M2 (PKM2) to optimise and increase the biosynthesis of precursors to macromolecules, thereby allowing survival and proliferation in the setting of variable nutrient supply. In a recent report, deficiency in the amino acid serine caused PKM2 inhibition, exerted negative influence on aerobic glycolysis and altered the utilization of glucose carbons to favor *de novo* serine biosynthesis. Metformin, a biguanide antidiabetic drug has displayed anti-neoplastic activity in preclinical and epidemiological studies. It functions primarily by inhibiting oxidative phosphorylation, the main cellular metabolic pathway for generating ATP. Prior work has revealed that cancer cells exposed to biguanides must effectively compensate for the resulting bioenergetic deficiency by increasing the rate of glycolysis and by AMPK-mediated downregulation of anabolic processes. Here, we show that biguanides have remarkably enhanced anti-neoplastic activity in the absence of serine. We provide evidence that cancer cells exposed to biguanides respond by upregulating glycolysis, which is prevented by the opposing influence of serine deprivation. By rendering compensation to energy stress by metabolic means impossible, this combination suppresses mTORC1 activity and forces cancer cells into a state of proliferation arrest. Extending these findings to an *in vivo* model, we show that dietary restriction of serine potentiates the antiproliferative effect of phenformin on aggressive MC38 tumors without causing greater host toxicity.

Résumé

L'altération du métabolisme a récemment réapparu comme une des capacités distinctives des cancers. De nouvelles preuves provenant de modèles précliniques et d'études épidémiologiques suggèrent que le ciblage du métabolisme des cancers est possible et pratique. Un aspect prometteur est la quasi-universalité de certaines caractéristiques déterminantes de l'altération du métabolisme. Notamment les cellules cancéreuses maintiennent des taux élevés de glycolyse aérobie et expriment le pyruvate kinase M2 (PKM2), afin d'optimiser et d'accroître la biosynthèse des précurseurs des macromolécules, ce qui permet la survie et la prolifération en milieu physiologique où l'apport en nutriments peut varier. Dans un rapport récent, la carence en sérine, l'acide aminé, a provoqué une inhibition du PKM2, a exercé une influence négative sur la glycolyse aérobie et modifié l'utilisation des molécules de glucose afin de favoriser la biosynthèse *de novo* de sérine. La metformine, un antidiabétique biguanide a démontré une activité antinéoplasique dans plusieurs études précliniques et épidémiologiques. Il fonctionne principalement par l'inhibition de la phosphorylation oxydative, la principale voie métabolique cellulaire pour la production d'ATP. Des travaux antérieurs ont montré qu'en présence de biguanides, les cellules cancéreuses doivent corriger efficacement la déficience bioénergétique en augmentant le taux de glycolyse et en freinant les processus anaboliques par activation d'AMPK. Ici, nous montrons qu'avec la carence en serine, l'activité antinéoplasique des biguanides est fortement amélioré. Nous fournissons la preuve que les biguanides créent chez les cellules cancéreuses une exigence pour un taux élevé de glycolyse, qui est empêché par l'influence adverse de la carence en sérine. En rendant impossible la compensation par les moyens métaboliques, cette combinaison supprime l'activité du complexe mTORC1 et freine la prolifération des cellules cancéreuses. Nous avons traduit ces résultats à un

modèle *in vivo*, où nous démontrons que la restriction alimentaire de la sérine potentialise l'effet antiprolifératif de la phenformine sur les tumeurs de cellules MC38 agressifs sans provoquer une toxicité accrue chez les animaux.

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ABBREVIATIONS

ADP, Adenosine diphosphate

AMP, Adenosine monophosphate

AMPK, AMP-activated protein kinase

ATP, Adenosine triphosphate

CAC, Citric acid cycle

FAD, Flavin adenine dinucleotide (fully oxidized or quinone form)

FADH2, Flavin adenine dinucleotide (fully reduced or hydroquinone form)

GSH, Glutathione (reduced)

IP, Intra-peritoneal

LKB1, Liver kinase B1

mTORC1, Mammalian target of rapamycin complex 1

NAD+, Nicotinamide adenine dinucleotide (oxidized)

NADH, Nicotinamide adenine dinucleotide (reduced)

NADP, Nicotinamide adenine dinucleotide phosphate (oxidized)

NADPH, Nicotinamide adenine dinucleotide phosphate (reduced)

NMR, Nuclear magnetic resonance

OXPHOS, Oxidative phosphorylation

PEP, Phosphoenolpyruvate

PET, Positron emission tomography

PHGDH, Phosphoglycerate dehydrogenase

PKL, Pyruvate kinase liver isoform

PKM1, Pyruvate kinase M1 isoform

PKM2, Pyruvate kinase M2 isoform

PKR, Pyruvate kinase erythrocyte isoform

PO, per os (Oral administration)

PPP, Pentose-phosphate pathway

SHMT, Serine hydroxymethyltransferase

SSP, Serine synthesis pathway

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CHAPTER 1

LITERATURE REVIEW

Introduction

Cancer cells are metabolically adapted to meet the bioenergetic and biosynthetic demands of proliferation. They exhibit elevated glucose uptake and shift the majority of glucose-derived carbons to lactate production rather than oxidation in the citric acid cycle. Although this metabolic phenotype is inefficient at producing ATP, it allows a greater availability of glycolysis intermediates which can be funnelled into biosynthesis pathways to support macromolecule production (Lunt & Vander Heiden 2011).

Key to the metabolic adaptation of tumour cells is the expression of the embryonic M2 isoform of pyruvate kinase (PKM2) (Christofk *et al* 2008a). This enzyme catalyses the last step in glycolysis, the conversion of phosphoenolpyruvate to pyruvate. In contrast with the constitutively active M1 isoform expressed in non-dividing tissues, the activity of PKM2 is tightly regulated. It can be inhibited in various ways in response to growth factor signalling and metabolic requirements (Christofk *et al* 2008b; Hitosugi *et al* 2009; Chaneton *et al* 2012). Low PKM2 activity causes a build-up of glycolysis intermediates which was shown to favour glucose carbon flux to precursors of macromolecular biosynthesis required for proliferation. As such, pharmacological activators of PKM2 have been synthesized and were found to interfere with tumor metabolism and impair tumor growth (Anastasiou *et al.* 2012).

Interestingly, pharmacological PKM2 activation was also found to cause a dependence on exogenous serine in vitro, since serine biosynthesis proceeds through a three-step pathway that utilizes 3-phosphoglycerate, a glycolysis intermediate (Kung *et al* 2012). The first enzyme in this

pathway, phosphoglycerate dehydrogenase has been found to be commonly overexpressed in melanoma (Mullarky *et al.* 2011) and 70% of estrogen receptor-negative breast cancers (Locasale *et al.* 2011; Possemato *et al*. 2011) which suggests that strategies targeting serine metabolism may be effective in these and other cancer types. Furthermore, serine acts as an allosteric activator of PKM2, reducing flux through the serine synthesis pathway in a feedback mechanism, and conversely, serine deprivation inhibits PKM2 (Chaneton *et al.* 2012) and limits glycolytic pathway flux (Ye *et al.* 2012).

Metformin is an antidiabetic drug which functions by inhibiting mitochondrial oxidative phosphorylation, thereby reducing hepatic gluconeogenesis and glucose output and attenuating hyperglycemia and hyperinsulinemia (Shaw *et al.* 2005). Various epidemiological studies have suggested a link between metformin use and decreased cancer-related mortality in diabetics (Pollak 2012). Further studies in *in vitro* models of cancer revealed that metformin may also act directly on cancer cells by inhibiting mitochondrial ATP generation, triggering AMPK activation-mediated antiproliferative effects (Zakikhani *et al.* 2006; Algire *et al* 2011) and increasing cellular utilization of glucose by aerobic glycolysis (Buzzai *et al.* 2007; Algire *et al.* 2011).

Various small molecule inhibitors targeting cancer cell metabolism have been proposed and are in trials. However, extensive studies have revealed the existence of multiple metabolic programmes that may allow cancer cells to evade single-target approaches (Vander Heiden 2011). We therefore elaborated two strategic combinations that exploit metabolic adaptations specific to cancer cells with the goal of inhibiting multiple essential pathways required for proliferation. Firstly, we developed a synthetically lethal approach by exposing cancer cells to metformin in the

absence of exogenous serine. We determined that, in cancer cells, this combination simultaneously increases the requirement for glycolysis while preventing its upregulation, then we replicated our findings in a murine allograft model. Secondly, we used serine deprivation *in vitro* and *in vivo* to potentiate the antiproliferative effects of a pharmacological activator of PKM2.

Overview of Cancer Metabolism

Warburg hypothesis

The study of cancer cell metabolism began with biochemist Otto Warburg's observations in 1924 that cancer cells display an atypical metabolic phenotype (Warburg 1956). Normal tissues uptake glucose and transform it through glycolysis to pyruvate, the majority of which then proceed to be fully oxidized through the citric acid cycle (CAC) and mitochondrial electron transport chain, generating carbon dioxide and up to 36 molecules of adenosine triphosphate (ATP) per glucose molecule in what is globally termed oxidative phosphorylation (OXPHOS). As this process requires oxygen, the final electron accepter in the mitochondrial electron transport chain, pyruvate does not proceed to the CAC in hypoxic conditions and instead, it is transformed to lactate (Berg *et al.* 2006). Warburg discovered that in contrast to normal tissues, tumors preferentially ferment glucose to lactate even when oxygen was available to support oxidative phosphorylation. He also observed markedly increased rates of glucose consumption by cancer cells relative to normal cells. This metabolic phenotype, termed aerobic glycolysis, was also observed in normal cells undergoing rapid proliferation. Therefore, Warburg postulated that his findings suggested a

primary causative role for mitochondrial dysfunction in oncogenesis (Warburg 1956). Although this postulation was refuted in later studies (Fantin *et al* 2006), his main observation of high rates of aerobic glycolysis in cancer cells underlies the use of the 2-deoxy-2-(^{18}F) fluoro-D-glucose positron emission tomography (PET) scan used for tumor diagnosis and staging (Upadhyay *et al* 2013) and has formed the basis of the modern study of cancer metabolism.

Aerobic glycolysis

Cancer cells will predominantly transform glucose by aerobic glycolysis rather than by oxidative phosphorylation. By favoring this metabolic pathway, cancer cells appear to generate energy inefficiently. In contrast with the complete oxidization of a glucose molecule to CO_2, in which 36 ATP molecules are produced, aerobic glycolysis has a net yield of 2 ATP (Berg *et al.* 2006). As this energetically disadvantageous process is a defining characteristic of altered metabolism in cancer, understanding why it arises and how it provides a selective advantage is essential for the elaboration of therapeutic strategies.

Aerobic glycolysis has been shown not to stem from selective pressure for oxygen-independent catabolism and growth in the context of progressively increasing hypoxia in the early microenvironment of solid tumors. Rather, it arises in a context of oxygen availability and confers a selective advantage without necessitating a hypoxic stimulus (Vander Heiden *et al.* 2009). This is exemplified by tumors arising in the lungs which, not only display elevated uptake of glucose characteristic of the Warburg effect, but in which doubling time was shown to correlate with the

degree of glucose uptake, despite the high oxygen saturation of their blood supply (Duhaylongsod *et al.* 1995). Conversely, leukemic cells, arsing in the bone marrow where they may initially be exposed to hypoxia, do not switch to oxidative phosphorylation when circulating in the blood; display elevated aerobic glycolysis (Tili *et al*. 2012) and are sensitive to glycolysis inhibitors (Akers *et al.* 2011).

Since the predominance of aerobic glycolysis in cancer cells is not thought to be a consequence of growth in the hypoxic tumor microenvironment, it is therefore believed to confer a selective advantage in supporting the requirements of rapid proliferation (Ward & Thompson 2012). Initially, aerobic glycolysis was thought to be favoured for its ability to rapidly produce ATP, with elevated uptake of glucose compensating for the reduced net bioenergetic yield relative to oxidative phosphorylation (Lunt & Vander Heiden 2011). Although, ATP is produced more rapidly through glycolysis, it was ascertained by reviewing the metabolic characteristics of several cancer models that, paradoxically, oxidative phosphorylation was predominantly responsible for producing ATP in cancer cells (Zu & Guppy 2004). Through such findings, it became evident that aerobic glycolysis does not confer energetic advantages and that mitochondrial oxidative phosphorylation, despite receiving a lesser fraction of glucose carbon flux, is not defective and still provides the bulk of cellular bioenergetic resources in cancer cells.

