Effects of metformin and Ataxia Telangiectasia Mutated kinase inhibition on cellular energy metabolism

By

Shiva Javeshghani

Faculty of Graduate Studies

Department of Experimental Medicine

McGill University, Montreal

A thesis submitted to McGill in partial fulfillment of the requirements of the degree of Master of Science in Experimental Medicine

Abstract

The use of the biguanide metformin for the management of type 2 diabetes has been associated with reduced cancer burden and mortality by several population studies. Our previous work has demonstrated that metformin has growth inhibitory effects *in vitro* that are related to AMPK phosphorylation, leading to mTOR inhibition. Prior evidence linked the activation of AMPK to a decline in ATP as a result of the inhibitory effects of metformin on complex 1 of the mitochondria. In view of this finding, we studied the inhibitory effects of metformin on proliferation as a function of carbon source in several transformed cell lines. Our results demonstrated that the consequences of exposure to metformin vary as a function of carbon source. The greatest inhibition by metformin resulted in the presence of glutamine and absence of glucose. This finding together with prior evidence linking *myc* expression to "glutamine addiction", led us to examine the effects of varying *myc* level on metformin sensitivity. Our results revealed the overexpression of this oncogene to be associated with sensitization to the antiproliferative effects of metformin. This suggests that for a subset of neoplasms in which *myc* is overexpressed, biguanides may provide significant antiproliferative activity.

In view of preliminary evidence suggesting defective mitochondrial function in patients with Ataxia-Telangiectasia, we examined whether the ATM inhibitor KU55933 resulted in similar antiproliferative effects to those of metformin. Our results indicated that similar to metformin, KU-55933 exposure resulted in inhibition of proliferation. Furthermore, analogous to the effects of metformin on metabolism,

the compound also increased AMPK activation, glucose uptake, and lactate production, while reducing mitochondrial membrane potential and coupled respiration. Our observations suggest a role for ATM in mitochondrial function, and reveal that both KU-55933 and metformin perturb the TCA cycle and oxidative phosphoryaltion.

Résumé

L'utilisation de la metformine, un biguanide, pour la gestion de diabète de type 2, a été associée à une réduction du risque de cancer et de mortalité due au cancer par plusieurs études de population. Nos travaux antérieurs ont démontré que la metformine a des effets inhibiteurs de la croissance cellulaire in vitro qui sont liés à la phosphorylation d'AMPK, conduisant à une inhibition de mTOR. De plus, des études précédentes ont montré que l'activation de l'AMPK est liée à une baisse de l'ATP suite aux effets inhibiteurs de la metformine sur le complexe 1 de la mitochondrie. Compte tenu de ces résultats, nous avons étudié les effets inhibiteurs de la metformine sur la prolifération en fonction de la source de carbone dans plusieurs lignées cellulaires transformées. Nos résultats ont démontré que les conséquences de l'exposition à la metformine varient en fonction de la source de carbone. La plus grande inhibition par la metformine se produit en présence de glutamine et en l'absence de glucose. Ces résultats, conjointement avec des observations antérieures liant l'expression de myc à la «dépendance à la glutamine», nous a conduits à examiner les effets de la variation des niveaux de myc sur la sensibilité à la metformine. Nos résultats ont révélé que la surexpression de cet oncogène est associée à une sensibilisation aux effets anti-prolifératifs de la

metformine. Cela suggère que pour un sous-ensemble des néoplasmes dans lequel myc est surexprimé, les biguanides peuvent apporter une importante activité anti-prolifération.

Compte tenu des preuves préliminaires suggérant une fonction mitochondriale défectueuse chez les patients atteints d'ataxie télangiectasie, nous avons examiné si l'inhibiteur de l'ATM KU55933 produit des effets antiprolifératifs similaires à ceux de la metformine. Nos résultats indiquent que comme la metformine, l'exposition à KU-55933 a entraîné une inhibition de la prolifération cellulaire. En outre, KU-55933 provoque des effets analogues à ceux de la metformine sur le métabolisme : soit une augmentation de l'activation de l'AMPK, de l'absorption du glucose et de la production de lactate, tout en réduisant le potentiel de membrane mitochondriale et la respiration couplée. Nos observations suggèrent un rôle pour l'ATM dans la fonction mitochondriale et révèle que KU-55933 et la metformine peuvent perturber le cycle de Krebs et la phosphorylation oxydative.

TABLE OF CONTENTS

List of Abbreviations

AMPK, adenosine mono-phosphate activated kinase

mTOR, mammalian target of rapamycin

ATP, adenosine tri-phosphate

ATM, ataxia telangiectasia mutated

TCA, tricarboxylic acid

DNA, deoxyribonucleic acid

pCR, pathological complete response

ACF, aberrant crypt foci

OCT1, organic cation transporter 1

LKB1, liver kinase B-1

TSC2, tuberous sclerosis complex 2

4E-BPs, 4E-binding proteins

S6Ks, S6 kinases

LDH, lactate dehydrogenase

Acetyl-CoA, Acetyl coenzyme A

PDH, pyruvate dehydrogenase

NAD+, nicotinamide adenine dinucleotide

NADH, reduced form of NAD+

Rib-5-P, ribose-5-phosphate

FAD, flavin adenine dinucleotide

$FADH_2$, reduced form of FADH

NADPH, Reduced form of Nicotinamide adenine dinucleotide phosphate

GLS, glutaminase

EAA, essential amino acids

NMR, nuclear magnetic resonance

Acknowledgements

This work would not have been possible without the guidance and assistance of several individuals who in one way or another contributed and extended their valuable support in the preparation and completion of this study.

First and foremost, I would like to express my sincere gratitude to Dr. Michael Pollak, for supervising me through my graduate studies. He always offered valuable advice, encouraged me to present my work at various conferences, and to engage in collaborative work. A heartfelt thank you goes to Dr. Zakikhani who not only offered guidance, knowledge, and assistance but was a true mentor and inspiration. I would also like to thank my colleagues who supported me throughout my academic program. Finally I would like to thank Lilliane Lui for her truly kind gesture of helping me with my references.

I am extremely grateful to my partner, David Ricella, who ceaselessly provided love and support and instilled in me the motivation to follow my dreams. I would like to thank my parents Soheila Eghtedari and Danesh Javeshghani who provided me with the opportunity to have a excellent education. Above all else, I am grateful to God for hearing my prayers when I lost hope and for giving me the strength to continue.

Chapter I

Literature Review

Introduction

The anti-diabetic drug metformin has been the subject of numerous studies regarding antineoplastic activity both *in vitro* and *in vivo*. These studies were supported by several population based findings suggesting that diabetic patients treated with metformin had an approximately 40% decrease in cancer burden as compared to diabetic patients not using metformin for the management of their diabetes (Evans *et al*. 2005; Libby *et al*. 2009).

Some proposed mechanisms regarding the actions of metformin include activation of the cellular energy sensor AMPK (Hardie *et al*. 2006). This activation is a consequence of a decline in ATP levels induced by the inhibitory effects of metformin on respiratory complex 1 of the mitochondria thus resulting in reduced oxidative phosphorylation (El Mir *et al*. 2000; Hardie *et al*. 2006)). While many *in vitro* models have shown metformin having cell-autonomous antineoplastic activity, metformin also inhibits indirectly through the liver as a consequence of the lowering of insulin levels (Algire *et al*. 2008; Goodwin *et al*. 2008). This may lead to an antineoplastic effect in cases where the cancer is insulin responsive in hyperinsulinemic patients (Pollak 2012). In view of prior work identifying host insulin (Algire *et al*. 2011) and expression of transport molecules for metformin uptake (Segal *et al*. 2011) as candidate predictors of metformin sensitivity, we

examined carbon source and myc expression as potential predictive markers for the antineoplastic effects of metformin.

Ataxia-Telangiectasia Mutated protein is associated with cell-cycle control and DNA repair (Lavin 2008). Despite its localization in the nucleus and cytoplasm (Watters *et al*. 1997), experimental findings have supported a role for ATM in the mitochondria. Studies examining fibroblasts from patients with ataxia-telangiectasia reported defective mitochondrial function in these cells (Ambrose *et al*. 2007). With both ATM and metformin having a similar site of action, being the mitochondria, we compared the effects of the ATM inhibitor, KU-55933, on cellular energy metabolism to those metformin.

1.1: Metformin

1.1.1 History and Clinical Use in Diabetes

Metformin, a drug widely used to treat diabetes mellitus (estimated 120 million prescriptions per year worldwide (Foretz *et al*. 2010)) has a fascinating history that dates back to ancient Egypt and Medieval Europe Witters, 2001 3521 /id}(Bailey & Day 2004; Hadden 2005). The herb *Galega officinalis*, also known as the French Lilac was used to relieve both excessive urination (polyuria) and halitosis (a sweet odor on breath) which are both well known symptoms of diabetes. During that period no discoveries had been made concerning the active ingredient of this plant or the disease responsible for polyuria, diabetes mellitus. In the 1920's the biguanides metformin, phenformin, and buformin were synthesized and were found to be the active compound in the French Lilac. During the 1950's, clinical studies showed that metformin (*N*',*N*'-dimethylbiguanide) had an excellent therapeutic index for the management of diabetes. Finally, in 1958, the drug was approved in Europe, and eventually gained approval in Canada and the United States in 1972 and 1995 respectively. Phenformin however was removed from the clinic in the 1970's after a rare association with lactic acidosis had been seen in patients treated with the drug (Dowling *et al*. 2011).

Today, metformin has been used for over 50 years for the treatment of type II diabetes (Pollak 2010). In patients with non-insulin dependent diabetes mellitus, metformin decreases hyperglycemia by reducing hepatic gluconeogenesis and stimulating glucose uptake in muscle (Cusi *et al*. 1996; Hundal *et al*. 2000). In the clinic, metformin is commonly used in combination with other anti-diabetic drugs such as sulfonylureas. While both drugs are effective in reducing blood glucose levels, metformin, unlike sulfonylureas, does not cause weight gain induced by increased circulating insulin levels (Bailey & Turner 1996).

Epidemiological studies regarding metformin and cancer 1.1.2

Interest in the use of metformin in oncology was sparked by retrospective epidemiological studies on diabetic cancer patients. Observational studies have associated metformin with decreased cancer incidence and cancer related mortality in patients receiving standard doses (1500 to 2250 mg/day in adults) of the drug for the management of their diabetes (Ben, I *et al*. 2010; Decensi *et al*. 2010; Evans *et al*. 2005; Landman *et al*. 2010; Libby *et al*. 2009). Other studies aimed at examining the incidence of all forms of cancer in type 2 diabetic patients reported that exposure to metformin was associated with a significant reduction in the risk of cancer (Libby *et al*. 2009; Monami *et al*. 2009). Metformin was also associated with lower cancer related mortality compared to other standard therapies for diabetes (Bowker *et al*. 2006). Additional studies examining 2529 woman receiving neoadjuvant

chemotherapy for early stage breast cancer reported that diabetic patients with breast cancer receiving metformin and neoadjuvant chemotherapy had higher pathological complete response rates (pCR 24%) compared to diabetics not receiving metformin (pCR 8%) and non-diabetic patients not receiving metformin (pCR 16%) (Jiralerspong *et al*. 2009).

While some pharmacoepidemiological studies have suggested that metformin may have antineoplastic activity, others have not supported this view. For example, studies aiming to investigate the relationship between diabetes and metformin use with outcomes after radical prostatectomy reported that metformin use did not prove to be beneficial (Patel *et al*. 2010). Other studies also concluded that metformin does not reduce the risk of prostate cancer in patients with type 2 diabetes (Azoulay *et al*. 2011). A study examining the relationship between metformin use and survival outcomes in patients with triple-negative breast cancer also suggested that metformin use during adjuvant chemotherapy did not significantly impact survival outcome (Bayraktar *et al*. 2012). Finally some have suggested the presence of other variables that might modify the anti-neoplastic effects of metformin. These studies suggest that exposure to both a statin and metformin may be necessary for an antineoplastic effect to be observed (Lehman *et al*. 2012) or that administration of proton pump inhibitors may inhibit metformin uptake (Nies *et al*. 2011).

With the appearance of these inconsistencies, attention has been given to methodology, confounding factors, and to the possibility that in the case where a

reduced cancer burden is seen by metformin, it may be confined to certain subpopulations and/or certain types of cancer (Pollak 2012).

There is also evidence suggesting that the diagnosis of diabetes may concomitantly influence cancer detection, (Carstensen *et al*. 2012) and that the decision to use metformin (compared to other anti-diabetic drugs) for the management of diabetes is influenced by other clinical and metabolic factors determining cancer risk and prognosis. In this situation, metformin use may be associated with reduced cancer burden, but not responsible for it (Pollak 2012).

Ongoing clinical studies in cancer 1.1.3

In addition to retrospective studies examining the role of metformin in oncology, further prospective clinical studies have also been carried out in non-diabetic patients. A pilot study evaluating the chemopreventative effect of metformin on rectal aberrant crypt foci (ACF), provided preliminary evidence that low doses of metformin (250 mg/day) suppresses colonic epithelial formation and rectal ACF formation in humans suggesting a role for metformin in prevention of colorectal cancer (Hosono *et al*. 2010).

A randomized control trial examining the biological effects of metformin in operable breast cancer in non-diabetic women (n=55) compared serum and tissue biomarkers obtained at baseline and following metformin administration. A decline

in tumor cell proliferation in patients receiving metformin as measured by Ki-67 staining (Hadad *et al*. 2009) was observed. Another study, larger in number (n=200), also conducted in non-diabetic women with operable breast cancer, compared the change in Ki-67 staining between pretreatment biopsy and post-treatment surgical specimens. While metformin before surgery did not significantly affect Ki-67 staining overall, significantly different effects were noted according to insulin resistance. These studies suggest that the benefits of metformin may be confined to subpopulations of women with distinct tumor or host metabolic characteristics (Bonanni *et al*. 2012).