Cancer cells fulfill their need for ATP with a small fraction of consumed glucose. To explain the need for elevated glucose uptake seen in the Warburg effect, the complex metabolic networks stemming from and converging upon central glucose metabolism must be considered. Glycolysis intermediates are precursors for several important *de novo* biosynthetic pathways

required for the production of various amino acids, nucleic acids, lipids and for metabolites involved in one-carbon metabolism (Lunt & Vander Heiden 2011, Vander Heiden *et al*. 2009). Therefore, it was proposed that maintaining high flux through central glucose metabolism would permit adequate shunting of carbons derived from glucose to vital biosynthetic pathways in order to generate biomass for growth and the doubling of cellular components ahead of cell division (Vander Heiden 2011, Cairns *et al.* 2011, Anastasiou *et al.* 2012). Furthermore, high glucose flux would allow the small proportion of total pyruvate that is used in mitochondrial oxidative phosphorylation to be sufficient to meet the ATP requirements of the cell, without greatly impacting the availability of carbons for biosynthesis (Lunt & Vander Heiden 2011). In light of its role in favoring *de novo* biosynthesis, the observations that aerobic glycolysis results in the production of lactate from the majority (up to 90% in some models, DeBerardinis *et al.* 2007) of glucose carbons seems paradoxical. However, it has been conclusively shown that cancer cells constantly maintain large pools of glycolytic intermediates (Anastasiou *et al*. 2012) that serve as precursors for biosynthesis due in part to a complex system of regulatory adaptations which allow the replenishment of these pools when demand is increased or nutrient supply is limited (Cairns *et al.* 2011, Mazurek 2011). As elevated glucose uptake is promoted by several commonly disregulated oncogenic signalling pathways, the resulting constitutive glycolytic flux in the absence of a severe deficit in biosynthetic precursors may require the production of lactate as an outlet for unused carbon (Ward & Thompson 2012, Mazurek 2011).

To summarize, the aerobic glycolysis phenotype seen in cancer cells allows the uptake and transformation of sufficient amounts of glucose in order to maintain a constant supply of biosynthetic precursors without compromising ATP production. Key to this balance are several

adaptations which form an effective system for budgeting nutrient resources to meet the requirements for proliferation (Mazurek 2011, Vander Heiden 2011). This review examines the biosynthesis pathways stemming from glycolysis below, and further, the regulation of the fate of glucose carbons is addressed.

Biosynthesis pathways diverging from glycolysis

The first step in glycolysis, the ATP-dependent phosphorylation of glucose generates glucose 6-phosphate, a substrate for the oxidative branch of the pentose-phosphate pathway (PPP) (Berg *et al.* 2006). Flux through the oxidative PPP produces NADPH, a reducing factor required for lipid and nucleotide biosynthesis and for maintaining cellular pools of reduced glutathione (GSH) for ROS detoxification (Tong *et al.* 2009; Anastasiou *et al.* 2011). Consequently, oxidative stress was not tolerated in cancer cells in which the ability to increase flux through the PPP was impaired or pharmacologically prevented (Anastasiou *et al.* 2011). Downstream glycolysis intermediates fructose 6-phosphate and glyceraldehyde 3-phosphate contribute to flux through the non-oxidative branch of the pentose phosphate pathway resulting in the production of ribose 5-phosphate, a key precursor for *de novo* nucleotide biosynthesis to support RNA transcription and DNA replication required for proliferation (Tong *et al.* 2009).

Further downstream, the glycolysis intermediate 3-phosphoglycerate is required for the *de novo* serine synthesis pathway (SSP). The importance of this pathway for cancer cells was highlighted in studies revealing amplifications of genomic region 1p12 which contains the gene

coding for phosphoglycerate dehydrogenase (PHGDH), the rate limiting enzyme of the SSP. This was shown to occur in 16% of cancers and was especially common in melanoma (Locasale *et al*. 2010). Another study found that these amplifications occurred in approximately 70% of estrogen receptor-negative breast cancers and were associated with poor prognosis (Possemato *et al.* 2011). Both studies found correlations between these amplifications and significant PHGDH overexpression as well as flux through the serine synthesis pathway. Furthermore, they also revealed that PHGDH suppression decreased proliferation in cells harbouring PHGDH amplification, which were highly and differentially sensitive to this approach relative to cells lacking amplification (Locasale *et al.* 2010, Possemato *et al.* 2011).

There are several proliferation advantages to high flux through the serine synthesis pathway. The production of high quantities of serine may offset a reduction in its exogenous availability (Possemato *et al*. 2011) and may help support the requirements of protein biosynthesis. Serine may also be converted to glycine in a reaction catalysed by serine hydroxymethyltransferase (SHMT) in which a methyl group is transferred to the folate cycle, an important driver of several biosynthesis reactions (Locasale 2013). Glycine is a contributor to protein biosynthesis and, more importantly, to purine biosynthesis. Metabolic profiling of the NCI-60 panel of cancer cell lines revealed that expression of glycine biosynthesis enzymes correlated with proliferation rates and that highly proliferating cells were sensitive to antagonism of glycine biosynthesis. This also revealed that proliferating cells utilized glycine predominantly for *de novo* purine biosynthesis (Jain *et al.* 2012).

Glucose carbons also contribute to phospholipid synthesis through the conversion of dihydroxyacetone phosphate to glycerol 3-phosphate. Lipid synthesis is also partially dependent on glucose carbons in the form of acetyl-CoA resulting from pyruvate decarboxylation in the mitochondria and is supported by NADPH generated in the oxidative branch of the pentose-phosphate pathway (Lunt & Vander Heiden 2011).

Regulation of carbon flux by Pyruvate Kinase M2

Pyruvate kinase catalyses the last step in glycolysis, the conversion of phosphoenolpyruvate (PEP) to pyruvate coupled with the generation of one molecule of ATP. Four isoforms of pyruvate kinase exist in human cells and are expressed in different tissues: PKL in the liver, PKR in erythrocytes, PKM1 in non-dividing tissues and PKM2 in all studied cancer cell lines and some proliferating tissues (Mazurek 2011). PKM1 displays constitutively high activity, and favours glucose flux to oxidative phosphorylation. This isoform is thought to contribute to generating adequate ATP in tissues with high energetic demands such as the heart, brain and muscle (Israelsen *et al*. 2013, Mazurek 2011). On the other hand, PKM2 is highly regulated, and is usually found in an inactive homodimeric conformation (Anastasiou *et al*. 2012, Anastasiou *et al.* 2011). The expression of PKM2 has been shown to be a positive regulator of aerobic glycolysis and of glucose flux to biosynthesis pathways (Ye *et al* 2012, Christofk *et al.* 2008a). Biosynthesis is favoured by low PKM2 activity because this allows the pooling of glycolysis intermediates. Consequently, replacing PKM2 with PKM1 in cancer cells caused an increased reliance on oxidative phosphorylation and resulted in an impaired ability to form tumors *in vivo* (Christofk *et al.* 2008a).

The proliferation advantage conferred by PKM2 is due to the multiple regulatory mechanisms governing its activity which allow a coordination of metabolic flux with specific requirements arising from internal and external stimuli. PKM2 is activated when its tetrameric form is stabilised by allosteric interaction with fructose 1,6-bisphosphate, an upstream metabolite of glycolysis (Christofk *et al*. 2008b, Wong *et al*. 2013). Interactions with phospho-tyrosine residues on proteins generated in several signalling pathways cause the release of FBP form PKM2 and stabilise the inactive conformation. This is believed to be an adaptation favouring the coordination of growth signals with metabolic reorientation to support proliferation with adequate shunting to biosynthesis pathways (Christofk *et al.* 2008b). PKM2 is also inhibited by ROS-mediated oxidation of Cys358, and this increases flux through the pentose-phosphate pathway, regenerating reduced glutathione for ROS scavenging. Interfering with this response by mutating Cys358 into the oxidation-resistant Ser358 residue or by forcing PKM2 activation with a pharmacological activator, results in enhanced sensitivity to oxidative stress (Anastasiou et al. 2011). Through an allosteric mechanism, PKM2 activity is lowered in the absence of serine, which allows rapid shunting of glucose carbons to the serine synthesis pathway (Chaneton *et al.* 2012, Ye *et al*. 2012). As a precursor for glycine, nucleotide, phospholipid and glutathione synthesis, as well as a methyl donor in one-carbon metabolism, serine is an important source for the biosynthesis of many macromolecules needed for proliferation (Locosale 2013). Therefore, as low intracellular serine may be an indicator of increased biosynthetic demand in anticipation of proliferation, its allosteric control of PKM2 activity provides further precise synchronisation of glucose utilisation and proliferation (Chaneton *et al.* 2012, Ye *et al*. 2012). In poorly vascularized microenvironments, cancer cells would benefit from PKM2-mediated shunting of glucose carbons to supplement their serine requirements with *de novo* biosynthesis since they would be unable to

obtain sufficient serine by uptake alone but may still be adequately supplied with glucose, given its approximately 20-fold higher concentration in the blood (Chaneton *et al*. 2012). Inversely, when uptake of exogenous serine adequately maintains intracellular stores, allosteric activation of PKM2 would prevent wasteful flux through the serine synthesis pathway and would allow redirection of glucose carbons to different biosynthesis pathways, citric acid cycle anaplerosis and ATP production (Chaneton *et al*. 2012, Mazurek 2011).

Cellular pyruvate kinase activity was also shown to be regulated by high glucose availability through PKM2 acetylation at K305. This inhibits PKM2 by reducing its affinity for its natural activator, fructose 1,6-bisphosphate, and by increasing its affinity for interaction with the chaperone protein HSC70 and subsequent lysosomal degradation (Lv *et al.* 2011). As with allosteric regulation, this was shown to increase the levels of several glycolysis intermediates. Degrading PKM2 in response to glucose sufficiency allows cells to increase the production of biosynthetic intermediates while avoiding consequent feedback allosteric activation of PKM2, thereby committing cells to sustained anabolic metabolism (Lv *et al.* 2011). However, when glucose is limiting, PKM2 acetylation and destruction are reduced, allowing allosteric regulatory mechanisms to redirect glucose carbon flux to meet the requirements for survival (Lv *et al.* 2011, Mazurek *et al.* 2011).

Taken together, the extensive work done to elucidate the importance of PKM2 in cancer metabolism have shown that its expression is driven by the need for metabolic flexibility in cancer cells at all stages of tumor development. Periods of nutrient deprivation will favor increased pyruvate kinase activity and glucose flux to oxidative phosphorylation in order to maintain

adequate ATP for survival. Tumor initiation and metastasis are examples of conditions where slow or non-dividing cancer cells may require high pyruvate kinase activity (Israelsen *et al.* 2013). With angiogenesis and adequate vascularisation, nutrient availability will decrease PKM2 activity and consequently, biosynthesis from glucose metabolites will be favoured (Lv *et al.* 2013, Chaneton *et al.* 2012). Phases of rapid proliferation require extensive coordination of cell division with macromolecule biosynthesis. This is made possible partly through PKM2 inhibition by phosphotyrosine residues downstream of growth signalling (Christofk *et al.* 2008b). Tumors are highly heterogeneous and may contain dividing and non-dividing cell populations. In PKM2 null tumors, re-expression of constitutively active PKM1 was found to occur in non-dividing cells, thereby confirming the importance of the regulability of PKM2 between active and inactive states to support the needs of heterogeneous cell populations existing simultaneously within the same tumor (Israelsen *et al.* 2013). Finally, PKM2 may alter metabolism to allow cancer cells to survive cytotoxic and anti-angiogenic therapies, making it a potential target in neoadjuvant therapy (Galluzzi *et al.* 2013, Israelsen *et al.* 2013).

Citric acid cycle and oxidative phosphorylation in cancer cell metabolism

In non-dividing adult cells, the primary role of the citric acid cycle (CAC) and the mitochondrial electron transport chain is to produce ATP. Glucose carbons enter the cycle as acetyl-CoA, which is condensed with oxaloacetate to produce citrate. Carbon flux through the CAC results in the reduction of NAD+ and FAD to NADH and $FADH_2$ and the production of CO_2. Electrons are transferred through NADH and $FADH_2$ to acceptors in the mitochondrial electron transport chain. There, subsequent reductive steps are coupled to the generation of a proton

gradient across the inner membrane of the mitochondria. The re-entry of protons through the H^+-ATPase complex generates ATP from ADP and inorganic phosphate (Berg *et al.* 2006).

Mitochondrial metabolism is not commonly impaired in cancer cells and is in fact the major source of ATP, as in normal tissues. However, the sources and flux of carbon in the CAC differ in cancer cells, which may depend heavily on glutamine as a carbon source for anaplerosis and may utilize CAC intermediates such as citrate for lipid biosynthesis and reducing equivalents as cofactors for various biosynthetic reactions (Lunt & Vander Heiden 2011, Wise & Thompson 2010).

Citrate is produced in the mitochondria through the condensation of oxaloacetate and acetyl-CoA. Oxaloacetate is made in the CAC and is the acceptor of carbon input into the cycle by acetyl-CoA, which is derived primarily from pyruvate produced in glycolysis. In normal cells, biosynthetic demand is low and the input of acetyl-CoA's two carbons is sufficient to offset the loss of two molecules of CO_2 in the course of one rotation of the CAC (Berg *et al.* 2006). However, in cancer cells with elevated biosynthesis needs, citrate is exported to the cytosol and reconverted to acetyl-CoA, where it is a major carbon source for the synthesis of essential lipid components of cellular membranes such as fatty acids, sphingomyelin and cholesterol (Jones & Thompson 2009). Inhibiting this process has been shown to decrease lipid biosynthesis and cell proliferation (Hatzivassiliou *et al.* 2005). Furthermore, other citric acid cycle intermediates contribute to the biosynthesis of non-essential amino acids (Lunt & Vander Heiden 2011). Extensive efflux or cataplerosis of CAC carbons must be offset with influx or anaplerosis to maintain the continued function of the cycle and to ensure adequate production of reductive cofactors and ATP (Lunt &

Vander Heiden 2011, Jones & Thompson 2009). In cancer cells, glutamine was found to be a major contributor to anaplerosis through conversion to glutamate catalysed by glutaminase, and subsequent transamination to alpha-ketoglutarate, which enters the CAC (Deberardinis *et al.* 2007). Consequently, many cancer cells were found to exhibit a requirement for exogenous glutamine in order to survive and proliferate (Wise & Thompson 2010). Furthermore, elevated expression of the *MYC* oncogene, which is seen in several aggressive forms of cancer, increases mitochondrial oxygen consumption while also inducing the utilization of glutamine for citric acid cycle anaplerosis by increasing glutaminase expression. (Wallace 2012, Wise *et al.* 2008).

Reductive carboxylation and lipid biosynthesis under hypoxia and mitochondrial impairment

De novo lipid biosynthesis from acetyl-CoA is essential for proliferation and survival in cancer cells. This process depends anaplerosis to maintain the function of the citric acid cycle. Under hypoxia or mitochondrial electron transport chain dysfunction, the reductive NADH cofactors generated by the various oxidative CAC reactions cannot be utilized and accumulate, inactivating the NAD+ dependant enzymes that catalyze these reaction (Metallo *et al.* 2011). However, cancer cells possess an alternative pathway, in which reductive metabolism of glutamine-derived alpha-ketoglutarate through highly reversible isocitrate dehydrogenase (IDH) allows the production of citrate for lipid biosynthesis (Mullen *et al*. 2012, Metallo *et al.* 2011). Interestingly, this reverse pathway is active alongside the oxidative CAC pathway in most cancer cell lines tested under normoxic conditions. However, it becomes the exclusive provider of carbons

for lipid synthesis under hypoxia (Mullen *et al*. 2012) or in cells harbouring mutations in genes coding for mitochondrial electron chain complexes (Metallo *et al.* 2011). When relying on this reductive pathway rather than the oxidative pathway, cells display even greater dependence on glutamine for anaplerosis, as glucose-derived carbon entry into the citric acid cycle is greatly decreased. Inhibiting glutaminase in the context of pharmacological inhibition of the mitochondrial electron transport chain was shown to synergistically lower proliferation in prostate cancer cells (Fendt *et al*. 2013).

The LKB1/AMPK/mTORC1 pathway in cancer cells

Although metabolic flux regulation in glycolysis by PKM2 and within the citric acid cycle allow the coordination of metabolic resources to meet biosynthetic and bioenergetic demands under various conditions and stages of nutrient deprivation, cancer cells must also regulate proliferation and other bio-energetically costly processes to ensure survival under energy stress. AMP-activated protein kinase (AMPK) is the main intracellular fuel sensor that regulates energy homeostasis. It is activated by LKB1 when the ratio of AMP to ATP is increased, and inhibits energy-consuming processes such as lipid and protein biosynthesis and cell proliferation (Hardie 2011). The LKB1/AMPK pathway plays an important role cellular stress resistance and in regulating cell proliferation. *LKB1* functions as a tumor-suppressor gene that is mutated in Peutz-Jegher's syndrome, an inherited predisposition for epithelial tumor formation, and is a commonly mutated during tumorigenesis in many subtypes of non-small cell lung cancer (NSCLC) and cervical cancer (Shackelford & Shaw 2009). Recently, the inactivation of AMPK was shown to

promote the metabolic transformation to aerobic glycolysis which is characterized by increased glucose uptake and carbon flux to lactate. This was associated with increased flux of glycolytic intermediates towards lipid biosynthesis, an increase in net biomass and greater tumor progression *in vivo* (Faubert *et al*. 2013).

However, LKB1/AMPK pathway inactivation plays a contradictory role by rendering deficient cancer cells incapable of sensing and responding to energy stress resulting in apoptosis due to a loss of energy homeostasis (Shackelford & Shaw 2009). The therapeutic implication of this was recently demonstrated in a preclinical model of non-small cell lung cancer, in which *LKB1* deficiency conferred *in vivo* hypersensitivity to phenformin, a biguanide inhibitor of mitochondrial ATP production (Shackelford *et al*. 2013).

The mammalian target of rapamycin complex 1 (mTORC1) is inhibited by AMPK in response to energy stress. Under normal conditions, mTORC1 coordinates protein synthesis by activating downstream effectors such as p70 S6 kinase and inhibiting the translational inhibitor 4E-BP1. mTORC1 also serves to coordinate protein translation with amino acid availability, by preventing translation when many essential and nonessential amino acids are deficient (Jewell *et al.* 2013, Jones & Thompson 2009). In one example of this, serine deficiency, induced in lung cancer cells by withdrawal of serine from the growth media and by concomitant impairment of *de novo* serine biosynthesis, was shown to severely suppress the activity of mTORC1 effectors S6 kinase and S6 (Ye *et al* 2012). A similar effect can be seen with the withdrawal of leucine, and many other amino acids (Jewell *et al.* 2013, Ye *et al.* 2012).

Metformin & Biguanides

Much emphasis has been put on finding metabolism-centered approaches to cancer therapy. Metformin is a widely-used antidiabetic drug that reduces hyperglycemia in non-insulin-dependent diabetes mellitus. There is evidence that metformin and other biguanides such as phenformin and buformin function by inhibiting complex I of the mitochondrial transport chain and thereby reduce mitochondrial ATP production (Owen *et al*. 2000, El-Mir *et al*. 2000). This causes AMPK activation in hepatocytes and consequently, the anabolic process of gluconeogenesis is inhibited, lowering blood glucose and insulin (Shaw *et al.* 2005). Pharmacoepidemiological studies have suggested that metformin use lowers cancer risk and improves cancer outcome in type II diabetics (Pollak 2012). Subsequent laboratory studies have established a role of metformin and other biguanides in countering the positive influence of hyperglycemia and hyperinsulemia on tumor progression (Algire *et al.* 2010, Algire *et al*. 2008). In addition, metformin was shown to be anti-proliferative in breast cancer cells *in vitro* through AMPK activation by direct action on the cells themselves (Zakikhani *et al.* 2006).

Recent studies have yielded additional therapeutically-relevant results pertaining to the action of biguanides. LKB1 deficiency was shown to sensitize lung cancer cells to phenformin *in vivo* by eliminating their ability to respond to energy stress (Shackelford *et al.* 2013). Two important compensatory mechanisms to the effects of biguanides were identified and targeted. Increased glucose uptake and flux through glycolysis allows cells to compensate for the reduction in mitochondrial ATP production. In this context, additional pharmacological inhibition of glycolysis by 2-deoxyglucose resulted in the induction of apoptosis and in severe inhibition of cell

proliferation (Cheong *et al.* 2011, Ben Sahra *et al.* 2010). Metformin also increased uptake of glutamine and its utilization by reductive carboxylation. Inhibiting glutaminase restricted this increase and exerted synergistic antiproliferative effect with metformin in prostate cancer cells (Fendt *et al.* 2013).

Rationale and Hypotheses

Our current understanding of cancer metabolism, and more specifically, the interplay between glycolysis, biosynthesis pathways and the production of chemical energy, yielded two strategies for targeted metabolic disruption of cancer cells. Both strategies hinged upon well-known manipulations of PKM2 activity (Wong *et al*. 2013) in order to produce novel and cancer-specific therapeutic interactions.

First, we show that the well-established inhibitory effect on glycolysis by serine deprivation-mediated PKM2 inhibition (Chaneton *et al.* 2012; Ye *et al* 2012) can be used to sensitize cancer cells to metformin, given the enhanced requirement for glycolytic flux arising from metformin treatment. Upon demonstrating that serine deprivation significantly enhanced the efficacy of metformin *in vitro*, our goal was to extensively study this novel effect with metabolic and signalling endpoints. We then tested the efficacy of a serine-deficient diet in reproducing this effect *in vivo*. We hypothesised that serine deprivation acting through PKM2 expressed in cancer cells would mimic a state of glucose deprivation or pharmacological inhibition of glycolysis in a tumor-specific way.

The second strategy was to enhance the antiproliferative activity of a pharmacological activator of PKM2 using serine deprivation *in vivo*. Given the allosteric activation of PKM2 by serine, and the importance of PKM2 inhibition-mediated build-up of 3-phosphoglycerate for *de novo* serine biosynthesis (Chaneton *et al*. 2012; Kung *et al.* 2012) we hypothesised that the resulting intracellular deficiency in serine would be severe enough to inhibit tumor growth.

CHAPTER 2

MANUSCRIPT

Serine Deprivation Enhances Anti-neoplastic Activity of Biguanides

Submitted to Cancer Discovery

(Pending review)

Serine Deprivation Enhances Anti-neoplastic Activity of Biguanides

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Abstract

Metformin, a biguanide widely used in the treatment of type II diabetes, has anti-neoplastic activity in experimental models and is currently being evaluated in clinical trials for indications in oncology. Its fundamental activity is as an inhibitor of oxidative phosphorylation. Here, we show that biguanides have enhanced antineoplastic activity in the absence of serine. Whereas serine synthesis is not inhibited by metformin, metabolic studies demonstrate a requirement for serine to allow cells to compensate for biguanide-induced decrease in oxidative phosphorylation by upregulating glycolysis. In vivo, dietary restriction of serine significantly enhanced the tumor growth-inhibitory actions of phenformin. Thus, we identify a dietary manipulation that enhances the efficacy of biguanides as metabolic antineoplastic agents.

Introduction

Metformin, like other biguanides, inhibits complex one of the mitochondrial electron transport chain (1). 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 (2, 3). Pharmaco-epidemiologic studies have generated the hypothesis that there are novel indications for metformin in oncology by associating its use with reductions in cancer risk and improvements in cancer prognosis (reviewed in (4)). These findings led to investigation of metformin in pre-clinical cancer models, most of which demonstrate clear antineoplastic effects (5). However, the methodology employed in some of the pharmaco-epidemiologic studies is controversial (6), and most laboratory models involve drug exposure levels considerably higher than those used in diabetes treatment (7). Thus, it is uncertain if conventional anti-diabetic 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 have multiple mechanisms of action (8-10) and that sensitivity to biguanides varies with genetic characteristics of tumors (11-13), nutrient conditions (14, 15), and other factors (16-17), the possibility of combination therapies involving a biguanide requires investigation.

Cancer cells are metabolically adapted to meet the bioenergetic and biosynthetic demands of proliferation. Disregulated metabolism in cancer cells presents potential therapeutic targets (18). Recent studies have highlighted the over-expression of serine synthesis pathway enzymes in melanoma and breast cancer (19, 20) and have determined a regulatory relationship between the level intracellular serine and the anabolic versus catabolic use of glucose carbons (21, 22).

Furthermore, serine deprivation was associated with reduced proliferation and an inhibition of glycolysis (21-23). As cancer cells treated with biguanides have a particularly stringent reliance on glycolysis to compensate for the inhibition of mitochondrial ATP production and inhibited glucose carbon flux to the citric acid cycle (17), we investigated the influence of serine deprivation on the antiproliferative effects of metformin and phenformin. The data presented here reveal that serine deprivation potentiates the anti-neoplastic effects of biguanides, whereby serine deprivation interferes with the metformin-mediated compensatory induction of glycolysis.

Methods

Cell lines and tissue culture

H1299 and A549 were obtained from ATCC. MC38 were generously provided by Dr. Pnina Brodt (McGill University). H1299 with AMPKα1/2 stable knockdown and control cells were a generous gift from Dr. Russell Jones (McGill University). Cell lines were cultured in RPMI 1640 with 10% (v/v) FBS (Wisent) and gentamycin. To generate transient *PHGDH* knockdown in MC38, we transfected cells with siRNA targeting PHGDH (Qiagen, cat. #: SI01377131) or negative control siRNA (Qiagen, cat. #: 1027280) using a pipette-type electroporator (MicroPorator MP-100, Digital Bio Technology Co., Ltd., Seoul, Korea) as described by the manufacturer. Knockdown of target was verified by immunoblot.