1.1.4 Laboratory studies and mechanism of action

Many investigators believe that the fundamental mechanism of biguanides may involve inhibition of oxidative phosphorylation (El Mir *et al*. 2000; FALCONE *et al*. 1962; Turner *et al*. 2008). In the case of metformin, this includes inhibition of respiratory complex 1 as shown by a study where metformin caused time-dependent inhibition of complex 1 in isolated mitochondria (Owen *et al*. 2000). While many studies have lead to a better understanding of complex 1 (Efremov & Sazanov 2011; Hirst 2010), no specific binding site on this complex has been proposed for biguanides (Pollak 2012). In addition, biguanides are not as toxic as other poisons disrupting oxidative phosphorylation such as cyanide (Pollak 2012). Some proposed mechanisms for this finding include biguanides requiring active transport into the mitochondria, and as increasing amounts are transported inside, mitochondrial

function is reduced to a greater degree leading to an equilibrium which limits the magnitude of their effect (Owen *et al*. 2000).

The mechanism of action of metformin in cancer involves both direct (insulin independent) effects and indirect (insulin dependent) effects (Dowling *et al*. 2011). The indirect effects of the drug primarily involve the liver because this organ is exposed to relatively high concentrations of the drug via the portal vein and because the cell surface transport molecule, organic cation transporter 1, is predominantly expressed in hepatocytes (Foretz *et al*. 2010; Pollak 2012). In the hepatocyte, metformin inhibits oxidative phosphorylation through its actions on respiratory complex 1 of the mitochondria (El Mir *et al*. 2000). As mitochondrial function is impaired, ATP levels decline and AMP levels rise, thereby leading to an activation of the cellular energy sensor AMPK (Hardie *et al*. 2006). The activation of AMPK downregulates anabolic processes such as gluconeogenesis in order to conserve ATP (Hardie *et al*. 2006). This in turn lowers hepatic glucose output and circulating glucose levels (in cases with high baseline circulating glucose levels), and as a result leads to a decline in circulating insulin levels. This may lead to an antineoplastic effect in cases where the cancer is insulin responsive in hyperinsulinemic patients (Pollak 2012). A study aiming to examine the effects of metformin on insulin reported metformin significantly lowered insulin levels by 22% in non-diabetic women with breast cancer (Goodwin *et al*. 2008), but it remains to be determined if this reduction is sufficient to reduce proliferation in the subset of cancers that are insulin dependent. *In vivo* models have also shown metformin suppressing the

stimulatory effects of obesity and hyperinsulinemia on lung tumor growth through lowering circulating insulin levels, reducing insulin signaling, and activating AMPK (Algire *et al*. 2008).

Many *in vitro* models have shown biguanides to have cell-autonomous antineoplastic activity in breast, prostate, colon, endometrial, ovarian, and glioma cell-lines (Ben Sahra *et al*. 2008; Buzzai *et al*. 2007; Cantrell *et al*. 2010; Dowling *et al*. 2007; Dowling *et al*. 2011; Gotlieb *et al*. 2008; Isakovic *et al*. 2007; Zakikhani *et al*. 2006). The first example was reported in 2006 where metformin was shown to act as a growth inhibitor, rather than an insulin sensitizer in breast cancer cells (Zakikhani *et al*. 2006). The direct, insulin-independent actions of metformin may be as a result of liver kinase B1 (LKB1) activation of AMPK and a reduction in mammalian target of rapamycin (mTOR) signaling leading to a decrease in protein synthesis of cancer cells (Dowling *et al*. 2011; Hardie 2007; Shaw *et al*. 2004). LKB1 and its substrate AMPK are intrinsically involved in maintaining cell growth and acting as energy sensors with changes in nutrient availability (Hardie *et al*. 2006). In conditions of energy stresss, when levels of AMP are high, AMPK reduces mTOR signaling via phosphorylation and activation of tumor suppressor tuberous sclerosis complex 2 (TSC2) which negatively regulates mTOR activity (Inoki *et al*. 2003). A number of different laboratory models have shown metformin to inhibit proliferation and protein synthesis via activation of the AMPK pathway leading to an inhibition in mTOR signaling, and consequently a reduction in phosphorylation of downstream effectors such as the eukaryotic initiation factor 4E-binding proteins

(4E-BPs) and ribosomal protein S6 kinases (S6Ks) (Alimova *et al*. 2009; Dowling *et al*. 2007; Dowling *et al*. 2011; Gotlieb *et al*. 2008; Zakikhani *et al*. 2006).

1.2: Glucose Metabolism in Cancer

1.2.1 The Warburg effect and glycolysis

The theory that cancer cells have defective energy metabolism, specifically in the mitochondria, was first proposed in 1921 by Otto Warburg. The "Warburg effect" supported the notion that tumor cells preferentially use glycolysis over oxidative phosphorylation by the mitochondria for ATP production even in the presence of ample oxygen (WARBURG 1956). This phenomenon is not only observed in cancer cells as many non-transformed cells also exhibit high aerobic glycolysis during proliferation (Lunt & Vander Heiden 2011). For example increased glucose uptake and lactate excretion has been observed in mitogen-stimulated normal human lymphocytes (Hedeskov 1968), mouse lymphocytes (Wang *et al*. 1976), and rat thymocytes (Brand 1985; Hume & Weidemann 1979).

The original observation of Warburg whereby cancer cells respired at lower rates compared to their non malignant counterparts led him to propose that "cancer cells are impaired in respiratory chain function and are very glycolytic"

{WARBURG, 1956 4421 /id. It is now clear that the driving force behind enhanced glycolysis in tumor cells is not an energy deficit caused by defective oxidative phosphorylation (Moreno-Sanchez *et al*. 2007; Zu & Guppy 2004), but rather the fact that glycolysis not only generates ATP but also provides via pentose phosphate pathway, many intermediates required for cell growth (Jones & Thompson 2009).

The switch to aerobic glycolysis is one of the important metabolic changes associated with proliferating tumor cells. Glucose serves as a critical nutrient for proliferating cells (Holley & Kiernan 1974; Pardee 1974). Pyruvate generated from glucose can be oxidatively metabolized to CO_2 in the TCA cycle producing large quantities of ATP through oxidative phosphorylation or it can reductively be metabolized to lactate (Lunt & Vander Heiden 2011).

The use of glycolysis to generate ATP instead of electron transport coupled oxidative phosphorylation appears to be a wasteful form of metabolism as the energetic yield per molecule of glucose is 2 ATPs with glycolysis and 36 ATPs with oxidative phosphorylation (Berg *et al*. 2007). However, when glucose is in excess and flux is high, glycolysis can produce ATP in greater quantities and at a faster rate (Guppy *et al*. 1993; Pfeiffer *et al*. 2001).

Pyruvate, the end product of glycolysis, can undergo three major pathways in mammalian cells: (a) converted into lactate via lactate dehydrogenase (LDH), (b) converted to alanine via alanine amino transferase, (c) converted into Acetyl-CoA in

the mitochondria via pyruvate dehydrogenase (PDH) (Lunt & Vander Heiden 2011). In highly proliferating cells, including cancer cells, the majority of the pyruvate (>90%) is turned into lactate via lactate deydrogenase (LDH-A) (Fantin *et al*. 2006). The reason high amounts of lactate and in some cases alanine are excreted from proliferating cells may be as a result of glycolytic flux rates exceeding that of maximal PDH activity leaving high concentrations of pyruvate to be excreted as lactate and alanine (Curi *et al*. 1988). In addition the conversion of pyruvate to lactate regenerates NAD+ which is required to maintain glycolysis as the conversion of glyceraldehyde-3-phosphate to 1,3-bisphosphate requires this metabolite (Fantin *et al*. 2006).

Glycolysis can also generate intermediates for cell growth and division such as nucleotides, amino acids, and lipids. The metabolism of glucose through the pentose phosphate pathway can generates ribose-5-phosphate (Rib-5-P) which is required for nucleotide biosynthesis. NADPH, also produced by the pentose phosphate pathway, serves as a key reducing equivalent for nucleotide and fatty acid synthesis. Intermediates in glycolysis such as 3-phosphoglycerate also supply carbon sources for amino acid and lipid synthesis (Jones & Thompson 2009; Vander Heiden *et al*. 2009). Therefore, a high glycolytic flux may be useful in maintaining precursors for biosynthesis for the rapidly proliferating tumor cell (Hume & Weidemann 1979; Vander Heiden *et al*. 2009).

Oxidative phosphorylation and requirements of proliferating cells 1.2.2

The remaining pyruvate that is not converted into lactate enters the mitochondria and is decarboxylated and oxidized by pyruvate dehydrogenase to acetyl-CoA (Jones & Thompson 2009). The acetyl-CoA then enters the TCA cycle where the two carbons are oxidized to CO_2. In addition, hydrogen atoms reduce NAD+ and FAD to NADH and $FADH_2$ which will be shuttled into complexes of the respiratory chain. The electron transport chain consists of four multimeric complexes (complexes I, II, III, and IV). NADH and $FADH_2$ reduce complex I and II respectively. Electrons flow down the respiratory chain (complex I to complex IV) and are finally transferred to molecular oxygen which is the final electron acceptor. This process is known as respiration. The transfer of electrons down the respiratory chain triggers the pumping of hydrogen ions across complexes I, III, IV which generates a proton gradient across the inner membrane of the mitochondria. This proton gradient is critical for the synthesis of ATP in a process known as oxidative phosphorylation. The reentry of the protons into the mitochondrial matrix through the proton channel of H^+-ATP synthase is the driving force behind phosphorylation of ADP into ATP. The complete oxidative of one glucose molecule can generate up to 36 ATPs (Ristow & Cuezva 2009; Srivastava & Moraes 2009).

The process of mitosis whereby a cell must replicate all of its cellular contents requires nucleotides, amino acids, and lipids. Glucose can generate biomass as well as produce ATP. While free energy provided by ATP hydrolysis is important for replication of cellular contents, additional requirements needs to be met. For

example, in order to synthesize paltimate, a major constituent of cellular membranes, in addition to 7 molecules of ATP, 8 molecules of acetyl-CoA and 14 molecules of NADPH are also required (Lehninger *et al*. 1993). One glucose molecule can generate up to 36 ATPs and 2 NADPHs or provide 6 carbons for synthesis of macromolecules. So while the ATP demands to produce paltimate are met in excess, the NADPH requirements are not fulfilled. Therefore for cells to meet the biosynthetic demands of proliferation, the bulk of the glucose cannot be used for ATP production (Vander Heiden *et al*. 2009).

1.3: Glutamine Metabolism in Cancer

1.3.1 Glutamine addiction

The fact that cancer cells have an increased dependence on glucose for growth and proliferation has been known for a long time. In fact the active utilization of glucose by cancer cells is what constitutes the basis for ^{18}fluoro-deoxy-glucose-positron emission tomography (Fowler & Ido 2002; Gambhir 2002). However glutamine, despite being classified as a non-essential amino acid, is a key ingredient in culture medium supporting cancer cell growth. In 1955, Harry Eagle, while studying the nutritional requirements of cell lines growing in vitro, observed that the rate of glutamine consumption of many cell lines exceeded that of any other amino acid by 10 fold (EAGLE 1955). He observed that not only could many of the cell lines not proliferate in the absence of exogenous glutamine, but they were unable to

remain viable. Interestingly, the glutamine requirement could not be met by supplying the cells with glutamic acid, a byproduct of glutamine metabolism. Subsequently, in 1971, glutamine was discovered as a substrate for respiration in isolated mitochondria (Kovacevic 1971).

This amino acid is abundant in serum and serves many functions including its intracellular conversion to glutamate, an intermediate that is consequently converted to α-ketoglutarate used in the TCA cycle. Glutamine also serves as a precursor for the biosynthesis of nucleic acids, some amino acids, and glutathione. The enzyme glutaminase (GLS), found in the mitochondria, is responsible for the conversion of glutamine to glutamate (DeBerardinis & Cheng 2010; Wise & Thompson 2010). Increased expression of this enzyme has been reported in tumors and rapidly dividing cells. It has been reported that c-Myc, an oncogenic transcription factor known to stimulate cell proliferation, increases expression of mitochondrial glutaminase in human P-493 B lymphoma cells and PC3 prostate cancer cells (Gao *et al*. 2009). Glutamine's role as a nitrogen donor in nucleotide and amino acid biosynthesis is not the sole reason some cancer cells appear to be glutamine dependent. Glutamine also plays a role in the uptake of essential amino acids and activation of mTOR. In addition to its role as a key mitochondrial substrate, glutamine also maintains mitochondrial membrane potential and integrity and contributes to NADPH production required for redox potential (Wise & Thompson 2010).

1.3.2 Regulation of glutamine metabolism by c-Myc

As mentioned previously, glutamine plays a critical role in nucleotide biosynthesis as it can act as a nitrogen donor. For example, five enzymatic reactions involved in the synthesis of purines and pyrimidines use glutamine as a source for nitrogen. Three of the five reactions including PRPP amidotransferase, carbamoyl phosphate synthetase II, CTP synthetase are directly regulated by the transcription factor c-Myc (Bush *et al*. 1998; Wise & Thompson 2010). In addition, multiple experimental systems have shown that the transcription factor, c-Myc, transactivates eleven genes involved in nucleotide synthesis (Liu *et al*. 2009; Wise & Thompson 2010).

Oncogenic levels of this transcription factor have been linked to increased glutaminolysis (Gao *et al*. 2009; Wise *et al*. 2008; Yuneva *et al*. 2007). In addition to promoting the metabolism of glutamine into glutamate (Wise *et al*. 2008) Myc also activates at the level of transcription two high affinity glutamine transporters namely SLC38A5 and SLC1A5 (DeBerardinis *et al*. 2007; Nicklin *et al*. 2009). Studies have suggested that Myc-overexpressing cells have an enhanced ability to convert glutamine into glutamate through an overexpression of the enzyme glutaminase thereby providing the cell with a large pool of carbon for anaplerosis and NADPH production (Gao *et al*. 2009).