To assess proliferation, cells were seeded in culture plates and incubated for 24h to allow attachment. Subsequently, seeding media was 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.

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 5–6 weeks of age. Animals were acclimatized for one week, after which, they were divided into two groups, one receiving a control diet and the other, a diet lacking serine and glycine (ser-/gly-) (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-/gly- 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 two weeks prior to the start of the allograft experiment in order 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 (5×10^5 per animal) were implanted by subcutaneous injection into left 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 intraperitoneal (IP) injections of 40 mg/kg phenformin, a biguanide with greater *in vivo* bioavailability than metformin; while control groups received twice-daily IP saline injections. In two-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, weighed and flash-frozen in liquid nitrogen.

Immunoblots

For protein analysis, cells were seeded and cultured in complete medium for 24h 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 phosphate-buffered saline (PBS) and lysed in lysis buffer containing 10 mM Tris-HCl (pH 7.3), 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X, 1 mmol/L EDTA, 10 mM β-glycerophosphate and 0.5 mmol/L NaF, supplemented with protease and phosphatase inhibitor pills (Roche). The crude lysates were centrifuged at 10,000 rpm for 15 min at 4ºC and the resulting soluble fractions were isolated. Protein concentration was assessed in all samples using a bicinchoninic acid (BCA) protein assay kit (Thermo Scientific). Loading buffer was added to purified lysates, which were subsequently boiled for 5 min. 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 ChemiDoc XRS+ system (Bio-Rad) using SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific). Image Lab software by Bio-Rad was used for densitometry analysis. Antibody to PHGDH was obtained from Novus Biologicals while those against phospho-S235/236 S6, phospho-T389 p70 S6K, phospho-T37/46 4E-BP1, phospho-T172

AMPKα, phospho-S79 ACC, S6, p70 S6K, 4E-BP1, AMPK, ACC and β-actin were obtained from Cell Signaling Technology.

Metabolic assays

Cells were seeded and cultured in complete medium for 24h in order to obtain 30% density. Medium was replaced with indicated treatment media, and cells were cultured for 24h. 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 (24). Total consumption was calculated by subtracting results from baseline glucose concentration, measured in samples from media incubated in identical conditions, without cells. These values were indexed to the relative amount of cells, obtained by assaying lysates for protein content. Lactate production was quantified using a commercial lactate assay kit (BioVision) and indexed to protein content of cell lysates.

Stable Isotope Labeling

MC38 grown up to 40-50% confluence in 35mm plates were subjected to ^{13}C-glucose (CML-1396, Cambridge Isotope Laboratories, Inc.) or ^{13}C-glutamine (CML-1822, Cambridge Isotope Laboratories, Inc.) pulse labeling along with metformin treatment, serine deprivation, or pyruvate supplementation for 24h. Basal medium used was RPMI-1640 lacking glucose, glutamine, glutamate, serine and glycine (Wisent) and supplemented with 2 %(v/v) dialyzed FBS (Wisent). ^{13}C-glucose and ^{13}C-glutamine were used at half the final concentration, with unlabelled counterparts kept at 5.55mM glucose and 2mM glutamine. When used as supplements, serine was added at 30 μg/mL and pyruvate was added at 1mM. 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 pre-chilled tubes. MeOH quenching and harvest was repeated once. Suspensions were sonicated for 10 minutes/4°C (30 sec ON and 30 sec OFF, high setting, using Diagenode's Bioruptor), vortexed and cleared by centrifugation (21,000g, 10min/4°C). Supernatants were transferred to pre-chilled tubes and 800ng ^{27}D-myristic acid diluted in pyridine was added to each sample. Samples were allowed to dry entirely in a Labconco CentriVap cold trap.

GC/MS

30µL pyridine containing 10mg/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 min in GC/MS injection vials. Samples were further incubated for 1h after the addition of 70µL N-Methyl-N-tert-butyldimethylsilyltrifluoroacetamide (MTBSTFA) (Sigma). 1µL was used per sample for GC/MS analysis. GC/MS installations and software were all from Agilent. The GC/MS method used was previously described elsewhere (25)

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 ^{13}C for this fragment, were transferred to a spreadsheet together with the integration of the internal standard ^{27}D-myristic acid. A correction matrix was generated for each metabolite using an in-house algorithm adapted from (26). Integration values for a given metabolite were multiplied to the corresponding correction matrix to remove the abundances of naturally occurring isotopes that

mask the labeling provided by exogenous ^{13}C-substrates. Values obtained for a given metabolite correspond to the proportional flux, e.g. the proportion of a labeled fraction within the pool of total ions. In order to assess relative amounts of labelled ions, values of the proportional flux for a given metabolite were multiplied by the abundance of this metabolite, defined as the sum of all ion integrations (m+0 to m+i) divided by the integration of the internal standard and further divided by protein content (mg) or cell number (million cells). Values obtained represent the relative flux, where the proportions of labelled ions are adjusted to the amount of the metabolite analyzed.

Statistical analyses

All experiments were independently performed at least 3 times unless specified. When biological replicates are shown, they are taken from a single experiment that was representative of multiple independent experiments. Student's t-tests were used for comparisons of individual treatments vs. control. Two-way analysis of variance (ANOVA) with Bonferroni's post-tests was used for multiple comparisons. Statistical tests were performed with Microsoft Excel, GraphPad Prism or GraphPad InStat.

Results

Proliferation is influenced by interactions between serine availability, phosphoglycerate dehydrogenase expression, and metformin exposure.

Either directly or through conversion to glycine, serine allows the replenishment of one-carbon units which play an important role in DNA methylation, in the maintenance of redox balance, and contribute to the biosynthesis of nucleotides, phospholipids and other amino acids (27). Consequently, serine has been shown to be indispensable for the growth and proliferation of cancer cells, which must obtain it through uptake or through *de novo* biosynthesis from glucose; thus, strategies combining the disruption of serine biosynthesis and serine deprivation are antiproliferative *in vitro* (23, 28). We therefore investigated factors influencing the ability of H1299 and A549 cells to proliferate when deprived of serine. Serine deprivation exerted a substantial inhibitory effect on the proliferation of A549 cells whereas it only weakly inhibited the proliferation of H1299 (Fig. 1A-B). This led us to hypothesize that these cell lines differ in their capacity for serine biosynthesis. As expected, relative to H1299 cells, A549 cells show reduced expression of phosphoglycerate dehydrogenase (PHGDH), the rate-limiting enzyme in the *de novo* serine synthesis pathway (Fig. 1C). To confirm a cause-and-effect relationship, we produced a pair of isogenic MC38 cell lines differing only in PHGDH expression, through siRNA-mediated knockdown (Fig. 2A). Cells transfected with siRNA targeting PHGDH displayed a significantly reduced ability to proliferate under serine deprivation relative to those that were transfected with non-targeting siRNA (Fig. 2B-C).

Cancer cells must coordinate the diversion of glucose metabolic flux into biosynthesis pathways to meet the macromolecular synthesis requirements for proliferation (18). Given the importance of serine for many biosynthetic pathways, its intracellular level may be an important indicator of cellular biosynthesis needs. Thus, PKM2, an essential regulator of the fate of glucose carbons (29, 30), is allosterically activated by serine. Low intracellular serine lowers PKM2 activity, favouring glucose carbon flux to biosynthesis (22). This has been shown to reduce the rate of glycolysis (23), which we hypothesized would increase sensitivity to the added metabolic stress induced by the inhibition of oxidative phosphorylation by biguanides. Therefore, we treated serine-deprived cells with metformin. At 2.5mM, the compound failed to significantly hinder proliferation of H1299 (Fig. 1A) and MC38 (Fig. 3A) in serine-replete conditions (Fig. 3A). 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 LKB1 expression (11). Under serine deprivation, metformin caused a marked inhibition in proliferation of both H1299 and MC38 not seen with either serine deprivation or metformin treatment individually (Fig. 1A and 3A, respectively). A timecourse of MC38 proliferation under serine deprivation combined with 2.5mM metformin revealed that arrest occurs within the first 24h of treatment and that neither serine deprivation nor the administration of 2.5mM metformin in the presence of serine affected the growth rate of MC38 cells (Fig. 3B). Furthermore, serine withdrawal increased the antiproliferative effectiveness of otherwise sub-optimal doses of metformin (1-2.5mM) (Fig. 3C).

Figure 1

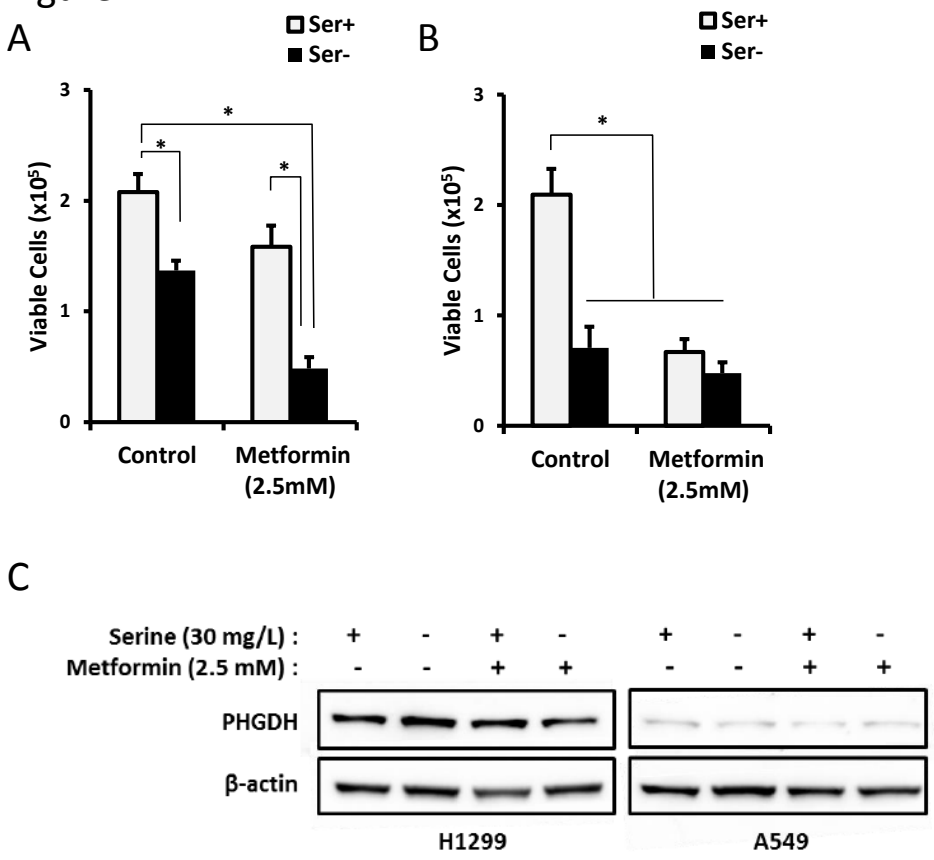

Figure 1

Serine deprivation causes greater growth inhibition in cells with low PHGDH expression.

(A) In H1299 cells, proliferation was only modestly inhibited by serine deprivation but greatly suppressed by the combination of serine withdrawal and metformin. Cells were treated with metformin (2.5 mM) in the presence or absence of serine (30 mg/L) for 72 hours and were then counted, and assessed for viability by trypan blue exclusion.

(B) A549 cells displayed great sensitivity to serine deprivation but showed little further growth inhibition by metformin. Cells were assessed after 72h treatment with metformin (2.5 mM) with or without serine (30 mg/L).

(C) Immunoblot of cell lysates reveals a much higher expression of PHGDH in H1299 relative to A549. Cells treated for 24h according to the conditions outlined in 1A-B.

Figure 2

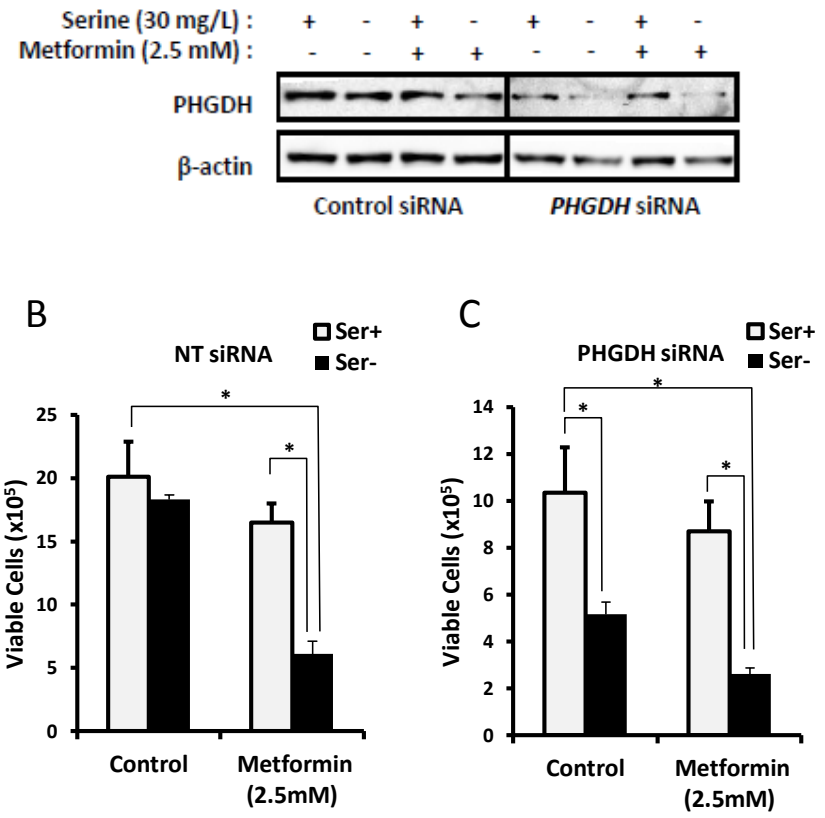

Figure 2

PHGDH knockdown induces sensitivity to serine deprivation in MC38 cells.

(A) Immunoblot of cell lysates from MC38 cells transfected with siRNA targeting PHGDH shows that partial knockdown was achieved.

(B) MC38 cells transfected with non-targeting siRNA were not sensitive to serine deprivation. However, the antiproliferative effect of metformin was greatly enhanced by serine withdrawal. Cells were treated with metformin (2.5 mM) for 72 hours while serine was either present (30 mg/L) or absent in the media.

(C) siRNA-mediated knockdown of PHGDH expression rendered MC38 cells sensitive to growth inhibition when they were deprived of serine. Treatment conditions and length were identical to those in 2B.

Figure 3

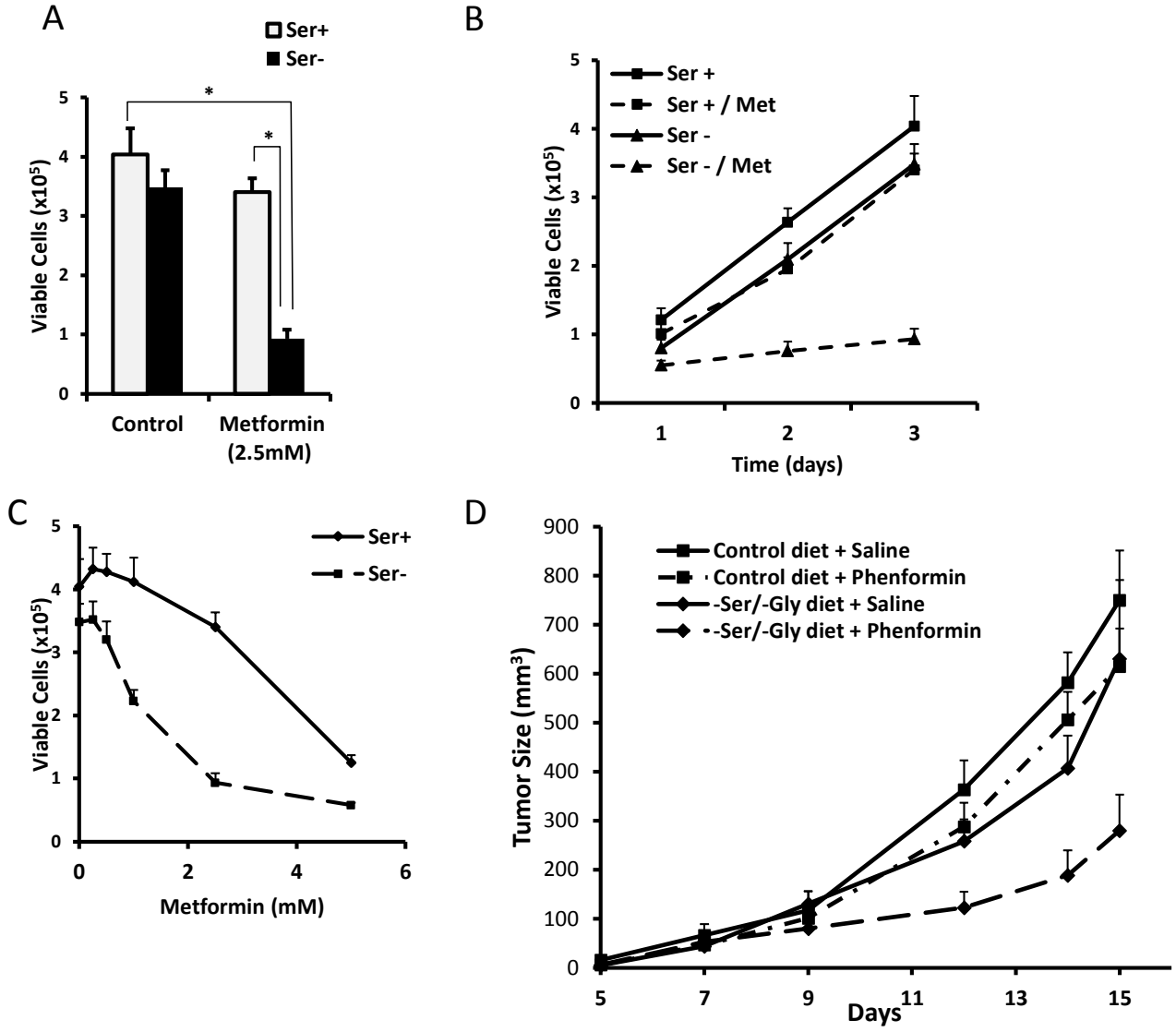

Figure 3

Serine deprivation enhances the antiproliferative effects of metformin *in vitro* and of phenformin *in vivo.*

(A) Proliferation of MC38 cells is unaffected by serine deprivation or metformin exposure alone. However, a severe inhibitory effect is observed when cells were simultaneously exposed to both. Cells were treated with metformin (2.5 mM) in the presence or absence of serine (30 mg/L) for 72 hours.

(B) The combination of serine deprivation and metformin (2.5mM) results in proliferation arrest in MC38 within 24h of exposure. Trypan blue-excluding cells were counted at 24, 48 and 72h timepoints.

(C) Metformin, at concentrations between 0.5 mM to 5mM substantially inhibited proliferation in MC38 cells cultured without serine. Cells were exposed to metformin (0.25, 0.5, 1, 2.5 and 5 mM) in the context of serine availability (30 mg/L) or deprivation for 72 hours.

(D) Administration of Serine and Glycine-free Diet Enhances Tumour Growth Suppression by Phenformin. We demonstrate an in vivo correlate of our in vitro observations in an MC38 allograft model. A serine and glycine free diet acted to sensitize tumours to an otherwise ineffective dose of phenformin. Mice (C57/BL6 male) were fed either control diet containing all amino acids, or an identical, isocaloric diet lacking serine and glycine. After 2 weeks on the diets, MC38 tumours were injected (day 0). On day 5, we began twice-daily IP injections of saline or 40 mg/kg phenformin (n = 8 for each diet / drug combination) Animals were sacrificed on day 15. Results are representative of two independent experiments. Data points indicate mean tumour volume ± Standard error.

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

It has been shown that a diet deficient in serine and glycine can significantly reduce the serum levels of those amino acids in mice (21). We sought to extend our *in vitro* findings by creating a similar diet-induced serine and glycine deficiency in C57BL/6 mice with MC38 allografts and treating with phenformin, a more bioavailable biguanide (7, 11). The control and serine/glycine deficient diets were isocaloric and were well tolerated by the animals, as we observed continuous weight gain in all groups. MC38 tumours grew rapidly regardless of the presence or absence of serine and glycine in the diet. Phenformin, dosed at 40mg/kg and given by intraperitoneal (IP) injection twice-daily did not impair tumor growth in mice fed the control diet. However, we observed a significant reduction in the growth and in the final size of the tumours in mice on the diet deficient in serine and glycine combined with phenformin treatment (Fig. 3D). All groups tolerated the combinations of diets and treatments administered. Constant weight gain was seen in all groups. Food intake was measured weekly and was not found to differ between each diet group.

Metformin abolishes the maintenance of mTORC1 activity under serine deprivation

The mammalian target of rapamycin complex 1 (mTORC1) is a key intracellular sensor that coordinates protein synthesis with growth signals and the availability of energy and nutrients (31). Accordingly, the depletion of intracellular serine has been shown to reduce the activity of key mTORC1 pathway effectors in a subset of cell lines (23). Serine withdrawal only marginally

reduced the phosphorylation of mTOR effectors S6 kinase 1 (S6K) and its downstream substrate ribosomal protein S6 (S6) in H1299 and MC38 as compared to control, whereas 2.5 mM metformin had no effect (Fig. 4A-B), consistent with retained proliferative capacity in these under these conditions. However, 2.5 mM metformin combined with serine withdrawal resulted in significant suppression of phosphorylated S6 and S6 kinase in both H1299 and MC38 cells; indicative of mTORC1 inhibition (Fig. 4A-B). A time-course of mTORC1 pathway activity in H1299 cells revealed that serine deprivation causes a time-dependant reduction in S6 phosphorylation (Fig. 4C). However, in 24h, untreated cells fully recover initial phosphorylated S6 levels, while those treated with 2.5 mM metformin did not (Fig. 4A). The data show that the potentiation of the anti-proliferative effects of metformin by serine withdrawal is paralleled by decreased mTORC1 signaling.

Figure 4

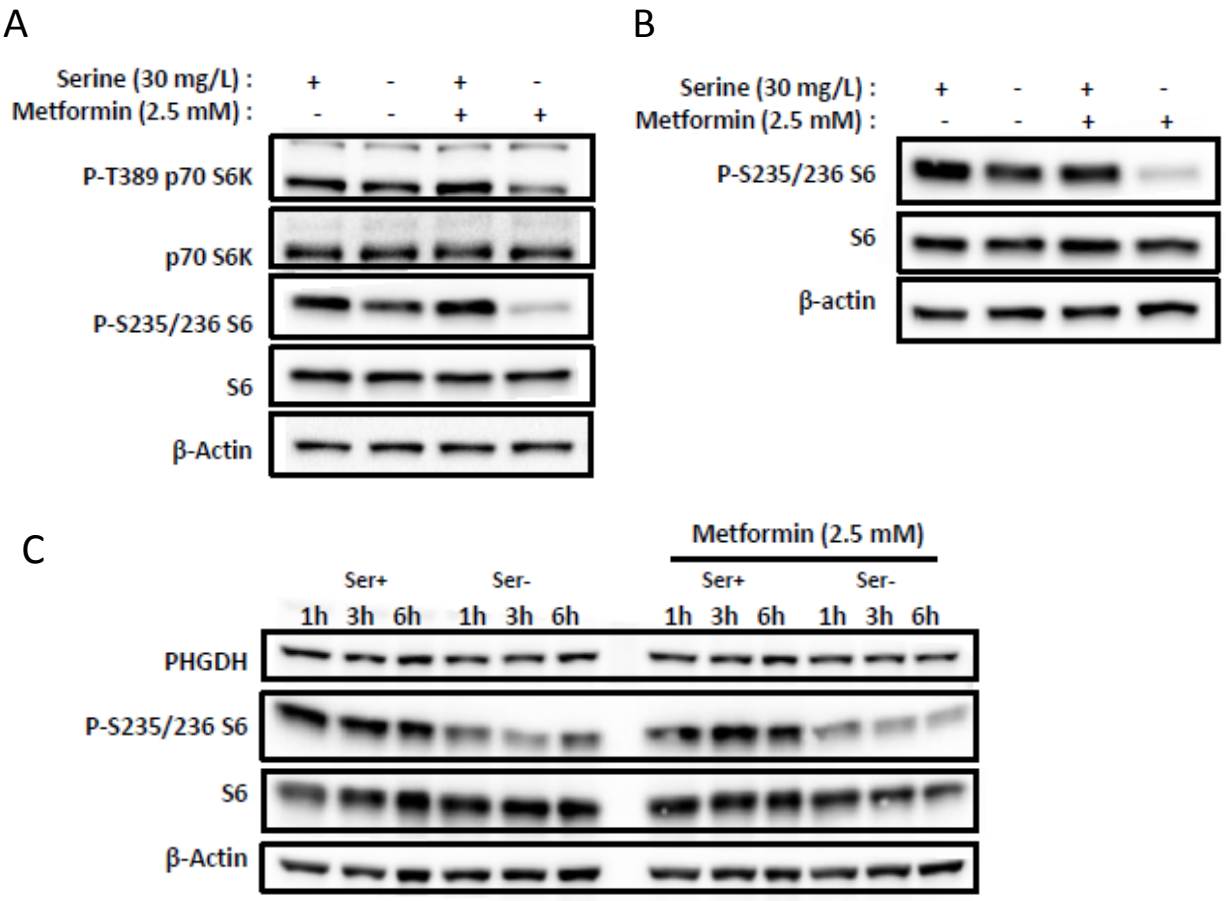

Figure 4

Metformin extends and significantly enhances mTORC1 inhibition caused by serine deprivation.