The induction of Myc triggers a reprogramming in the cell making it dependent on exogenous glutamine. In fact depletion of glutamine, and not glucose, induced apoptosis depending on Myc activity (Yuneva *et al*. 2007). Interestingly glutamine depletion in Myc transformed cells causes a significant decrease in TCA cycle metabolites, despite glucose availability, which supports a role for glutamine in mitochondrial anaplerosis (Yuneva *et al*. 2007).

1.3.3 Glutamine activation of mTOR

While glutamine is a key ingredient in amino acid biosynthesis and protein translation, it also supports activation of mammalian target of rapamycin 1 (mTORC1) (Nicklin *et al*. 2009). mTORC1 is a key activator of protein translation and inhibits autophagy in response to abundant amino acid levels (Wullschleger *et al*. 2006). While the most important amino acid responsible for mTOR activation is leucine, glutamine is also necessary as the cellular uptake of glutamine and its subsequent rapid efflux in the presence of essential amino acids (EAA) is the rate-limiting step that activates mTOR. Evidence provided by Nicklin et al. demonstrates that glutamine may not necessarily support protein translation, but is instead shuttled out of the cell through a bidirectional transport mechanism in exchange for essential amino acids that directly activate mTOR (Nicklin *et al*. 2009).

1.3.4 Glutamine metabolism in the mitochondria

The primary site of action of glutamine is the mitochondria. Before glutamine can enter the tricarboxylic acid cycle (TCA) cycle it must first be converted into glutamate through the loss of an amide group and then to α-ketoglutarate through the loss of an amine group (Wise & Thompson 2010). Studies using ^{13}C NMR spectroscopy in glioblastoma cells exhibiting high rates of aerobic glycolysis have shown that the TCA cycle activity in these cells is used to support the efflux of substrates for use in biosynthetic pathways. High rates of glutamine metabolism were shown to sustain NADPH levels as well as maintaining oxaloacetate pools for continued function of the TCA cycle (anaplerosis) (DeBerardinis *et al*. 2007). Oxaloacetate and citrate can condense to from citrate which can donate acetyl-CoA groups for cholesterol and fatty acid synthesis (Hatzivassiliou *et al*. 2005). The process of anaplerosis whereby the mitochondrial carbon pool is replenished by glutamine allows for the integrity of the mitochondrial membrane potential, as well as lipid, protein, and nucleotide synthesis (DeBerardinis *et al*. 2007). However in non-transformed, non-proliferating cells including liver, kidney, muscle and brain, glucose and not glutamine is thought to support anaplerosis through the actions of pyruvate carboxylase (Gibala *et al*. 2000; Hasan *et al*. 2008; Hassel 2000; Owen *et al*. 2002).

1.4: Ataxia-telangiectasia Mutated Protein (ATM)

Role of ATM in DNA repair 1.4.1

Ataxia-telangiectasia (A-T), an autosomal recessive human disorder, is associated with predisposition to cancer, hyperglycemia, and other abnormalities (Chenevix-Trench *et al*. 2002; Miles *et al*. 2007). Studies have mapped this syndrome to a specific gene, Ataxia Telangiectasia Mutated (ATM). The ATM gene product is involved in cellular responses to DNA damage and cell cycle checkpoints (Enoch & Norbury 1995).

The Mre11-Rad50-Nbs1 (MRN) complex senses double strand breaks and recruits ATM to the site of DNA damage (Lee & Paull 2005). Ataxia-telangiectasia mutated protein is activated as a result in an attempt to initiate the phosphoryation of intermediates involved in cell-cycle control and DNA repair checkpoints such as p53 (Bakkenist & Kastan 2003). While the mechanism of activation of ATM remains incompletely described, studies have shown that autophosphorylation may play a significant role. These studies have demonstrated that ATP can specifically induce activation of ATM and not other DNA dependent protein kinases (Kozlov *et al*. 2003). Other studies show that ATM is held inactive in unirradiated cells as a dimer with the kinase domain bound to a region contiguous to serine 1981. When the cell is

irradiated, autophosphorylation at the serine 1981 region causes dissociation of the ATM dimer and initiation of ATM kinase activity (Bakkenist & Kastan 2003).

Studies have revealed the ATM protein to be constitutively expressed in the nucleus and the level and localization of this protein remaining constant throughout all stages of the cell-cycle, consistent with the role of ATM in DNA repair (Brown *et al*. 1997). Subcellular fractionation, immunoelectronmicroscopy, and immunofluorescence have targeted the ATM protein to the nucleus and cytoplasmic vesicles (Watters *et al*. 1997). The role of ATM in the cytoplasm may involve binding to β-adaptin, one of the components of the AP-2 adaptor complex, which is involved in clathrin-mediated endocytosis of receptors (Lim *et al*. 1998). In addition, the absence of cytoplasmic ATM protein in neurons has led to an increase in lysosomal number, suggesting a role against abnormalities of cytoplasmic organelles (Barlow *et al*. 2000). It has been demonstrated that ATM levels are not regulated in response to UV or ionizing radiation, and that it plays an essential role as a component of the DNA damage response and not that of a downstream effector (Lakin *et al*. 1996).

1.4.2 Mitochondrial function in Ataxia-Telangiectasia

Studies conducted by Ambrose and colleagues have revealed findings supporting the role of ATM in the mitochondria (Ambrose *et al*. 2007). Firstly, through the use of mitochondrial specific stains examining the differences between

wild-type and A-T lymphoblastoid cells, they demonstrated poor staining and polarization of mitochondria to one end of the cell in A-T cells. A reduction in mitochondrial respiration and oxidation rate was noted in A-T cells. The percentage of mitochondria with decreased membrane potential was increased in A-T cells. Furthermore, the expression level of an integral outer mitochondrial membrane electron carrier, CYB5B, was significantly declined in A-T cells. Induced expression of ATM in stably transfected A-T cells restored respiration rates back to levels seen in wild-type suggesting the possible role of ATM in mitochondrial function (Ambrose *et al*. 2007). Alterations in redox state as noted by increased levels in reactive oxygen species were also found in ATM-deficient mice suggesting the absence of functional ATM leading to oxidative stress (Kamsler *et al*. 2001).

1.4.3 Small molecule kinase inhibitor KU-55933

Early studies regarding the nature of defect in individuals with Ataxia-Telangiectasia revealed that A-T cells irradiated in the G1 or G0 phase appear to have an increased frequency of residual chromosome fragments that appear in mitosis. Therefore while the initial frequency of breaks and rejoining of breaks appeared to be similar in normal and A-T cells the fraction of breaks that did not rejoin was five to six times greater in A-T cells (Cornforth & Bedford 1985). This observation along with a slower capacity in double strand break rejoining

(Coquerelle *et al*. 1987) and a deficiency in the repair of DNA double strand break (Foray *et al*. 1997) seen in A-T cells provided insight into the increased radiosensitivity of A-T cells to ionizing radiation. These findings gave rise to the hypothesis that ATM might be a useful strategy for radiosensitization (Hickson *et al*. 2004).

The screening of a small molecule library of compounds for the phosphatidyl-inositol kinase family of proteins, led to the discovery of the ATP competitive inhibitor, 2-morpholin-4-yl-6-thianthren-1-yl-pyran-4-one (KU55933). KU-55933 is a specific inhibitor of the kinase activity of ATM as demonstrated by an absence of phosphorylation in a wide range of downstream effectors including p53 when exposed to ionizing radiation (Hickson *et al*. 2004). Cells exposed to KU-55933 were also sensitized to the cytotoxic effects of ionizing radiation and did not display an ionization induced cell cycle arrest. The drug candidate, however, had no effect on potentiating the cytotoxic effects of ionizing radiation in A-T cells (Hickson *et al*. 2004). This drug candidate, as an inhibitor of ATM, may enforce efficacies of radiation therapy by inhibiting DNA repair in radiation-damaged cancer cells; however, these are concerns that the inhibition of DNA repair in normal cells could increase cancer risk. In our work described in "Chapter 3", we use this compound as a research reagent to study the role of ATM in cellular energy metabolism.

Chapter II

Manuscript A

Cancer Research

(Pending Revision)

Carbon source and *myc* expression influence the antiproliferative actions of metformin

Shiva Javeshghani[4], Mahvash Zakikhani[1,2], Shane Austin[3,5], Miguel Bazile[2], Marie-José Blouin[1,2], Julie St-Pierre[3,5], Michael N. Pollak[1,2,3,4*]

[1] Department of Oncology, [2] Segal Cancer Centre, [3] Goodman Cancer Centre, [4] Division of Experimental Medicine, [5] Department of Biochemistry, McGill University, Montreal, Quebec, Canada.

Running title: Metformin, carbon source, and *myc*

Keywords: Metabolism, glutamine, metformin, biguanide, respiration, glucose

This work was supported by a grant (00921-000) from the Canadian Institutes of Health Research in partnership with The Terry Fox Foundation.

* Corresponding author:

Cancer Prevention Center, Jewish General Hospital,
3755 Cote Ste-Catherine, Montreal, Quebec, Canada, H3T 1E2
E-mail: michael.pollak@mcgill.ca
Phone: 514-340-8222 ext 4139
Fax: 514-340-8600
The authors disclose no potential conflicts of interest.

Word count: 4245

Number of figures: 7

Abstract

Epidemiologic and experimental data have led to increased interest in possible roles of biguanides in cancer prevention and/or treatment. Prior studies suggest that the primary action of metformin is inhibition of oxidative phosphorylation, resulting in reduced mitochondrial ATP production and activation of AMPK. *In vitro,* this leads to AMPK-dependent growth inhibition if AMPK and its effector pathways are intact, or to an energetic crisis if these are defective. We now demonstrate that the effect of exposure of several transformed cell lines to metformin varies with carbon source: in the presence of glutamine and absence of glucose, a 75% decrease in cellular ATP and an 80% decrease in cell number are typical; in contrast, when glucose is available, metformin exposure leads to increased glucose uptake and increased lactate production, indicating increased glycolysis, with only a modest reduction in ATP level and cell number. As *myc* expression has been associated with "glutamine addiction", we examined the effects of varying *myc* level on metformin sensitivity. Overexpression of this oncogene was associated with sensitization to the antiproliferative effects of metformin. Our results reveal previously unrecognized factors that influence metformin sensitivity and suggest that metformin-induced increase in glycolysis attenuates the antiproliferative effects of the compound.

Précis

There is intense interest in the hypothesis that the antidiabetic drug metformin may have utility as an antineoplastic agent; our research provides new insights into factors that define subsets of tumors that may be treated with this agent, and provides novel details concerning mechanism.

Introduction

Metformin is a partial inhibitor of complex I of the mitochondrial electron transport chain (1,2). By decreasing mitochondrial ATP production, metformin activates the AMP-activated protein kinase signaling pathway, a key regulator of cellular energy homeostasis (3). When this pathway is activated, energy-consuming processes such as protein synthesis and fatty acid synthesis are down regulated, which tends to lessen energetic stress but limits anabolic metabolism and proliferation (4-9). In cells with defective AMPK signaling or effector pathways, metformin exposure decreases oxidative phosphorylation without a compensatory decrease in energy expenditure, leading to an energetic crisis (10,11).

Metformin is widely used in the treatment of type II diabetes (12). In the special case of hepatocytes, the energetic stress leads to down-regulation of gluconeogenesis (13,14), which represents energy export from the liver as glucose. This, in turn, reduces the hyperglycemia of type II diabetes, with secondary reduction in the hyperinsulinemia seen in this condition (15,15). These systemic actions may contribute to the antineoplastic effects of metformin seen in some *in vivo* models (10,16). Cell autonomous actions, such as those described below, may also play critical roles in antineoplastic actions of biguanides, provided adequate drug concentrations are achieved *in vivo.*

In addition to glucose, glutamine is a major metabolic substrate for cancer cells, providing not only a carbon source for generating energy, but also precursors

for the synthesis of nucleic acids, proteins and lipids by replenishment of TCA cycle intermediates via anapleurosis (17,18). Oncogenic levels of *myc* induce a transcriptional program that increases glutamine consumption and reliance on glutamine as a bioenergetic substrate (19,20).

Some pharmacoepidemiologic studies have suggested that metformin may have antineoplastic activity (21-27) but others have not supported this view (28-31). Similarly, some (for example (10)) but not all (for example (32)) laboratory studies suggest that metformin has antineoplastic activity. It is possible that these inconsistencies may result from unrecognized genetic and/or metabolic characteristics of the tumor or the host that determine metformin sensitivity. Prior work has identified host insulin levels (10) and expression of transport molecules for metformin uptake (33) as candidate predictors of metformin sensitivity. Here we examined carbon source and *myc* expression as candidate predictive markers for antineoplastic activity of metformin.

Materials and Methods

Chemicals. Cell culture materials were obtained from Invitrogen (Burlington, ON, Canada). Metformin (1, 1-Dimethylbiguanide hydrchloride), glucose, oligomycin, and myxothiazol were purchased from Sigma-Aldrich (Oakville, ON, Canada). L-glutamine, dialyzed FBS and Dulbecco's modified Eagle's medium without D-glucose, sodium pyruvate & L-glutamine were purchased from Wisent. HEPES was purchased from EMD chemicals. Anti-phospho-mTOR (Ser ^{2448}), anti-phospho-p70S6K (S6K1) (Thr ^{389}), and anti-ß-actin were purchased from Cell Signaling

Technology (Beverly, MA). Horseradish peroxidase-conjugated anti-rabbit IgG, anti-mouse IgG, and enhanced chemiluminescene (ECL) reagents were from Pharmacia-Amersham (Baie d'Urfé, QC, Canada).

Cell lines and culture conditions. MC38 colon carcinoma, a mouse tumor cell line derived from a C57BL/6 mouse was generously donated by Dr Pnina Brodt. TGR1 rat fibroblasts, isogenic HO15.19 cells with targeted disruption of both alleles of *c-Myc*, and HOmyc cells in which c-Myc expression has been restored in the knockout cells (34) were kindly provided by Dr. Sedivy (Brown University, RI, USA) and maintained with DMEM supplemented with 10% FBS and 100 units/ml gentamycin at 37^{o} C and 5% CO_2. The mouse embryonic fibroblasts were generously donated by Dr. Miller and maintained with DMEM supplemented with 10% FBS and 100 units/ml gentamycin at 37^{o} C and 5% CO_2. The cell line P493-6 (generously donated by Dr. Dang) was established by stable transfection of EREB2-5 cells with the construct p*myc*-tet (35,36). Cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 units/ml gentamycin, 2 mM L-glutamine (Wisent). For repression of *myc*, 0.1 μg/ml tetracycline was added to culture medium. In order to reinduce *myc*, cells were washed three times with tetracycline-free, prewarmed phosphate-buffered saline (PBS) containing 10% FBS (37). Uninduced cells (0 h), which were arrested by tetracycline for 72 h, served as a control. The control cells were washed similarly to the induced cells, except that tetracycline was present in all washing solutions. All experiments were performed in the absence of EBNA2 function (without estrogen) (36).

Cell Treatments. Cells were plated in 96-well plate (3500 cells/well), 12-well plate (10^5 cells/well), 6-well plate (3×10^5 cells/well), or in 10-cm petri dish (1.5×10^6 cells) for 24 h. Medium was changed for fresh medium containing either glucose or glutamine (20mM or 5mM) or both in the absence or presence of metformin (5mM) for another 12, 48, or 72 hours depending on the experiment. After treatment, supernatant was collected and stored at −80 °C until use and cells were trypsinized and counted (Trypan blue exclusion) for extraction or colorimetric assays. Other treatments included increasing concentrations of carbon source with or without metformin. All experiments were performed at least twice and each condition was in triplicates or four replicates.

Cell proliferation assay. We used the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma Chemical) assay to determine the effects of various carbon sources and metformin on cell growth (cell viability). After appropriate treatment time (48 h), MTT was added to a final concentration of 1 mg/ml, and the reaction mixture was incubated for 3 h at 37 °C. The resulting crystals were dissolved in 0.04% HCl in isopropanol and the absorbance was read at 562 nm. Triplicates were used for each treatment, and each experiment was repeated twice. Cell proliferation in presence of various concentrations of glucose and glutamine was also evaluated by 0.4% Trypan blue exclusion cell counting. Duplicates were used for each condition and the experiment was repeated three times (38). A Bromodeoxuridine Assay Kit (Calbiochem) was also used to assess

proliferation. The kit was used as per the manufacturer's instructions, with 2×10^5 cells per well.

ATP measurement. Cellular ATP levels were measured using the Invitrogen ATP Determination Kit A22066, (Invitrogen, Burlington, ON, Canada). Cells were treated in 5% FBS DMEM in the absence or presence metformin and under various substrate conditions, for 48 hrs. The kit was used as per the manufacturer's instructions, with 2 x 10^5 cells per well. Measurements were done in triplicate.

Measurements of glucose consumption. Cells were cultured in complete medium with 10% FBS. After 24 h, the complete medium was replaced with test medium in the absence or presence of metformin. Cells were incubated for 48 hrs and the culture medium was then collected and analyzed for measurement of glucose and lactate concentrations using colorimetric kits according to manufacturer's instructions. Glucose levels were determined using a Glucose assay kit (Eton Bioscience, Inc**.**, Cambridge, MA). Results were indexed to cell-free media and to the number of cells.

Lactate production assay. Lactate levels were determined in 10µl culture medium collected from treated cells and results were standardized with the number of cells. Lactate was calculated using a Lactate Kit (BioVision, Inc., San Francisco, CA).

Cellular respiration. Cells were rinsed, trypsinized and spun twice at 1200 rpm for 5 min, the final pellet was resuspended in Dulbecco's Modified Eagle's Medium, 5%

dialyzed FBS, 25mM HEPES with either 20mM glucose or glutamine. Oxygen consumption was measured by a Clarke type electrode (Rank Brothers, Cambridge, UK) using 4 $x10^6$ cells/ml suspended in respiration medium at 37 °C. Total respiration was assessed without the presence of any inhibitors, while proton leak was measured using the ATP synthase inhibitor oligomycin (2.5 μg/ 1 x 10^6 cells) and non-mitochondrial respiration measured using myxothiazol (12 μg/ 1 x 10^6 cells).

NAD+/NADH Quantification. Cellular NAD+ and NADH levels were measured using the NAD+/NADH Quantification Kit, (BioVision, CA, USA). Cells were treated in 5% FBS DMEM in the absence or presence of metformin and under various substrate conditions, for 48 hrs. The kit was used as per the manufacturer's instructions, with $2x10^5$ cells per well. Measurements were done in triplicate.

Protein extraction and western blot analysis. Cells were washed three times with ice-cold phosphate-buffered saline (PBS) and lysed in 100-400μl lysis buffer (20 mM Tris HCl (pH 7.5)), 150 mM NaCL, 2.5 mM sodium pyrophosphate, 1 mM ß-glycerol phosphate, 1 mM Na_3VO_4, 1 mM EGTA, 1% Triton, and Complete Protease Inhibitor Cocktail Tablet from Roche Diagnostic (Laval, QC, Canada). Cell debris was removed by centrifugation at 14,000X rpm for 20 minutes at 4 ºC. Following the assay for total protein (Bio-Rad, Mississauga, ON, Canada), clarified protein lysates from each experimental condition (40-50 μg) were boiled for 5 minutes and subjected to electrophoresis in denaturing 10% SDS-PAGE. Separated proteins were

transferred to a nitrocellulose membrane and after blocking, the membranes were probed with antibodies of interest. In some cases, developed blots were stripped in stripping buffer (62mM Tris HCL (pH 6.8), 100mM ß-mercaptoethanol, 2% SDS) to confirm equal protein loading. Horseradish peroxidase-conjugated anti-rabbit IgG and anti-mouse IgG were used as secondary antibodies. The position of proteins was visualized using the enhanced chemiluminescene reagent ECL.

Statistical Analysis. Data are presented as means ± s.e.m. The distribution of variables was tested for normality. The significance of differences between paired or unpaired sets of values was assessed using GLM or Mixed Procedures. Two-way and one-way analysis of variance (ANOVA) was used to determine the effects of different variables. Additionally, Least-squares means post hoc for multiple unpaired comparisons of means (LSMEANS statement with Bonferroni correction) was applied. All statistical analyses were performed using Statistical Analysis System software, version 9.2 (SAS Institute, Cary, NC), with P values ≤ 0.05 considered significant.

Results and discussion

Carbon source influences the antiproliferative effect of metformin. Figure 1a shows proliferation of MC38 colon carcinoma cells grown under increasing concentrations of glucose-only, glutamine-only, and both glucose and glutamine. Proliferation was considerably higher when media contained glutamine (20 mM) as compared to growth under the glucose-only (20mM) condition (proliferation in

glutamine, 0.71 ± 0.04 AU vs. proliferation in glucose, 0.22 ± 0.08 AU, $P<0.0001$) **(Fig 1a)**. Following 48 hour exposure, we observed that metformin (5mM) had no significant inhibitory activity for cells growing in glucose, but inhibited cells using glutamine as a carbon source (inhibition by meformin in glucose containing media, 7% vs. inhibition by metformin in glutamine-only media, 78%, $P<0.0001$) **(Fig 1b)**. Furthermore, increasing concentrations of glutamine in the absence of glucose did not diminish the inhibitory effects of metformin as shown in **Figure 1b**. When both glucose and glutamine were present, the inhibitory activity of metformin was intermediate between the glucose-only and glutamine-only conditions, but increasing glucose concentrations did attenuate the metformin effect (inhibition by meformin in glutamine containing media, 78% vs. inhibition by metformin in glutamine and glucose containing media, 20%, $P<0.0007$).

To confirm these results, which were determined by MTT assay, we counted Trypan blue excluding cells **(Figure 1c)**, and the same trends were observed (inhibition by meformin in glucose containing media 20mM, 12% vs. inhibition by metformin in glutamine containing media 20mM, 89%, $P<0.0001$). Furthermore, similar effects of metformin were seen with the Trypan blue exclusion assay when glucose and glutamine were provided at 5 mM (Results not shown). In order to further confirm and establish the generality of this observation, Mouse Embryonic Fibroblasts were studied using Brdu labeling **(Figure 1d)**. Again, metformin was a better growth inhibitor in glutamine containing media than glucose containing media not only in terms of absolute decrease in viable cells, but also in terms of percentage

growth inhibition relative to control (inhibition by meformin in glucose containing media 20mM, 0% vs. inhibition by metformin in glutamine-only media 20mM, 94%, $P<0.0001$).

Effect of metformin on mTOR signaling as a function of carbon source. Our previous findings demonstrated that *in vitro*, metformin, like rapamycin, decreases mTOR phosphorylation and prevents phosphorylation of S6K1 (8,10,39), consistent with the previously described role of AMPK activation as an inhibitor of mTOR (7). In our experimental system, glutamine availability was associated with increased mTOR activation, as expected (40). While metformin attenuated mTOR activity when cells were grown on glucose alone or both nutrients to a slight degree, it drastically repressed mTOR activity when cells grown on glutamine. Similarly, metformin inhibited phosphorylation of S6 kinase, a downstream effector of mTOR, in cells provided with glutamine, as shown in **Figure 2**.

Effect of metformin on ATP level, glucose consumption, lactate production, and NAD+/NADH ratio as a function of carbon source. Having established that metformin is a more effective growth inhibitor when cells utilize glutamine rather than glucose as a fuel, we examined additional metabolic end points. **Figure 3a** shows a significant drop (~75%) in ATP level when cells in glutamine-only media were exposed to metformin, while a metformin-induced decrease in ATP level was not observed when glucose was available. As metformin inhibits mitochondrial ATP production (2), this is in keeping with the fact that the utilization of glutamine to

generate ATP is more dependent on functional mitochondria than utilization of glucose, because glucose can provide ATP via glycolysis or oxidative phosphorylation, but generation of ATP from glutamine requires the TCA cycle and oxidative phosphorylation.

Glucose consumption increased (P<0.0001) and lactate production increased (P<0.0001) **(Figure 3b and 3c)** when cells with access to glucose were exposed to metformin in the presence or absence of glutamine. This is consistent with a decrease in oxidative phosphorylation and an increase in glycolysis. Interestingly, when both glutamine and glucose are available, in either the presence or absence of metformin, cells consume less glucose than in the glucose-only condition (glucose consumption with both glutamine and glucose, 8.59 ± 0.02 μmoles/10^6 cells/48 hours vs. the glucose alone condition, 18.55 ± 0.11 μmoles/10^6 cells/48 hours, P<0.0002; both glutamine and glucose in the presence of metformin, 22.14 ± 0.05 μmoles/10^6 cells/48 hours vs. glucose alone in the presence of metformin, 31.83 ± 0.25 μmoles/10^6 cells/48 hours, P<0.0001), consistent with a contribution of glutamine to ATP generation via anapleurosis.

Figure 3c shows higher lactate production in the presence or absence of metformin when glutamine is excluded from media, consistent with increased glycolysis under such conditions. Metformin exposure was associated with increased lactate production in both glucose-only and glucose plus glutamine media. Maximal lactate production was seen in the absence of glutamine and the presence of

metformin. Lactate was not detected when cells were provided with glutamine in either the presence or absence of metformin, indicating that in these cells glutamine is not converted into pyruvate and then to malate in order to produce lactate.

When both glutamine and glucose are present, lactate production is substantially less than in the glucose-only condition. This is observed in both the presence or absence of metformin (lactate production in media with both glucose and glutamine, 10.08 ± 0.08 μmoles/10^6 cells/48 hours vs. glucose-only media, 39.24 ± 2.78 μmoles/10^6 cells/48 hours, P=0.0003; media with both glucose and glutamine in the presence of metformin, 24.34 ± 0.42 μmoles/10^6 cells/48 hours vs. glucose-only media in the presence of metformin, 56.96 ± 0.61 μmoles/10^6 cells/48 hours, P= 0.0005). This provides further evidence that glutamine availability leads to reduced glycolysis.

A metformin-induced decline in the NAD+/NADH ratio is only observed in the absence of glucose (41.06% percent decline in NAD+/NADH ratio in the glutamine-only condition, P=0.0045 vs. a 0% decline in conditions where glucose was available) **(Figure 3d)**. This is expected because we observe that in the absence of glucose, no lactic acid is produced, indicating minimal reductive metabolism of pyruvate to lactate by LDH which would result in reduced generation of NAD+.

Myc overexpression leads to sensitization to metformin. In the context of prior evidence that glutamine influx and metabolism are increased by *myc* (41) and our

observation that glutamine utilization sensitizes cells to metformin, we examined the influence of *myc* on metformin action. We used TGR-1 Rat1 fibroblasts expressing physiological levels of *myc* (designated Myc +/+), the isogenic *myc* null HO15.19 cell line (designated Myc -/-), and the isogenic HOmyc cell line which overexpressed *myc* (designated Myc +++). The highest proliferation was seen in the Myc +++ cell line using glutamine as a carbon source, consistent with evidence that oncogenic levels of *myc* lead to glutamine addiction (40) (**Figure 4a**).

We observed that as *myc* levels decrease, proliferation when glutamine was the carbon source also declined (Myc+++, $1.50x10^6$ vs. Myc+/+, $0.97x10^6$, P= 0.0049), whereas *myc* level had no effect on proliferation when glucose was the carbon source. While *myc* has been reported to increase glycolysis and LDH expression (42) as well as glutamine utilization (40), this observation suggests that functionally, the effect of *myc* on glutamine utilization is dominant.

In order to better understand the relation between *myc* expression and metformin inhibition, a more physiologic replete media consisting of 5mM glucose and 5mM glutamine was used and *myc* expression was varied **(Figure 4b)**. BrdU incorporation measurements revealed that even in media where both glucose and glutamine are available, metformin reduced proliferation to the greatest degree when *myc* was overexpressed (% inhibition for Myc+++ cells, 62%, P<0.0001, % inhibition for Myc+/+ cells, 14% P= NS). The fact that metformin decreased proliferation in *myc* overexpressing cells growing in replete media and glutamine-

only media but not in the glucose-only media suggests that when *myc* is overexpressed, the inhibitory effects of metformin mostly involve glutamine utilization.