(A) Under serine deprivation, metformin increased the magnitude of S6 and S6 kinase dephosphorylation in H1299 cells, indicating marked inhibition of mTORC1. Cells were treated with metformin (2.5 mM) in the presence or absence of serine (30 mg/L) for 24 hours.

(B) Similarly, in MC38 cells, S6 phosphorylation was suppressed only when cells were treated with metformin (2.5 mM) in the absence of serine. MC38 cells were treated for 24 hours with metformin (2.5 mM) with or without serine (30 mg/L) in the culture media.

(C) Serine deprivation causes a temporary decrease in S6 phosphorylation in H1299 cells. However, a marked suppression of phosphorylated S6 is seen in all timepoints in cells exposed to combined metformin and serine withdrawal. Cells were treated with metformin (2.5 mM) for 1, 3 or 6 hours in the presence or absence exogenous serine (30 mg/L).

Metformin does not inhibit serine biosynthesis

The inability of serine deprivation to significantly impair growth and inhibit mTORC1 in MC38 and H1299 suggests that these cells may be able to endogenously support their serine requirements through the serine synthesis pathway (19, 23). We therefore sought to determine whether the enhanced effects of metformin or phenformin under serine deprivation were due to an inhibition of serine biosynthesis. In order to evaluate the flux of glucose into serine under metformin and/or serine deprivation, we performed stable isotope tracer analysis using ^{13}C-glucose (Fig.2C). ^{13}C-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 (PSAT) and 3-phosphoserine phosphatase (PSP). Serine is reversibly converted into glycine m+2, thus, total serine isotopomers derived from ^{13}C-glucose will contain m+1, m+2 and m+3 enrichments. Metformin did not inhibit ^{13}C-glucose contribution to the serine pool, while serine deprivation drastically reduced it (Fig.5A). This suggests that serine deprivation impairs the flow of glycolytic intermediates into the serine biosynthesis pathway, but that this is not further inhibited by metformin. While the presence of an active serine synthesis pathway favours maintenance of proliferation and mTORC1 signalling in cells deprived of serine

(19), these results show that a mechanism other than inhibition of serine synthesis is responsible for the decrease in proliferation upon metformin exposure in serine-deprived MC38 and H1299 cells.

Figure 5
A

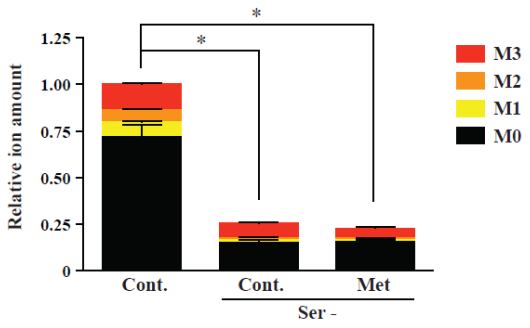

Figure 5

De novo serine biosynthesis is unaffected by metformin

Under serine deprivation, the level of intracellular serine is significantly reduced. Metformin did not reduce intracellular serine levels, nor did it affect serine biosynthesis from glucose. MC38 cells were cultured in U-^{13}C-glucose and treated with metformin in the absence of serine. Mass isotopomer distribution analysis of TBDMS-derivatized serine was performed following GC/MS acquisition. Signal was normalized to protein content. Results are representative of 3 independent experiments. Data represent mean relative ion amount; error bars, SEM. *P < 0.05, Student's *t* test.

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

Evidence from preclinical models suggests that biguanides exert antineoplastic effects through several mechanisms, including AMPK activation (4-5, 7-10). AMPK inhibits anabolic processes and proliferation, in part through suppression of mTORC1 activity (32). Serine availability was shown to also influence mTORC1 activity (23). We hypothesised that combining biguanides and serine deprivation may result in converging inhibitory signals to mTORC1, leading to greater inhibition of mTORC1 and consequently, enhanced inhibition of proliferation (31). To this end, we activated AMPK pharmacologically using AICAR while varying serine availability in MC38 cells. At 50 μM, AICAR stimulated AMPK phosphorylation to a comparable extent as in cells treated with 2.5 mM metformin (Fig. 6A). AICAR-induced AMPK activation was not influenced by serine availability. Furthermore, we did not observe greater reduction in proliferation (Fig. 6B) nor an enhancement of mTORC1 inhibition (Fig. 6A) attributable to serine deprivation in AICAR-treated MC38 cells. Instead, recognizing that the context of precipitating metabolic stress associated with AMPK activation by metformin is absent in AICAR-treated cells, the data suggest that metabolic stress plays an important primary role in the interaction of biguanides and serine deprivation.

To further exclude the involvement of AMPK, we measured the effects of metformin and serine withdrawal on the proliferation of H1299 cells depleted of AMPKα1/2 by shRNA (Fig. 6C). AMPK depletion does not prevent the inhibition of proliferation by metformin in cells deprived of serine (Fig. 6D). Taken together, these findings show that serine withdrawal bolsters the anti-proliferative effects of biguanides independently of AMPK.

Figure 6

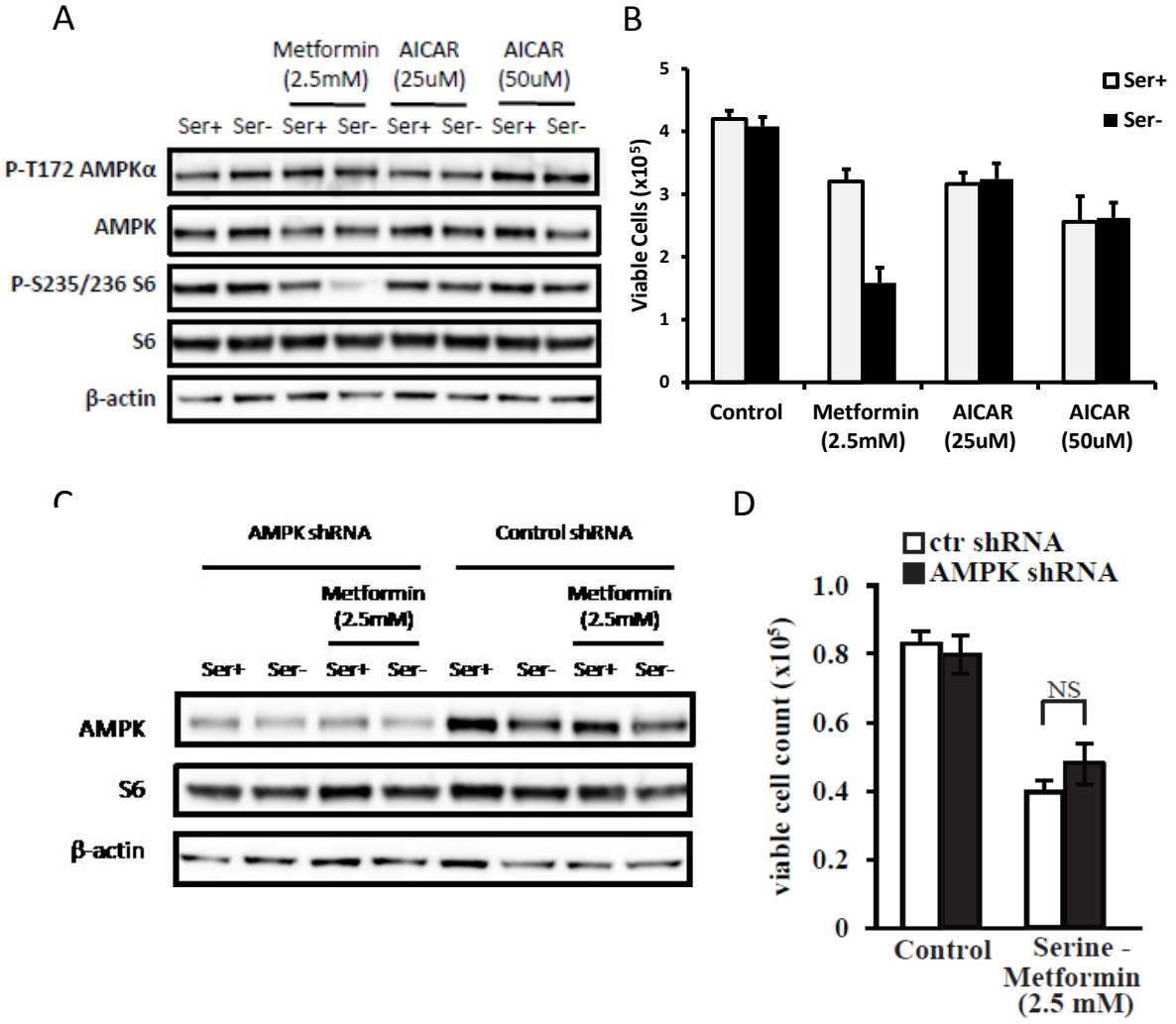

Figure 6

Enhanced antineoplastic activity of metformin under serine deprivation is independent of AMPK activation

(A) Immunoblot of lysates of MC38 cells that were treated with metformin (2.5 mM) or AICAR (25 μM or 50 μM) in the presence or absence of serine (30 mg/L) for 24h demonstrate that the activation of AMPK by AICAR, which is not influenced by serine availability, is insufficient for mTORC1 inhibition under serine deprivation.

(B) Pharmacological activation of AMPK by AICAR in the setting of serine deprivation fails to mimic the antiproliferative effect of metformin. MC38 cells were treated for 72 hours with metformin (2.5 mM) or AICAR (25 μM or 50 μM) in the presence or absence of serine. Trypan blue-excluding cells were counted using an automated cell counter.

(C) H1299 cells transfected with shRNA targeting AMPK display markedly reduced levels of AMPK in all conditions tested. Lysates were obtained from cells treated with metformin (2.5 mM) in the presence or absence of serine (30 mg/L).

(D) H1299 Cells depleted of AMPK were equally sensitive as their wild-type counterparts to the antiproliferative effect of combined serine deprivation and metformin (2.5 mM) in 72 hour assays of proliferation with viable cell counts as endpoints.

Serine deprivation reduces metformin-induced upregulation of glycolysis

In the setting of complex 1 inhibition by biguanides, changes in metabolic fluxes occur. Primarily, pyruvate oxidation in the citric acid cycle is decreased, while glycolytic flux to lactate is upregulated (17). Serine is a critical modulator of glycolysis (23), likely via allosteric activation of PKM2 (22). We therefore sought to explore the effects of simultaneous serine deprivation and metformin treatment on glucose metabolism. We treated MC38 and H1299 cells with metformin for 24h and measured glucose consumption and lactate secretion over that time period. As expected, we observed substantial increases in both, indicative of upregulated glycolysis. Compared to cells in the untreated and serine-replete control condition, cells under serine deprivation exhibited a reduced rate of glycolysis. Furthermore, serine deprivation effectively inhibited the metformin-induced increases in glucose consumption and lactate secretion (Fig. 7A-B). These results confirm that cells respond to metformin-induced inhibition of mitochondrial oxidative phosphorylation by upregulating glycolysis (17) and demonstrate that serine deprivation is a potent inhibitor of this compensatory response. To obtain further insight into changes in glucose metabolism, we cultured MC38 cells in ^{13}C-glucose and varied serine availability and metformin treatment over 24h. Interestingly, serine deprivation did not reduce relative citrate levels (Fig. 7C) and the proportion ^{13}C labelled citrate (Fig. 7D) despite the observed decrease (Fig. 7B) in the rate of glycolysis, indicating that a greater proportion of available glycolytic carbon flux is diverted to citrate in serine-deprived cells. As expected, due to inhibition of oxidative phosphorylation and resulting impairment of the citric acid cycle, metformin greatly reduced glucose carbon flux to citrate (Fig. 7D), reducing relative citrate levels, irrespective of serine availability (Fig. 7C).

Glutamine consumption and its utilization in reductive citric acid cycle metabolism increase in the setting of biguanide exposure (17). We therefore considered the possibility that this compensatory mechanism may be inhibited by serine deprivation. Our data confirm the induction by metformin of reductive metabolism of glutamine in the CAC as shown by the predominance of m+5 labeled citrate ions derived from ^{13}C-glutamine over other labeled ions in the setting of metformin exposure (Fig 7E). This occurs regardless of serine availability (Fig. 7E), demonstrating that serine deprivation does not interfere with glutaminolysis and reductive carboxylation induced by metformin exposure.