Effect of metformin on glucose consumption and lactate production as a function of carbon source availability and *myc* expression. Previous reports have shown that c-*myc* upregulates the expression of glucose transporter 1 (GLUT1), phosphofructokinase, and enolase A as well as directly regulating LDH-A (35,37,43,44). We therefore carried out experiments to determine if varying *myc* expression influences the effects of metformin on glucose consumption and lactate production. **Figure 5a** shows that metformin increases glucose consumption regardless of *myc* expression level when cells were grown in media without glutamine, and that the amount of glucose consumed on metformin exposure varied significantly with *myc* expression level (glucose consumed in glucose-only media on metformin exposure for Myc +++ cells, 27.43 μmoles/10^6 cells/48 hours vs. for Myc+/+ cells, 10.54 μmoles/10^6 cells/48 hours vs. for Myc-/- cells, 2.67 μmoles/10^6 cells/48 hours; $P<0.0001$ for all comparisons). M*yc* expression was associated with both higher fold increase in glucose consumption (~ 3 fold) and higher absolute glucose consumption levels on metformin exposure as compared to no *myc* expression (~ 1.5 fold). Similar trends were seen in the amount of lactate produced **(Figure 5b)**. The high amount of lactate released in conditions where glucose was the only carbon source available indicates that a considerable amount of pyruvate

generated from glucose through glycolysis does not support oxidative phosphorylation but is instead reduced to lactate.

In cell lines expressing *myc*, in the presence of both glucose and glutamine, the increase in glucose consumption with metformin exposure was not as marked as in the absence of glutamine (% increase in glucose consumed with metformin exposure in Myc+/+: Both 20mM, 69% vs. glucose 20mM, 300%) in keeping with some residual glutamine utilization for ATP production despite the presence of metformin.

Effect of metformin on ATP level and oxygen consumption as a function of *myc* expression and carbon source. We observed higher baseline ATP concentrations when cells were grown on glutamine as compared to glucose, independent of *myc* status (~3 fold higher in glutamine compared to glucose in Myc +++ and 2.25 fold higher in Myc -/-) **(Figure 6a)**. As discussed previously, a substantial amount of lactate is released from cells when glucose is the only carbon source **(Figure 5b)**. Thus much of the pyruvate generated from glucose by glycolysis does not contribute to energy generated by oxidative phosphorylation. However, when cells are provided with only glutamine, no lactate is produced and glutamine carbon is all available for oxidative phosphorylation, explaining the high amounts of ATP and oxygen consumption (see below) generated in this condition.

Previous reports have demonstrated that biguanides reduce mitochondrial ATP production (1,2). Following 48 hours metformin exposure (5mM) we observed a substantial decrease (~95%) in cellular ATP levels when cells were grown in glutamine-only media (20mM), regardless of *myc* expression, as shown in **Figure 6a**. In contrast, little decrease in ATP level was detected when cells were grown on glucose-only media (20mM) (~25% decrease in Myc+++ and no decrease in Myc-/-). These results suggest that increased ATP production via glycolysis represents a mechanism that partially compensates for the metformin-associated decrease in ATP production by oxidative phosphorylation, and that this mechanism operates in cells with direct glucose availability.

In order to better understand the relevance of carbon source on oxidative phosphorylation as a function of *myc* status and metformin exposure, oxygen consumption was measured as *myc* status and carbon source were varied in the presence or absence of metformin. **Figure 6b** shows basal level of oxygen consumption was consistently higher when cells were using glutamine rather than glucose as a carbon source provided *myc* was expressed (-/- myc 1.3 fold P=NS; +/+ myc 1.8 fold P=0.0097; myc+++ 1.8 fold P=0.0011). The observation that lactate was not released when cells were grown on glutamine, yet oxygen consumption and ATP levels remained high, suggests that when glutamine is the only carbon source provided, cells rely on oxidative phosphorylation to supply their energy needs. When cells were grown on glucose-only, metformin decreased oxygen consumption (P<0.0001) but this had minimal effect on ATP levels, in keeping with a high

contribution of glycolysis to ATP generation. This is also in keeping with the metformin induced increase in glucose consumption and lactate production under these conditions. Cells in glutamine-only media also displayed decreased oxygen consumption, resulting in an important decrease in ATP but in this case an increase in glycolysis is not possible, and a major decline in ATP is observed. This supports the hypothesis that ATP provided by glycolysis protects against metformin induced energy stress, consistent with a prior report showing enhanced ATP depletion when metformin was combined with 2-deoxyglucose, a glycolysis inhibitor (45).

Metformin sensitization in a tetracycline repressible Myc expression model. Finally, in order to further investigate the relationship between *myc* expression and the antiproliferative effect of metformin, we used the cell line P493-6 carrying a conditional, tetracycline-regulated *myc (36).* **Figure 7** shows that in the presence of both glucose and glutamine, the cells overexpressing the *myc* oncogene (tetracycline absent) were significantly inhibited by metformin (37% inhibition, P=0.02) while cells with low levels of *myc* (tetracycline present) were not affected, consistent with our prior observation that high levels of *myc* expression sensitize cells to metformin inhibition.

There is increasing interest in developing cancer therapies that target energy metabolism as compared to current drugs that target signaling pathways or function as cytotoxic agents. Many strategies have been proposed (46) and interesting that biguanides, widely used in treating diabetes mellitus, deserve investigation in this context (9).

Some proposed metabolic therapies for cancer seek to down-regulate the "Warburg" phenomenon, which is characterized by rapid proliferation in the setting of increased glycolysis and lactate production, regardless of oxygen availability. Metformin and similar compounds paradoxically further increase the substantial rates of glycolysis and lactate production in cancer cells, but inhibit their proliferation. Our data suggest that the increase in glycolysis represents compensation to the metformin-induced decrease in oxidative phosphorylation which attenuates but does not eliminate metformin-induced energy stress. It is of interest to contrast the metabolic effects of metformin to those recently described with PTEN over-expression. PTEN elevation decreased glycolysis and increased oxidative phosphorylation, resulting in reduced cell proliferation (47), while metformin increases glycolysis and reduces oxidative phosphorylation but also leads to a decline in proliferation.

Oxidation of the carbon backbone of glutamine in the mitochondria is a key metabolic fate of glutamine and a major source of energy for proliferating cells (48,49). Our findings, using various experimental systems, demonstrate that overexpression of *myc,* previously shown to be associated with increased glutamine utilization (40) increases sensitivity to metformin. This finding identifies *myc* overexpresing tumors as particularly attractive targets for biguanides such as metformin, either alone or in combination with pharmacologic strategies to inhibit glycolysis.

However, careful attention needs to be given to pharmacokinetic considerations, as the conventional metformin doses used in diabetes treatment may not lead to adequate tumor concentrations of metformin, particularly for tumors that do not express the cell surface transport proteins required for drug influx (50).

Acknowledgments

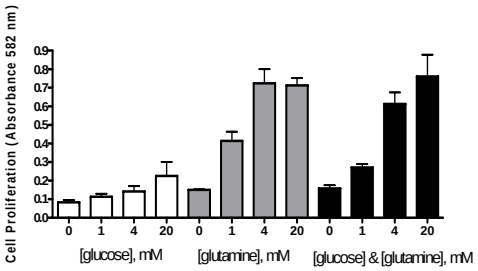
We thank Drs Ernesto Schiffrin for sharing laboratory resources, and Dr. Elena Birman and Miss Lilliane Lui for assistance.

Fig. 1. Carbon source influences the antiproliferative effect of metformin. (a) Effect of carbon source on proliferation: MC38 colon cancer cells were exposed to increasing concentrations of glucose, glutamine, or both for 48 hours. Cell growth in each well was measured by MTT. Proliferation was higher when media contained either glutamine (20 mM) or both nutrients as compared to growth under the glucose-only (20mM) condition (proliferation in glutamine 20mM, 0.71 ± 0.04 AU vs. proliferation in glucose 20mM, 0.22 ± 0.08 AU, $P<0.0001$). Data are presented as mean ± S.E. from 3 independent experiments. In each experiment, triplicates were used for each dose combination. **(b)** Effect of metformin on growth inhibition as a function of concentration of carbon source: MC38 colon cancer cells were exposed to increasing concentrations of glucose, glutamine, or both in the presence or absence of metformin (5mM) for 48 hours. Increasing concentration of glutamine in the absence of glucose did not diminish the inhibitory effect of metformin (inhibition by meformin in glucose 20mM containing media, 7% vs. inhibition by metformin in glutamine 20mM containing media, 78%, $P<0.0001$). **(c)** Effect of metformin on cell number: MC38 colon cancer cells were exposed to 20 mM glucose, glutamine or both in the presence or absence of metformin (5mM) for 48 hours. Cell growth in each condition was measured by counting Trypan blue excluding cells. **(d)** Mouse embryonic fibroblasts were exposed to 5mM glucose, glutamine or both in the presence or absence of metformin for 48 hours. Cell growth in each condition was measured by BrdU labeling. Metformin was a better growth inhibitor in glutamine-only media than glucose-only media (inhibition by meformin in glucose containing

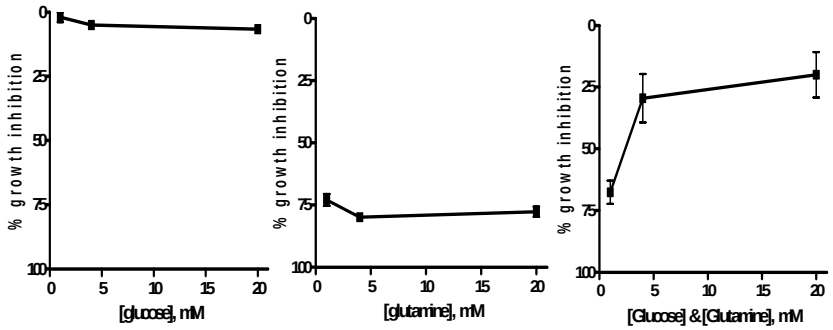
media 20mM, 0% vs. inhibition by metformin in glutamine containing media 20mM, 94%, $P<0.0001$).

Figure 1

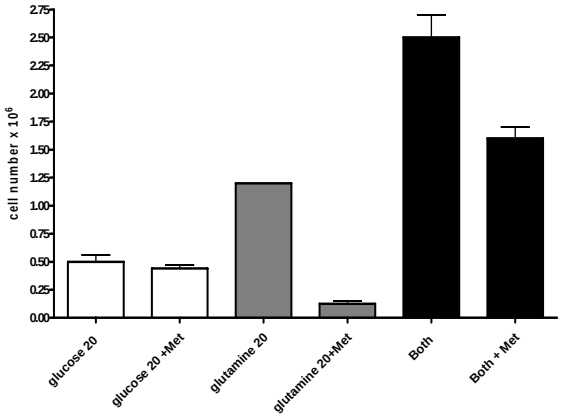
a



b



c



d

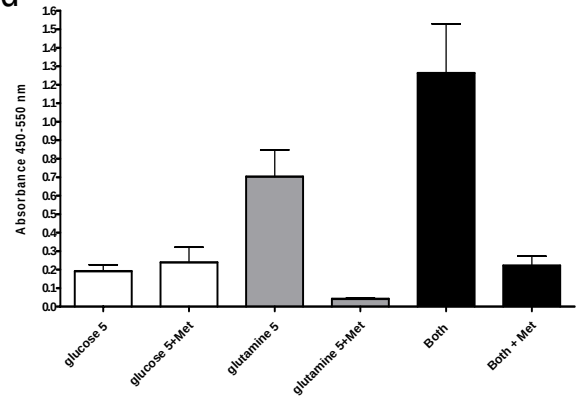


Fig. 2. Effect of metformin on mTOR signaling as a function of carbon source. Western blot analysis using antibodies against *P*-S6K1 and *P*-mTOR of MC38 colon cancer cells exposed to 20 mM glucose, glutamine or both in the presence or absence of metformin (5mM) for 48 hours. Results were quantified by densitometry.

Figure 2

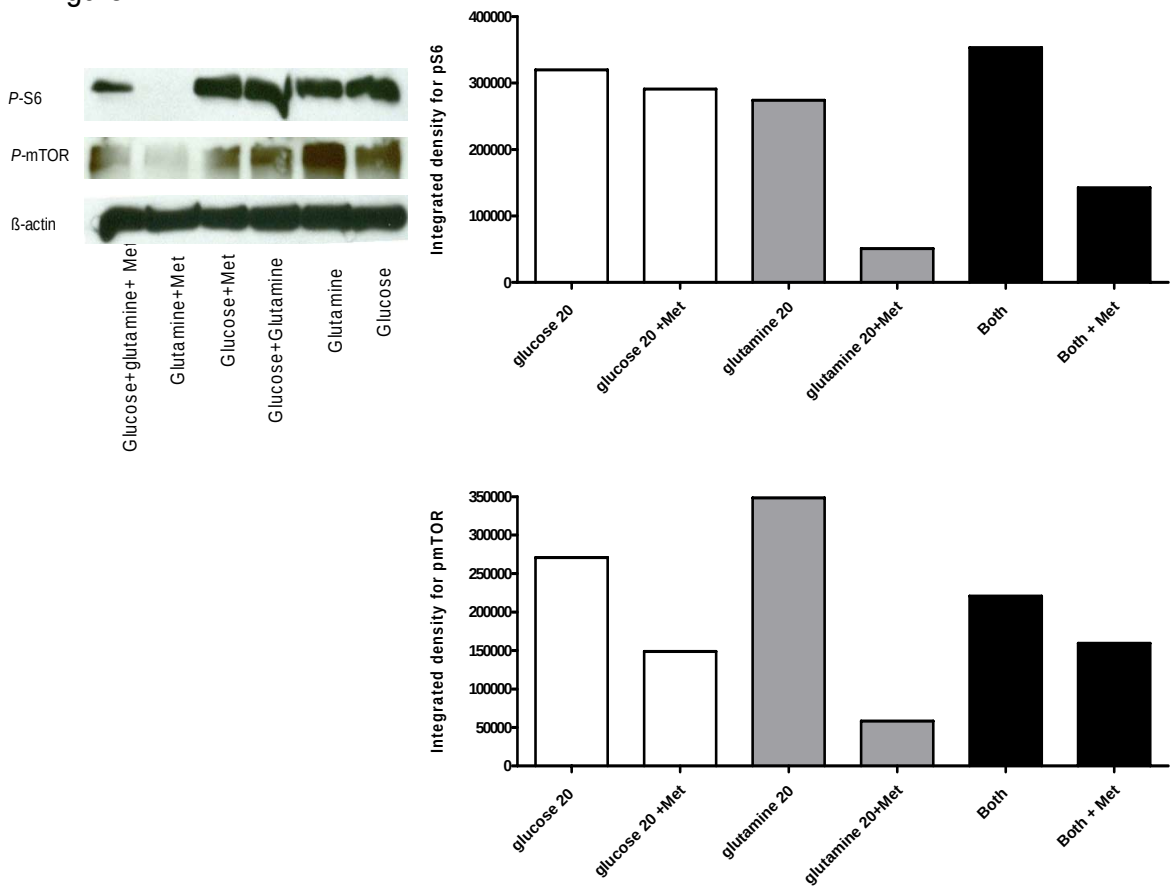

P-S6
P-mTOR
ß-actin
Glucose+glutamine+ Met
Glutamine+Met
Glucose+Met
Glucose+Glutamine
Glutamine
Glucose
Integrated density for pS6
400000
300000
200000
100000
0
glucose 20
glucose 20 +Met
glutamine 20
glutamine 20+Met
Both
Both + Met
Integrated density for pmTOR
350000
300000
250000
200000
150000
100000
50000
0
glucose 20
glucose 20 +Met
glutamine 20
glutamine 20+Met
Both
Both + Met

Fig. 3. Effect of metformin on ATP level, glucose consumption, lactate production, and NAD+/NADH ratio as a function of carbon source. MC38 colon cancer cells were exposed to 20 mM glucose, glutamine or both in the presence or absence of metformin (5mM) for 48 hours. **(a)** ATP levels: Exposure to metformin (5mM) resulted in a decrease (75%) in ATP level in the glutamine-only condition whereas this decline in ATP level was not observed in the glucose-only condition. **(b)** Glucose consumption: Metformin exposure also led to an increase in glucose consumption consistent with an increase in glycolysis. Less glucose was consumed when cells were exposed to both nutrients in the presence or absence of metformin ($P<0.0001$). **(c)** Lactate production: Metformin exposure resulted in an increase in lactate production when cells were either grown on glucose or both nutrients ($P<0.0001$). Lactate was not detected when cells were exposed to glutamine either in the presence or absence of metformin. **(d)** NAD+/NADH ratios: Metformin exposure resulted in a 41.06% decrease ($P=0.0045$) in NAD+/NADH ratio however this decline was not observed in glucose-only media or media containing both nutrients.

Figure 3

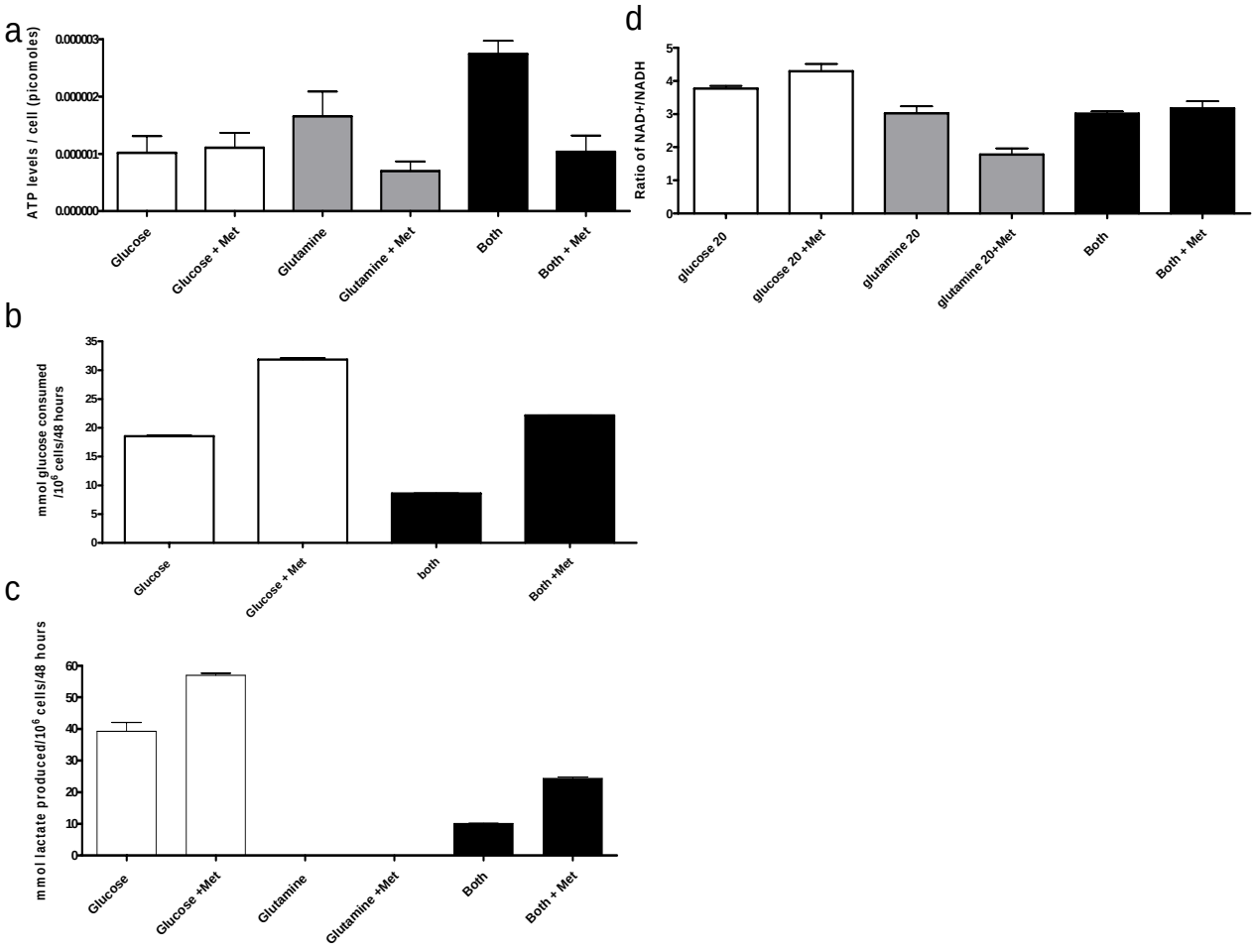

a
ATP levels / cell (picomoles)
0.000003
0.000002
0.000001
0.000000
Glucose
Glucose + Met
Glutamine
Glutamine + Met
Both
Both + Met
d
Ratio of NAD+/NADH
5
4
3
2
1
0
glucose 20
glucose 20 +Met
glutamine 20
glutamine 20+Met
Both
Both + Met
b
mmol glucose consumed /10^6 cells/48 hours
35
30
25
20
15
10
5
0
Glucose
Glucose + Met
both
Both +Met
c
mmol lactate produced/10^6 cells/48 hours
60
50
40
30
20
10
0
Glucose
Glucose +Met
Glutamine
Glutamine +Met
Both
Both + Met

Fig. 4. Myc overexpression leads to sensitization to metformin. (a) Isogenic HOmyc Rat1 fibroblast cells expressing high levels of Myc, TGR1 cells expressing physiological levels of Myc and HO15.19 cells in which *myc* was knocked out were grown with glucose and/or glutamine (20mM) in the presence or absence of metformin for 48 hours. Cell number was determined by Trypan Blue exclusion. As *myc* levels decrease, proliferation when glutamine was the carbon source also declined (proliferation in Myc+++, $1{,}50x10^6$ cells vs. proliferation in Myc+/+, $9{,}7x10^6$ cells, $P= 0.0049$), whereas *myc* had no effect on proliferation when glucose was the carbon source. **(b)** Rat1 fibroblasts exposed to replete media consisting of 5mM glucose and 5 mM glutamine in the presence or absence of metformin (5mM) for 48 hours. Cell proliferation measured by BrdU labeling. Metformin reduced growth to the greatest degree when *myc* was over-expressed (% inhibition in Myc+++, 62%, $P<0.0001$, % inhibition in Myc+/+, 14%, $P=$ NS).

Figure 4

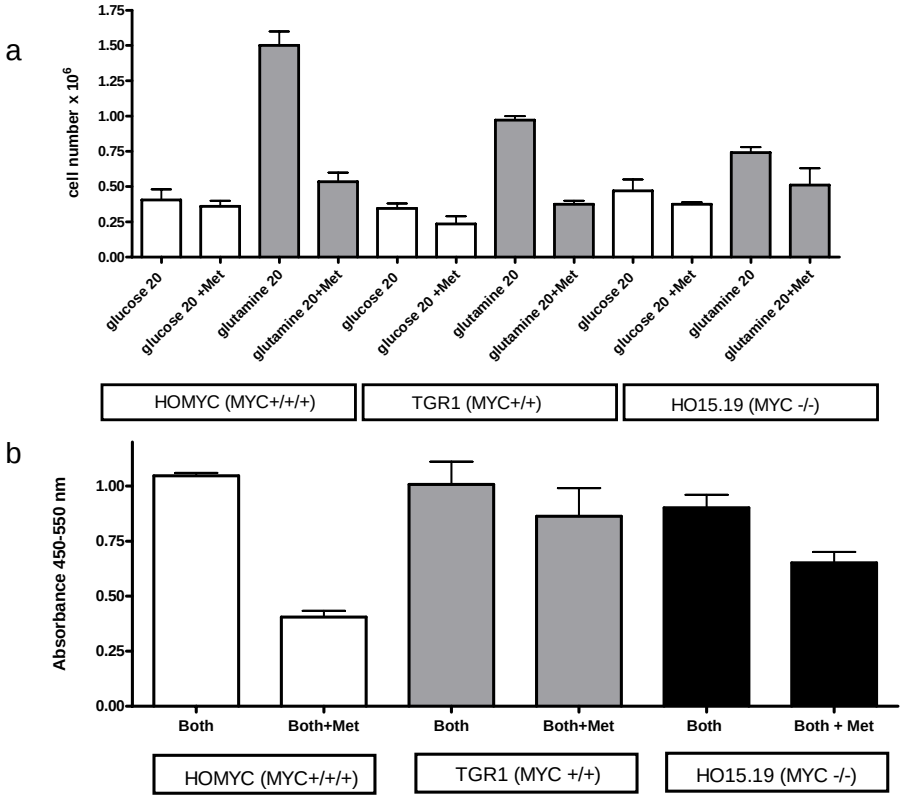
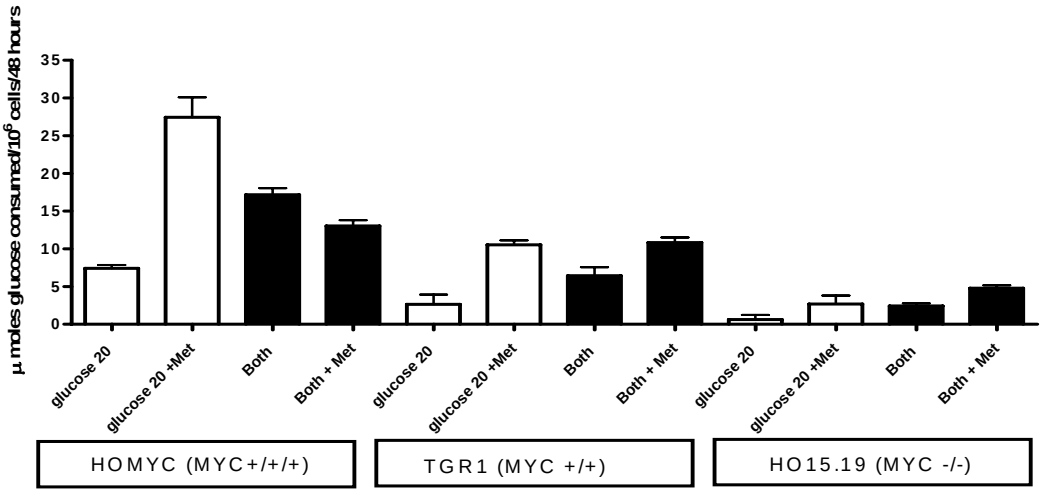

a
cell number x 10^6
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
glucose 20
glucose 20 +Met
glutamine 20
glutamine 20+Met
glucose 20
glucose 20 +Met
glutamine 20
glutamine 20+Met
glucose 20
glucose 20 +Met
glutamine 20
glutamine 20+Met
HOMYC (MYC+/+/+)
TGR1 (MYC+/+)
HO15.19 (MYC -/-)
b
Absorbance 450-550 nm
1.00
0.75
0.50
0.25
0.00
Both
Both+Met
Both
Both+Met
Both
Both + Met
HOMYC (MYC+/+/+)
TGR1 (MYC +/+)
HO15.19 (MYC -/-)

Fig. 5. Effect of metformin on glucose consumption and lactate production as a function of carbon source and *myc* expression. Isogenic HOmyc Rat1 fibroblast cells expressing high levels of *myc*, TGR1 cells expressing physiological levels of *myc* and HO15.19 cells in which *myc* was knocked out were grown with glucose and/or glutamine (20mM) in the presence or absence of metformin for 48 hours. **(a)** Metformin increased glucose consumption regardless of *myc* level when cells were grown in media without glutamine. The amount of glucose consumed with metformin exposure varied significantly according to *myc* expression (glucose consumed in the glucose-only condition with metformin exposure in Myc +++, 27.43 μmoles/10^6 cells/48 hours vs. Myc+/+, 10.54 μmoles/10^6 cells/48 hours vs. Myc-/-, 2.67 μmoles/10^6 cells/48 hours; $P<0.0001$ for all *myc* states). **(b)** Similar trends were also observed in the amount of lactate produced regardless of carbon source (lactate produced in the glucose-only condition with metformin exposure in Myc +++, 66.32 μmoles/10^6 cells/48 hours vs. Myc+/+, 57.64 μmoles/10^6 cells/48 hours vs. Myc-/-, 19.67 μmoles/10^6 cells/48 hours).