To further explore whether serine deprivation potentiates the anti-proliferative effects of biguanides by impeding the compensatory switch to glycolysis, we supplemented MC38 and H1299 cells with 1mM pyruvate. Given that in the presence of metformin, pyruvate entry into the citric acid cycle will be limited (17), pyruvate supplementation will lead to the conversion of pyruvate into lactate by lactate dehydrogenase and the generation of NAD+ that is required for the activity of the glycolytic pathway. Accordingly, pyruvate allowed metformin-induced upregulation of glycolysis despite serine deprivation (Fig. 7B) and this was associated with an almost complete rescue of proliferation (Fig. 7F). Taken together, these results suggest that serine deprivation enhances the antiproliferative effect of metformin by inhibiting compensatory upregulation of glycolysis.

Figure 7

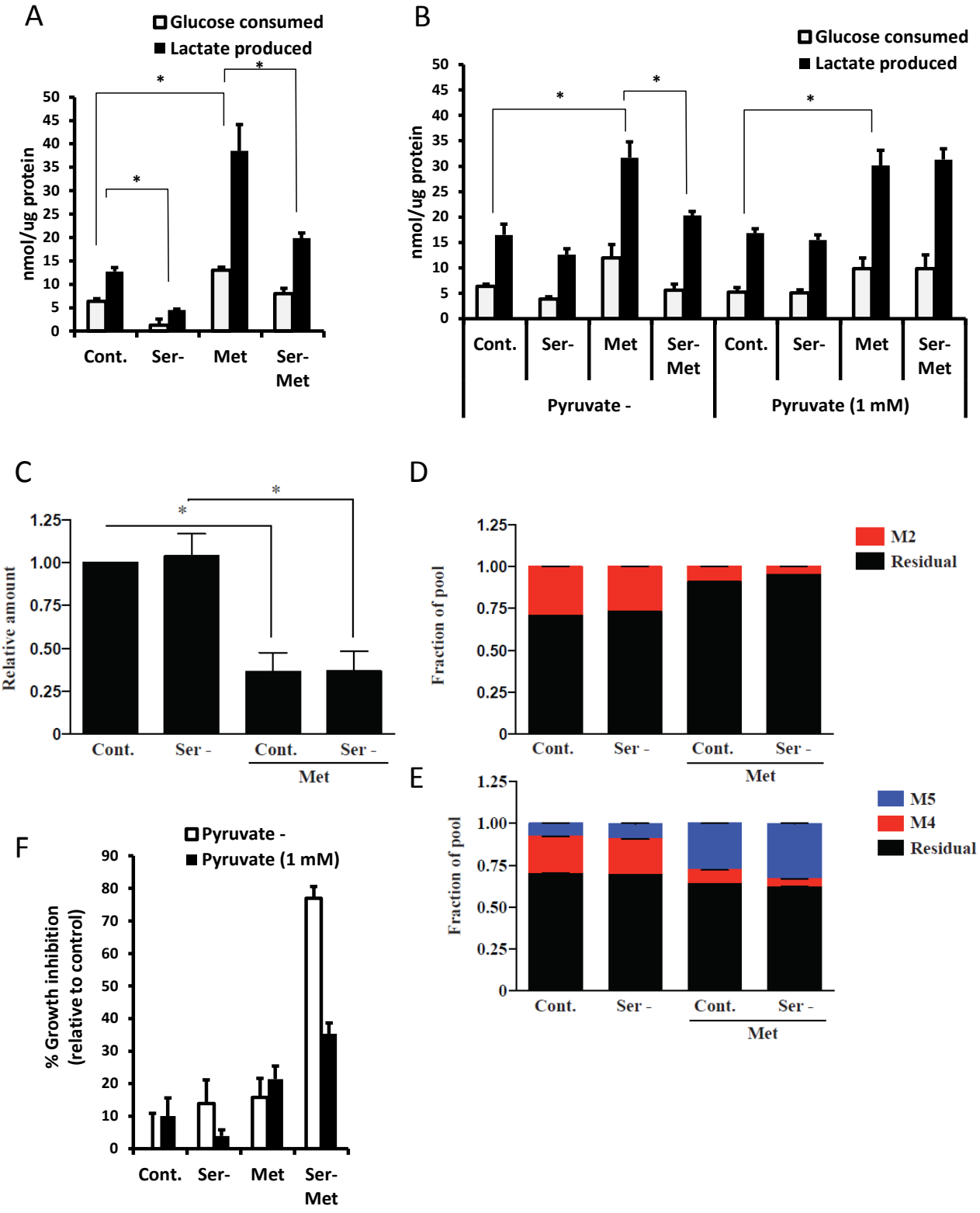
A
Glucose consumed
Lactate produced
nmol/ug protein
Cont.
Ser-
Met
Ser-Met
B
Glucose consumed
Lactate produced
nmol/ug protein
Cont.
Ser-
Met
Ser-Met
Pyruvate -
Pyruvate (1 mM)
C
Relative amount
Cont.
Ser -
Met
D
Fraction of pool
M2
Residual
E
Fraction of pool
M5
M4
Residual
F
Pyruvate -
Pyruvate (1 mM)
% Growth inhibition (relative to control)

Figure 7

Metabolic alterations underlie enhanced antineoplastic action of metformin under serine deprivation

(**A**) H1299 cells exhibit substantial inhibition of glycolysis when deprived of serine. Furthermore, they are unable to upregulate glycolysis in response to metformin exposure in the absence of serine. Conditions are as follows: Cont: control (Serine 30 mg/L); Ser-: absence of serine; Met: metformin (2.5 mM); Ser- Met: metformin (2.5 mM) and absence of serine.

(**B**) In MC38 cells, serine deprivation inhibits the metformin-induced upregulation of glycolysis. Supplementing with a supraphysiological concentration of pyruvate restores this compensatory mechanism in the setting of combined serine deprivation and metformin treatment. Cells (H1299 and MC38) were treated for 24h with vehicle (PBS) or metformin (2.5 mM) in either the presence or absence of serine. These conditions were repeated for MC38 cells with the addition of pyruvate (1 mM). Media samples were collected and assayed for glucose and lactate. Levels were subtracted from cell-free controls and normalized to protein content. $*P < 0.05$, indicates conditions that differ significantly in their glucose and lactate parameters.

(C) Metformin decreases relative citrate levels. Citrate levels form MC38 cells was analyzed by GC/MS. Data represent mean of relative values from three different experiments; error bars, SEM, *P < 0.05, Student's *t* test.

(D) Proportional ^{13}C-glucose flux into citrate is reduced by metformin in MC38 cells, as indicated by a decreased proportion of M+2 labelled citrate (M2).

(E) Metformin induces reductive glutamine metabolism, irrespective of serine deprivation. Proportional ^{13}C-glutamine flux into citrate is reduced by metformin in MC38 cells, as indicated by an increased proportion of M+5 labelled citrate (M5)

(F) Pyruvate reduces growth inhibition resulting from combined biguanide exposure and serine deprivation. Growth inhibition indicates percent decrease in proliferation relative to pyruvate -, serine + control. MC38 cells were treated for 72h with metformin (2.5 mM) in the presence or absence of serine, with or without pyruvate (1 mM).

Discussion

Various studies using preclinical models have explored the use of biguanides as antineoplastic agents and have raised the possibility that *in vivo* they act directly on cancer cells rather than by modulating host glucose and insulin levels to indirectly hinder tumor growth (4, 7). Furthermore, there is considerable evidence that sensitivity to the antineoplastic effect of biguanides can be modulated by host-related nutritional factors and by inherent tumor characteristics that influence cellular detection and responses to metabolic stress (11-15). A recent study supports the requirement for increased consumption of exogenous glutamine to salvage citric acid cycle function in metformin-treated cells (17). Although, the *de novo* serine synthesis pathway generates the CAC intermediate alpha-ketoglutarate as a by-product, exogenous serine is not known to contribute directly to CAC cycle anaplerosis or to catabolic processes that may alleviate biguanide-induced metabolic stress (27).

Nevertheless, 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, where a level of biguanide exposure that is well tolerated but insufficient to achieve antineoplastic activity under standard conditions inhibited tumor growth when mice were fed a diet deficient in glycine and serine. In our model, this dietary manipulation was well tolerated and was not by itself sufficient to impair the growth of the aggressive MC38 allografts. A recent report demonstrated that dietary restriction of serine and glycine inhibited proliferation of the slower growing HCT116 tumor model over a period of four weeks (21). This important discrepancy between the aforementioned study and our observations with regards to the antineoplastic activity of the serine

and glycine deficient diet is likely explained by differences in cell type, growth rates and exposure lengths.

Serine is directly involved in folate and methionine cycles, and is thus important for nucleotide biosynthesis, NADPH production and ROS clearance (27). In some preclinical models, serine deprivation is antiproliferative *in vitro* and *in vivo*, and this effect is influenced by the expression of serine synthesis pathway enzymes (19), PKM2 (23), and p53 (21). We initially suspected that our central observation was due to a novel action of metformin as an inhibitor of *de novo* serine biosynthesis but disproved this possibility by showing that the amounts of glucose-derived carbon entering the serine synthesis pathway are not reduced by metformin treatment. Therefore, we surmised that serine withdrawal likely enhances a known mechanism of antineoplastic activity of biguanides.

In cancer cells, metformin inhibits oxidative phosphorylation and requires compensation by metabolic changes and AMPK activation-dependent inhibition of anabolic processes and proliferation (8, 10-11, 14, 17). However, our findings preclude the possibility that serine deprivation enhances these effects of AMPK activation. Instead, we provide evidence that the newly-discovered role of serine as a modulator of glycolysis (22, 23) is responsible for the interaction of serine withdrawal with biguanides. The significant antiproliferative effect of this combination is associated with a notable absence of the compensatory upregulation of glycolysis that normally occurs to alleviate the bioenergetic impact of biguanide-induced inhibition of oxidative phosphorylation (17). By inhibiting glycolysis, serine deprivation provides a context

wherein the inhibition of oxidative phosphorylation by biguanides is particularly effective in inhibiting proliferation (33).

Our findings reveal that serine deprivation significantly enhances antineoplastic activity of biguanides in a manner that does not add toxicity *in vivo*. While clinical use of the diet we employed would be difficult to implement, the precedent of L-asparaginase therapy in leukemia treatment (34) suggests that enzymatic strategies to reduce serine bioavailability may be possible. More than 100 clinical trials of biguanides for indications in Oncology are underway (5), but most of these involve administration of metformin in a conventional anti-diabetic dosing regime, without the use of rational combinations that might enhance activity. While the results of these trials are eagerly anticipated, preclinical research, including results reported here, provides novel strategies that may deserve clinical evaluation, whether or not ongoing trials detect clinical benefit.

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CHAPTER 3

ADDITIONAL RESULTS

CONCLUSIONS AND FUTURE DIRECTIONS

Supplementary Results

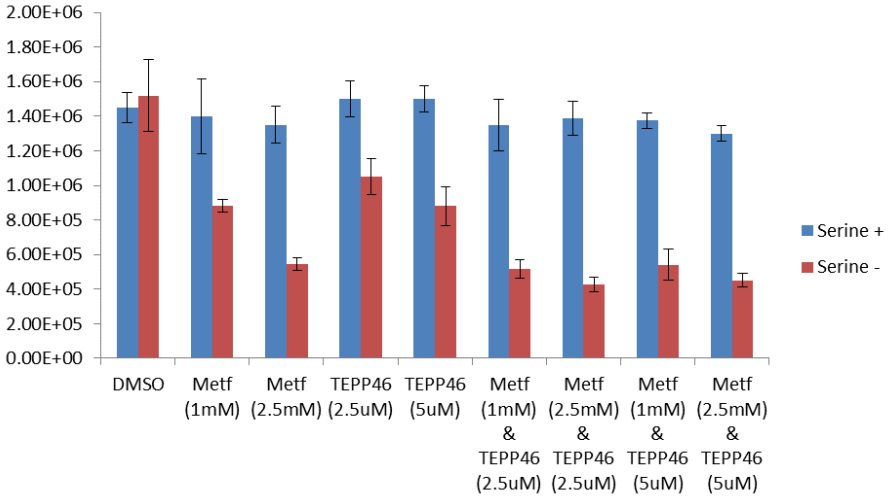
Serine deficiency potentiates the antineoplastic effect of PKM2 activators

PKM2 is a key regulator of the utilization of glucose carbon and is vital for diverting glycolytic intermediates for use in anabolic processes (Christofk *et al*. 2008a). Inactive PKM2 confers resistance to serine deprivation by enhancing flux through the *de novo* serine synthesis pathway *in vitro* (Kung *et al* 2012, Ye *et al* 2012). We sought to combine serine deficiency and TEPP-46, a pharmacological activator of PKM2 in an *in vivo* model. To this effect, we first tested this combination *in vitro*. Serine deprivation did not influence proliferation in MC38, nor did TEPP-46 at 2.5 and 5 μM (Fig. S1A). In combination with serine deprivation, both doses of TEPP-46 were significantly antiproliferative (Fig. S1A). In accordance with a prior report, combining serine withdrawal with forced activation of pyruvate kinase (in their case, by substituting PKM2 for PKM1, the constitutively active isoform of pyruvate kinase) inhibited mTORC1 activity as shown by suppression of S6 phosphorylation in MC38 and H1299 (Fig. S1B-C). Notably, we demonstrate that low-dose metformin (1 mM) and TEPP-46 (2.5 μM) exert additive antiproliferative effect under serine deprivation, while all combinations thereof in serine-replete conditions failed to impair proliferation.