Figure 5

a


µ moles glucose consumed/10^6 cells/48 hours
35
30
25
20
15
10
5
0
glucose 20
glucose 20 +Met
Both
Both + Met
glucose 20
glucose 20 +Met
Both
Both + Met
glucose 20
glucose 20 +Met
Both
Both + Met
HOMYC (MYC+/+/+)
TGR1 (MYC +/+)
HO15.19 (MYC -/-)

b

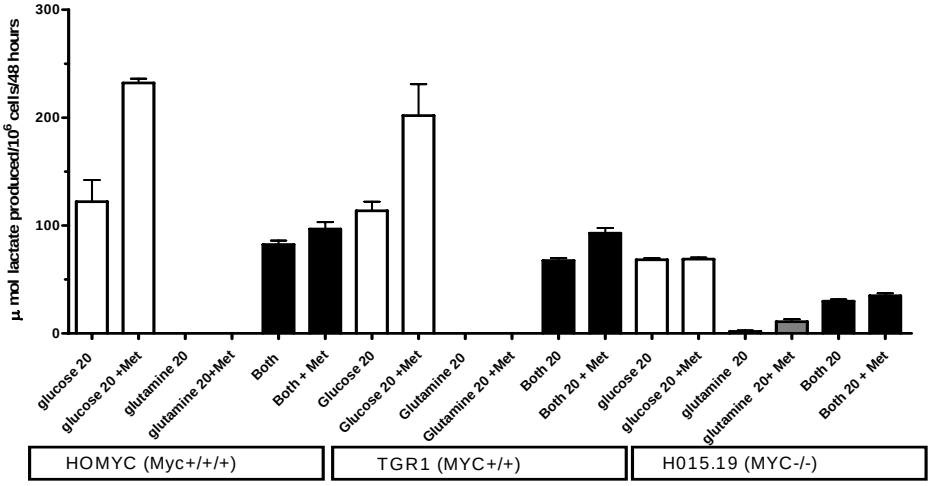
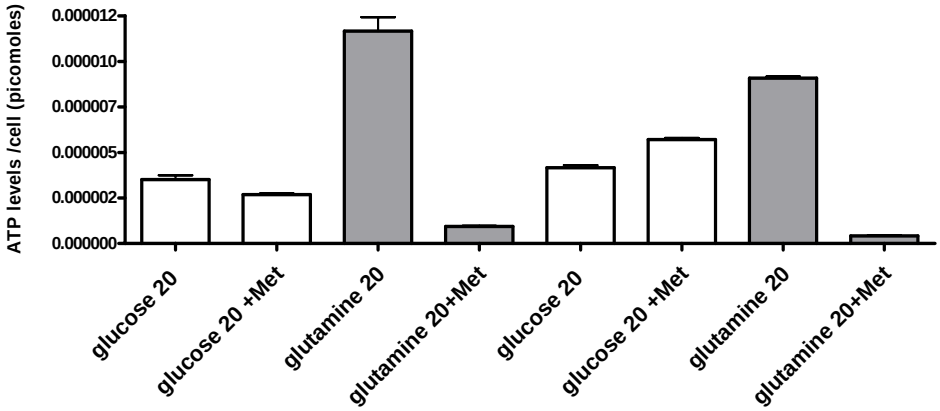

µ mol lactate produced/10^6 cells/48 hours
300
200
100
0
glucose 20
glucose 20 +Met
glutamine 20
glutamine 20+Met
Both
Both + Met
Glucose 20
Glucose 20 +Met
Glutamine 20
Glutamine 20 +Met
Both 20
Both 20 + Met
glucose 20
glucose 20 +Met
glutamine 20
glutamine 20+ Met
Both 20
Both 20 + Met
HOMYC (Myc+/+/+)
TGR1 (MYC+/+)
H015.19 (MYC-/-)

Fig.6. Effect of metformin on ATP level and oxygen consumption as a function of carbon source and Myc expression. Isogenic HOmyc Rat1 fibroblast cells expressing high levels of *myc*, TGR1 cells expressing physiological levels of *myc* and HO15.19 cells in which *myc* was knocked out were grown with glucose and/or glutamine (20mM) in the presence or absence of metformin for 48 hours. **(a)** ATP level: Higher baseline ATP concentrations were observed when cells were grown on glutamine as compared to glucose, independent of *myc* status (~ 3 fold higher in glutamine compared to glucose in Myc+++ and 2.25 fold higher in Myc-/-). Metformin exposure resulted in a decrease (95%) in cellular ATP levels when cells were grown on glutamine-only media. In contrast, little or no decrease in ATP level was detected when cells were grown in glucose-only media (~25% decrease in Myc+++ and no decrease in Myc-/-). **(b)** Oxygen consumption: The basal level of oxygen consumed was consistently higher, when glutamine as compared to glucose was the carbon source provided *myc* was expressed (-/- myc 1.3 fold *P*=NS; +/+ myc 1.8 fold *P*=0.0097; myc+++ 1.8 fold *P*=0.0011). Metformin significantly inhibited oxygen consumption in either nutrient condition regardless of *myc* expression level.

Figure 6

a


ATP levels /cell (picomoles)
0.000012
0.000010
0.000007
0.000005
0.000002
0.000000
glucose 20
glucose 20 +Met
glutamine 20
glutamine 20+Met
glucose 20
glucose 20 +Met
glutamine 20
glutamine 20+Met

b

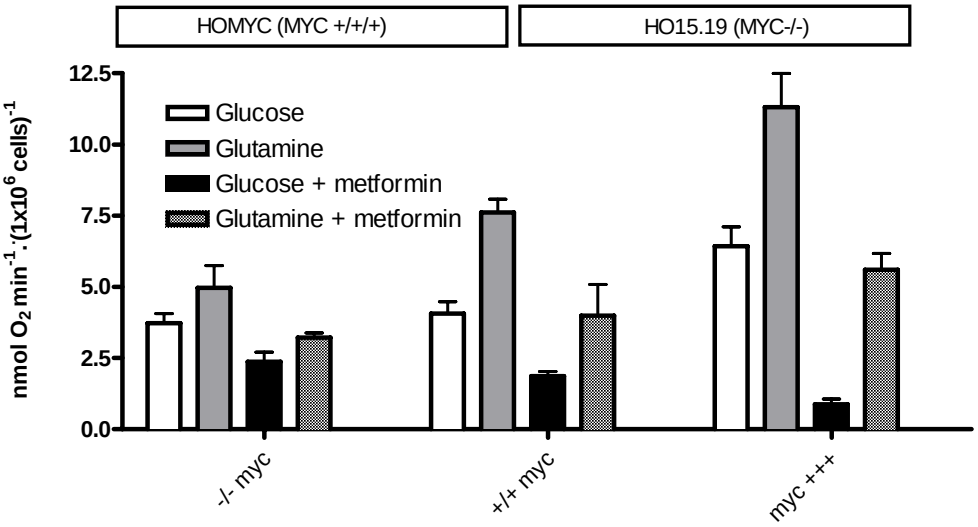
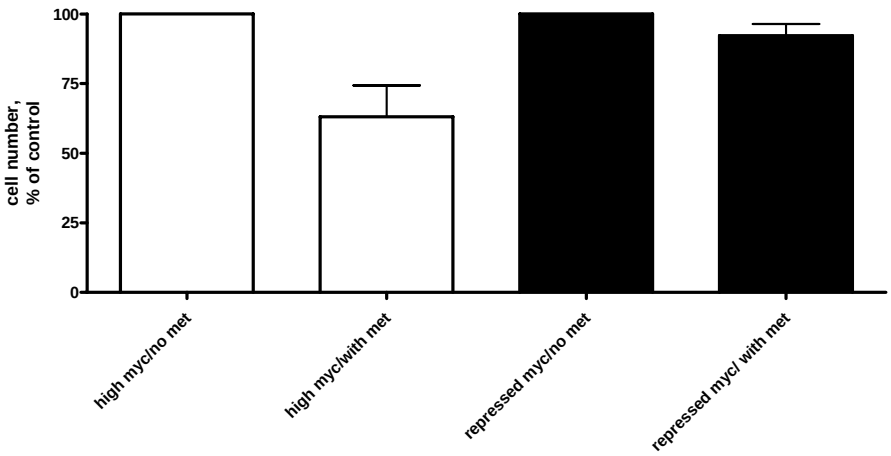

HOMYC (MYC +/+/+)
HO15.19 (MYC-/-)
nmol O2 min-1.(1x10^6 cells)-1
12.5
10.0
7.5
5.0
2.5
0.0
Glucose
Glutamine
Glucose + metformin
Glutamine + metformin
-/- myc
+/+ myc
myc +++

Fig. 7. Metformin sensitization in a tetracycline repressible Myc expression model P493-6 Burkitt Lymphoma cells carrying a conditional, tetracycline-regulated *myc* were exposed to replete media consisting of 5mM glucose and 5mM glutamine in the presence or absence of metformin (5mM). Cells overexpressing *myc* (tetracycline absent) were inhibited by metformin (37% inhibition, P=0.02) while cells with low levels of *myc* (tetracycline present) were not affected.

Figure 7


100
75
50
25
0
cell number,
% of control
high myc/no met
high myc/with met
repressed myc/no met
repressed myc/ with met

Reference List

1. El Mir MY, Nogueira V, Fontaine E, Averet N, Rigoulet M, Leverve X. Dimethylbiguanide inhibits cell respiration via an indirect effect targeted on the respiratory chain complex I. J Biol Chem 2000;275:223-8.

2. Owen MR, Doran E, Halestrap AP. Evidence that metformin exerts its anti-diabetic effects through inhibition of complex 1 of the mitochondrial respiratory chain. Biochem J 2000;348 Pt 3:607-14.

3. Hardie DG, Ross FA, Hawley SA. AMPK: a nutrient and energy sensor that maintains energy homeostasis. Nat Rev Mol Cell Biol 2012;13:251-62.

4. Shaw RJ, Kosmatka M, Bardeesy N, Hurley RL, Witters LA, Depinho RA, et al. The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress. Proc Natl Acad Sci U S A 2004;101:3329-35.

5. Jones RG, Plas DR, Kubek S, Buzzai M, Mu J, Xu Y, et al. AMP-activated protein kinase induces a p53-dependent metabolic checkpoint. Mol Cell 2005;18:283-93.

6. Hardie DG. AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy. Nat Rev Mol Cell Biol 2007;8:774-85.

7. Shackelford DB, Shaw RJ. The LKB1-AMPK pathway: metabolism and growth control in tumour suppression. Nat Rev Cancer 2009;9:563-75.

8. Zakikhani M, Dowling R, Fantus IG, Sonenberg N, Pollak M. Metformin is an AMP kinase-dependent growth inhibitor for breast cancer cells. Cancer Res 2006;66:10269-73.

9. Pollak M. Metformin in cancer prevention and treatment: the end of the beginning. Cancer Discov 2012;In press.

10. Algire C, Amrein L, Bazile M, David S, Zakikhani M, Pollak M. Diet and tumor LKB1 expression interact to determine sensitivity to anti-neoplastic effects of metformin in vivo. Oncogene 2011;30:1174-82.

11. Buzzai M, Jones RG, Amaravadi RK, Lum JJ, DeBerardinis RJ, Zhao F, et al. Systemic treatment with the antidiabetic drug metformin selectively impairs p53-deficient tumor cell growth. Cancer Res 2007;67:6745-52.

12. Bailey CJ, Turner RC. Metformin. N Engl J Med 1996;334:574-9.

13. Shaw RJ, Lamia KA, Vasquez D, Koo SH, Bardeesy N, Depinho RA, et al. The kinase LKB1 mediates glucose homeostasis in liver and therapeutic effects of metformin. Science 2005;310:1642-6.

14. Foretz M, Hebrard S, Leclerc J, Zarrinpashneh E, Soty M, Mithieux G, et al. Metformin inhibits hepatic gluconeogenesis in mice independently of the LKB1/AMPK pathway via a decrease in hepatic energy state. J Clin Invest 2010;120:2355-69.

15. Pollak M. Metformin and other biguanides in oncology: advancing the research agenda. Cancer Prev Res (Phila) 2010;3:1060-5.

16. Appleyard MV, Murray KE, Coates PJ, Wullschleger S, Bray SE, Kernohan NM, et al. Phenformin as prophylaxis and therapy in breast cancer xenografts. Br J Cancer 2012;106:1117-22.

17. Mullen AR, Wheaton WW, Jin ES, Chen PH, Sullivan LB, Cheng T, et al. Reductive carboxylation supports growth in tumour cells with defective mitochondria. Nature 2011;481:385-8.

18. DeBerardinis RJ, Cheng T. Q's next: the diverse functions of glutamine in metabolism, cell biology and cancer. Oncogene 2010;29:313-24.

19. Wise DR, DeBerardinis RJ, Mancuso A, Sayed N, Zhang XY, Pfeiffer HK, et al. Myc regulates a transcriptional program that stimulates mitochondrial glutaminolysis and leads to glutamine addiction. Proc Natl Acad Sci U S A 2008;105:18782-7.

20. Dang CV. MYC, microRNAs and glutamine addiction in cancers. Cell Cycle 2009;8:3243-5.

21. Noto H, Goto A, Tsujimoto T, Noda M. Cancer risk in diabetic patients treated with metformin: a systematic review and meta-analysis. Plos One 2012;7:e33411.

22. Lai SW, Chen PC, Liao KF, Muo CH, Lin CC, Sung FC. Risk of hepatocellular carcinoma in diabetic patients and risk reduction associated with anti-diabetic therapy: a population-based cohort study. Am J Gastroenterol 2012;107:46-52.