We extend these observations to an *in vivo* model by demonstrating that TEPP-46 displays significant antiproliferative activity on MC38 tumors in animals fed a serine/glycine free diet, and that this is absent with a standard diet (Fig. S2 Tumor growth and Tumor weight). Although enhanced effectiveness of PKM2 activators under serine deprivation has already been documented (Kung et al. 2012), here we show that this can be applied *in vivo*.

Figure S1

A



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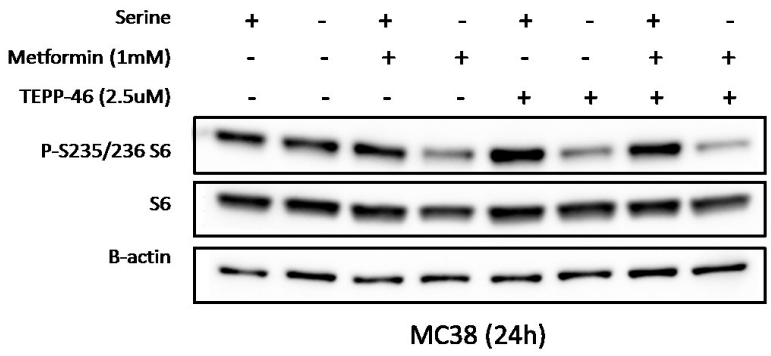

C

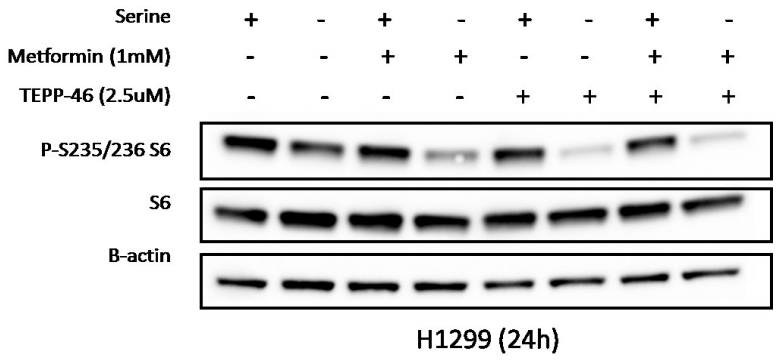

Figure S1

Metformin and PKM2 activation by TEPP-46 exert additive and enhanced effects under serine deprivation.

(A) Serine deprivation enhanced the antiproliferative effects of metformin and of TEPP-46 (2.5uM and 5uM). Here, MC38 cell proliferation is measured at 72h by viable cell counts.

(B-C) MC38 and H1299 cells are unable to maintain mTORC1 activity when treated for 24 hours with either TEPP-46 (2.5 uM) or metformin (1mM) or both, only when those are combined with serine deprivation. S6 phosphorylation is used to estimate mTORC1 activity.

Figure S2

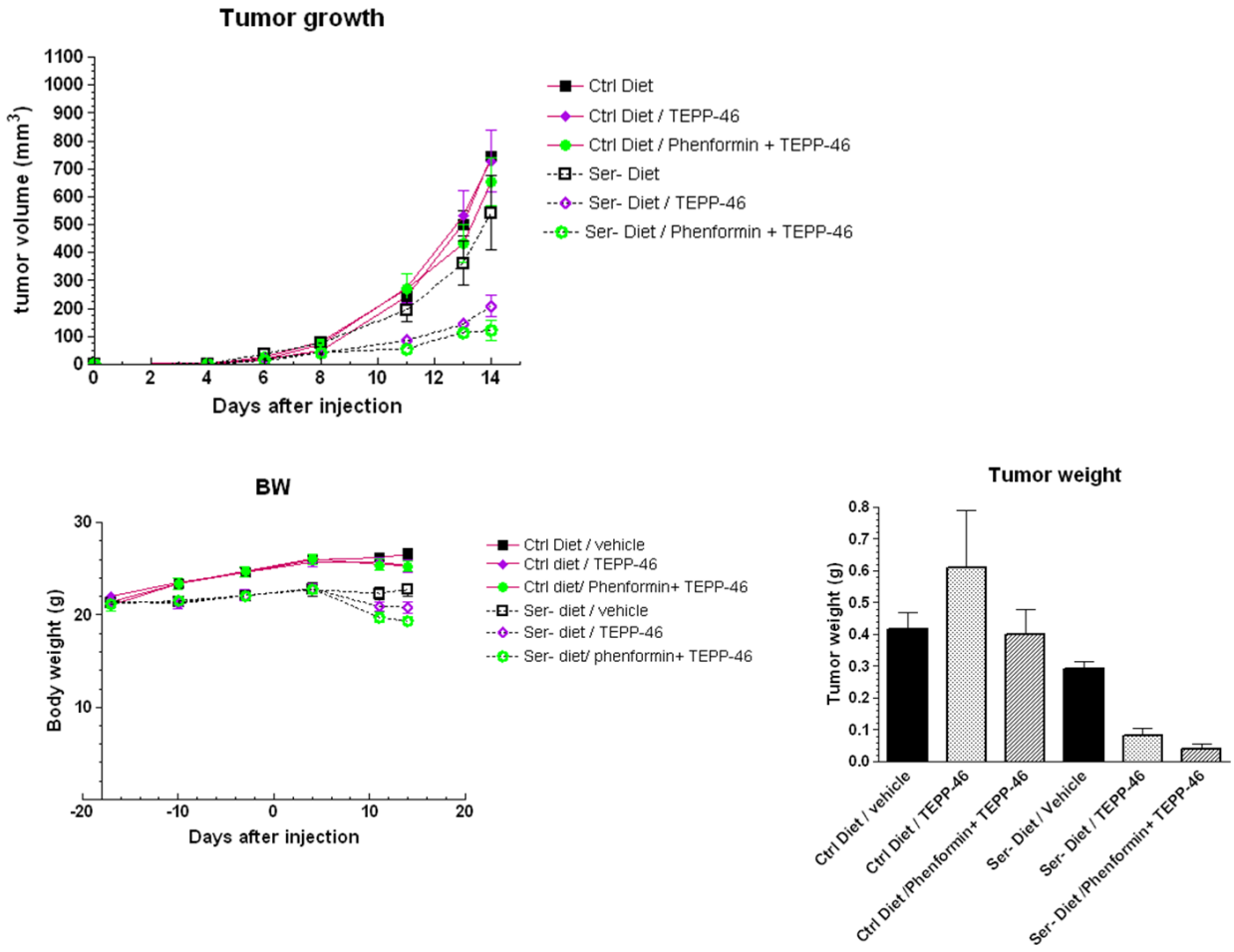

Figure 2

Dietary restriction of serine enhances antiproliferative effect of TEPP-46 *in vivo*

Tumor growth and final weight are severely impaired by the combination of the pharmacological PKM2 activator TEPP-46 and dietary serine and glycine deprivation. It is inconclusive whether combining these with phenformin further decreases growth. Neither drug given to the group on the control diet, nor the serine restricted diet produced a significant anti-tumor effect. The deficient diet was responsible for a small deficit in weight gain.

Mice, C57BL/6 male, were obtained from Charles River Laboratories. One week after reception, cages, each containing 3 mice were randomly divided into two groups, according to which they would be fed either a control diet or a serine and glycine deficient diet. Briefly, these diets were isocaloric, comprising of: Sucrose (25.9%), corn starch (41.8%), corn oil (5.0%) Baker amino acid mix (16.0%), Baker amino acid mineral mix (10.0%). The composition of the two diets differed only in composition of the amino acid mix, specifically, one lacking serine and glycine. Animals were acclimatized to the diets for a period of two weeks. Body weight measurements were taken once a week to verify adequate nutrition.

On day 0, MC38 cells (500,000 per animal) were injected into right flank subcutaneously. Treatments began on day 4. In each diet group, three sub-groups each comprising of 6 mice, were treated as follows: 1- Vehicle IP and PO (saline solution and 0.5% methyl cellulose / 0.1% tween 80, respectively); 2- TEPP-46 (30 mg/kg) PO twice-daily; 3- TEPP-46 (30 mg/kg) PO twice-daily and phenformin (40 mg/kg) IP twice-daily. Measurements of tumor volume were taken using an electronic caliper, every two days and prior to sacrifice on day 15. Tumors were excised and flash frozen in liquid nitrogen.

Conclusions and future work

These preliminary results and those outlined in the proposed manuscript are attempts to exploit metabolic adaptations unique to cancer cells in order to exert novel antineoplastic activities. Both approaches require elements of cancer metabolism that are largely absent from normal tissues, notably, PKM2 expression and the predominance of aerobic glycolysis. This likely accounts for the lack of observed toxicity and the promising results in animal models. However, the main problem with clinical application would be the induction of serine deprivation in humans. A diet may not be feasible given the likelihood of a lack of patient adherence. However other approaches geared towards reducing a specific amino-acid in circulation have been successfully used in the past. The best example of this is the L-asparaginase recombinant enzyme used to deplete the blood of asparagine for therapeutic effect in certain types of leukemia. Developing a similar enzyme to deplete serine would be more feasible than dietary restriction since it would also negate potential physiological mechanisms that may increase the level of serine in the blood in response to dietary deprivation.

Future preclinical research is required to refine and build upon the results presented here. Of greatest importance is the identification of specific tumor characteristics that confer vulnerability to the combination of serine deprivation and biguanides. Studies have independently provided evidence that *Trp53*-mutant tumors are sensitive to biguanides (Buzzai *et al.* 2007) and to serine deprivation (Maddocks *et al*. 2013). In light of these reports, we propose comparative studies of paired $p53^{+/+}$ and $p53^{-/-}$ tumor models in the context of the combination outlined in our work.

We provide considerable evidence that serine withdrawal exacerbates metformin-induced metabolic stress. A recent report demonstrated that metabolic stress is especially deleterious in cells with impaired cellular energy sensing. Indeed, *LKB1*-mutant tumours were highly sensitive to phenformin *in vivo* (Shackleford *et al.* 2013). Given that inactivating mutations in *LKB1* occur in many types of cancer; notably in 15% to 30% of non-small cell lung cancers, it is of great therapeutic relevance to make this gene an important variable in further *in vitro* and *in vivo* testing of combined biguanides and serine deficiency. We are optimistic that we will find *LKB1*-mutant tumors to be highly vulnerable to this strategic combination.

Given these promising avenues for future research, we remain hopeful that the work outlined in this thesis may, at least, improve our understanding of cancer metabolism and, at most, humbly contribute to the development of metabolism-specific antineoplastic therapies.

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APPENDIX

Contributions of Authors to Manuscript

Serine Deprivation Enhances Anti-neoplastic Activity of Biguanides

Nader Toban, Simon-Pierre Gravel, Laura Hulea, Elena Birman, Marie-José Blouin, Mahvash Zakikhani, Ivan Topisirovic, Julie St-Pierre, Michael Pollak

Nader Toban:
- Conceived study, designed and performed experiments, analysed data and wrote the manuscript.

Simon-Pierre Gravel:
- Designed and performed Stable isotope labeling and GC/MS experiments, contributed to design of other experiments; analysed data and edited manuscript.

Laura Hulea:
- Edited and contributed to manuscript.

Elena Birman:
- Carried out animal experiments.

Marie-José Blouin:
- Assisted with animal experiments and edited manuscript.

Mahvash Zakikhani:
- Performed statistical analyses, assisted with siRNA-mediated knockdown of PHGDH and edited manuscript.

Ivan Topisirovic:
- Contributed to conception of study and edited manuscript.

Julie St-Pierre:
- Contributed to conception of study, designed experiments and edited manuscript.

Michael Pollak:
- M.Sc. Supervisor and senior author: conceived and directed study; designed experiments and edited manuscript.