23. Chlebowski RT, McTiernan A, Wactawski-Wende J, Manson JE, Aragaki AK, Rohan T, et al. Diabetes, metformin, and breast cancer in postmenopausal women. J Clin Oncol 2012;-In press.

24. Sadeghi N, Abbruzzese JL, Yeung S-CJ, Hassan M, Li D. Metformin use is associated with better survival of diabetic patients with pancreatic cancer. Clinical Cancer Research 2012;18:2905-12.

25. Lee GE, Aung T, Lim KH, Tan WS, Tai WMD, Suhaimi N-AB, et al. Examining the effects of metformin on survival outcome in stage II/III colorectal cancer patients with diabetes mellitus. J Clin Oncol 2012;30:(suppl; abstr 3589).

26. Garrett CR, Hassabo HM, Bhadkamkar NA, Wen S, Baladandayuthapani V, Kee BK, et al. Survival advantage observed with the use of metformin in patients with type II diabetes and colorectal cancer. Br J Cancer 2012;106:1374-8.

27. Currie CJ, Poole CD, Jenkins-Jones S, Gale EA, Johnson JA, Morgan CL. Mortality after incident cancer in people with and without type 2 diabetes: impact of metformin on survival. Diabetes Care 2012;35:299-304.

28. Azoulay L, Dell'Aniello S, Gagnon B, Pollak M, Suissa S. Metformin and the incidence of prostate cancer in patients with type 2 diabetes. Cancer Epidemiol Biomarkers Prev 2011;20:337-44.

29. Bayraktar S, Hernadez-Aya LF, Lei X, Meric-Bernstam F, Litton JK, Hsu L, et al. Effect of metformin on survival outcomes in diabetic patients with triple receptor-negative breast cancer. Cancer 2012;118:1202-11.

30. Cossor FI, Adams-Campbell LL, Chlebowski RT, Gunter MJ, Johnson K, Martell RE, et al. Diabetes, metformin use, and colorectal cancer survival in women: A retrospective cohort study. J Clin Oncol 2012;30:(suppl; abstr e14005).

31. Bodmer M, Becker C, Meier C, Jick SS, Meier CR. Use of metformin is not associated with a decreased risk of colorectal cancer: a case-control analysis. Cancer Epidemiol Biomarkers Prev 2012;21:280-6.

32. Martin MJ, Hayward R, Viros A, Marais R. Metformin Accelerates the Growth of BRAFV600E-Driven Melanoma by Upregulating VEGF-A. Cancer Discov 2012;2:344-55.

33. Segal ED, Yasmeen A, Beauchamp MC, Rosenblatt J, Pollak M, Gotlieb WH. Relevance of the OCT1 transporter to the antineoplastic effect of biguanides. Biochem Biophys Res Commun 2011;414:694-9.

34. Mateyak MK, Obaya AJ, Adachi S, Sedivy JM. Phenotypes of c-Myc-deficient rat fibroblasts isolated by targeted homologous recombination. Cell Growth Differ 1997;8:1039-48.

35. Schuhmacher M, Staege MS, Pajic A, Polack A, Weidle UH, Bornkamm GW, et al. Control of cell growth by c-Myc in the absence of cell division. Curr Biol 1999;9:1255-8.

36. Pajic A, Spitkovsky D, Christoph B, Kempkes B, Schuhmacher M, Staege MS, et al. Cell cycle activation by c-myc in a burkitt lymphoma model cell line. Int J Cancer 2000;87:787-93.

37. Schuhmacher M, Kohlhuber F, Holzel M, Kaiser C, Burtscher H, Jarsch M, et al. The transcriptional program of a human B cell line in response to Myc. Nucleic Acids Res 2001;29:397-406.

38. Blouin MJ, Zhao Y, Zakikhani M, Algire C, Piura E, Pollak M. Loss of function of PTEN alters the relationship between glucose concentration and cell proliferation, increases glycolysis, and sensitizes cells to 2-deoxyglucose. Cancer Lett 2010;289:246-53.

39. Zakikhani M, Blouin M-J, Piura E, Pollak M. Metformin and rapamycin have distinct effects on the AKT pathway and proliferation in breast cancer cells. Breast Cancer Res Treat 2010;123:271-9.

40. Wise DR, Thompson CB. Glutamine addiction: a new therapeutic target in cancer. Trends Biochem Sci 2010;35:427-33.

41. Dang CV. Rethinking the Warburg effect with Myc micromanaging glutamine metabolism. Cancer Res 2010;70:859-62.

42. Dang CV. c-Myc target genes involved in cell growth, apoptosis, and metabolism. Mol Cell Biol 1999;19:1-11.

43. Osthus RC, Shim H, Kim S, Li Q, Reddy R, Mukherjee M, et al. Deregulation of glucose transporter 1 and glycolytic gene expression by c-Myc. J Biol Chem 2000;275:21797-800.

44. Shim H, Dolde C, Lewis BC, Wu CS, Dang G, Jungmann RA, et al. c-Myc transactivation of LDH-A: implications for tumor metabolism and growth. Proc Natl Acad Sci U S A 1997;94:6658-63.

45. Ben S, I, Laurent K, Giuliano S, Larbret F, Ponzio G, Gounon P, et al. Targeting cancer cell metabolism: the combination of metformin and 2-deoxyglucose induces p53-dependent apoptosis in prostate cancer cells. Cancer Res 2010;70:2465-75.

46. Vander Heiden MG. Targeting cancer metabolism: a therapeutic window opens. Nat Rev Drug Discov 2011;10:671-84.

47. Garcia-Cao I, Song MS, Hobbs RM, Laurent G, Giorgi C, de Boer VC, et al. Systemic elevation of PTEN induces a tumor-suppressive metabolic state. Cell 2012;149:49-62.

48. Reitzer LJ, Wice BM, Kennell D. Evidence that glutamine, not sugar, is the major energy source for cultured HeLa cells. J Biol Chem 1979;254:2669-76.

49. DeBerardinis RJ, Lum JJ, Hatzivassiliou G, Thompson CB. The biology of cancer: metabolic reprogramming fuels cell growth and proliferation. Cell Metab 2008;7:11-20.

50. Nies AT, Hofmann U, Resch C, Schaeffeler E, Rius M, Schwab M. Proton pump inhibitors inhibit metformin uptake by organic cation transporters (OCTs). Plos One 2011;6:e22163.

Linking Statement

Using various experimental systems, we demonstrated that in the presence of glutamine and the absence of glucose, metformin was a better growth inhibitor. As the inhibitory actions of metformin mainly involve the mitochondria, this is in keeping with the fact that the utilization of glutamine to sustain the metabolic needs of the cell is more dependent on functional mitochondria than that of glucose. We further went on to show that cells associated with "glutamine addiction" where *myc* was overexpressed were sensitized to the antiproliferative actions of metformin.

With a new class of metabolic therapies seeking to down-regulate the Warburg effect such as the use of glycolysis inhibitors in combination with chemotherapy and radiotherapy (Maschek et al., 2004), it is paradoxical that metformin and similar compounds increase rates of glycolysis and lactate production in cancer cells, yet inhibit their proliferation. Attention is being given to the possibility that a reduction in oxidative phosphorylation may be useful in cancer treatment.

We therefore decided to look into the inhibition of the tumor suppressor gene, Ataxia Telangiectasia Mutated (ATM), in view of prior preliminary evidence suggesting defective mitochondrial function in patients with Ataxia Telangiectasia. A specific inhibitor of the kinase activity of the protein encoded by Ataxia Telangiectasia Mutated (KU-55933) was used to assess whether this would mimic a

reduction in mitochondrial function and hence a decline in oxidative phosphorylation. This was of interest for if KU-55933 resulted in similar metabolic and antiproliferative effects to that of metformin, despite the fact that a tumor suppressor gene was being inhibited, evidence would be provided for a reduction in mitochondrial function as being potentially useful in treating cancer.

Chapter III

Manuscript B

PloS ONE

(Pending Revision)

Alterations in Cellular Energy Metabolism Associated with the Antiproliferative Effects of the ATM Inhibitor KU-55933 and with Metformin

Mahvash Zakikhani, PhD[1,2], Miguel Bazile, BSc[2], Sina Hashemi[2], Shiva Javeshghani, BSc[4], Daina Avizonis, PhD[3], Julie St Pierre, PhD[3,5], Michael N. Pollak, MD[1,2,3,4*]

[1] Department of Oncology, [2] Division of Cancer Prevention, [3] Goodman Cancer Centre, [4] Division of Experimental Medicine, [5] Department of Biochemistry, McGill University, Montreal, Quebec, Canada.

* Corresponding author:

Cancer Prevention Center, Jewish General Hospital,
3755 Cote Ste-Catherine, Montreal, Quebec, Canada, H3T 1E2
E-mail: michael.pollak@mcgill.ca
Phone: 514-340-8222 ext 4139
Fax: 514-340-8600

Abstract

KU-55933 is a specific inhibitor of the kinase activity of the protein encoded by *Ataxia telangiectasia mutated* (ATM), an important tumor suppressor gene with key roles in DNA repair. Unexpectedly for an inhibitor of a tumor suppressor gene, KU-55933 reduces proliferation. In view of prior preliminary evidence suggesting defective mitochondrial function in cells of patients with Ataxia Telangiectasia (AT), we examined energy metabolism of cells treated with KU-55933. The compound increased AMPK activation, glucose uptake and lactate production while reducing mitochondrial membrane potential and coupled respiration. The stimulation of glycolysis by KU-55933 did not fully compensate for the reduction in mitochondrial functions, leading to decreased cellular ATP levels and energy stress. These actions are similar to those previously described for the biguanide metformin, a partial inhibitor of respiratory complex I. Both compounds decreased mitochondrial coupled respiration and reduced cellular concentrations of fumarate, malate, citarate, and alpha-ketogluterate. Succinate levels were increased by KU-55933 levels and decreased by metformin, indicating that the effects of ATM inhibition and metformin are not identical. These observations suggest a role for ATM in mitochondrial function and show that both KU-55933 and metformin perturb the TCA cycle as well as oxidative phosphorylation.

Introduction

DNA repair deficiency facilitates accumulation of mutations and accelerates carcinogenesis. These are features of the ataxia-telangiectasia syndrome, seen in patients with loss of function of ataxia telangiectasia mutated protein (ATM) [1,2]. On the other hand, robust DNA repair capacity by cancer cells leads to resistance to therapies such as ionizing radiation that are intended to cause lethal DNA damage [3]. Small molecule ATM inhibitors [4] were developed in the context of the classic role of ATM in DNA repair, with the rationale that inhibition of DNA repair would increase efficacy of radiation therapy or cytotoxic drugs. The finding that inhibition of ATM by the small molecule kinase inhibitor KU-55933 has an antiproliferative effect [5] was unexpected in the context of the classic role of ATM as a tumor suppressor gene. However, there is recent evidence for novel functions of ATM [6], including participation in insulin signalling by an effect on protein translation regulator 4E-BP1 [7], regulation of response to oxidative stress (8-10), regulation of ribonucleotide reductase [11], and activation of the pentose phosphate pathway [12,13]. Recent results (14,15) provide evidence that KU- 55933 also inhibits the function of the organic cation transporter 1 (OCT1), which is known to be involved in cellular influx of several drugs, including metformin. In view of a prior report (16) that mitochondrial function is defective in fibroblasts from patients with ataxia-telangiectasia, we studied the effects of the small molecule inhibitor KU-55933 on cellular energy metabolism. We compared the effects of the ATM inhibitor to those metformin, because this biguanide is known to be a growth inhibitor with a

mitochondrial site of action, at respiratory complex I (17-21). Other biguanides also inhibit mitochondrial function through incompletely described mechanisms (22).

Materials & Methods

Chemicals. Cell culture materials were obtained from Invitrogen (Burlington, ON, Canada). Anti-phospho AMPKα (Thr^{172}), anti-AMPKα, anti-ATM, anti-phospho p53, anti-p53, anti-VDAC (voltage-dependent anion channel), anti-α-tubulin, and anti-ß-actin were purchased from Cell Signaling Technology (Beverly, MA), anti-phospho-ATM (Ser^{1981}) and anti-SCO2 from Abcam (Cambridge, MA), anti-Ki67 from Novus Biologicals (Oakville, ON, Canada) Horseradish peroxidase-conjugated anti-rabbit IgG, anti-mouse IgG, and enhanced chemiluminescene (ECL) reagents were from Pharmacia-Amersham (Baie d'Urfé, QC, Canada). Metformin (1, 1-Dimethylbiguanide hydrchloride), rotenone and FCCP, (Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone) were purchased from Sigma-Aldrich (Oakville, ON, Canada), and KU-55933 from Calbiochem-EMD Biosciences, Inc (La Jolla, CA). siRNA against ATM and LKB1 and negative control siRNA (Alexa Fluor 488) were purchased from QIAGEN (Mississauga, ON, Canada), JC-1 (5,59,6,69-tetramethylbenzimidazolcarbocyanine iodide) from eBiosience (San Diego, CA).

Cell lines and culture conditions. MCF-7(breast), HeLa (cervical) and HepG2 (hepatom) cell lines were purchased from American Tissue Culture Collection (ATCC) (Manassas, VA). HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ (colorectal)

(generously provided by Dr. Russell Jones, McGill University and have been described previously in (46)) were cultured in RPMI 1640 or DMEM, supplemented with 10% fetal bovine serum (FBS) and 100 units/ml gentamycin at 37^{o} C and 5% CO_2. Cells were passaged by 0.25% Trypsin-EDTA when they reached ~ 80% confluence. Complete medium containing both glucose and glutamine was used in all conditions.

Cell proliferation assay. The effect of metformin or KU-55933 on cell lines was evaluated by the resazurin assay to measure overall mitochondrial respiration rates (Alamar Blue), (Biosource International, Camarilo, CA). Cells were plated at 3-$5x10^3$ per well in triplicate in 96-well plates and incubated in medium containing 10% FBS. After 24 hrs, the complete medium was replaced with test medium containing vehicle control or various doses of metformin or KU-55933 for 72 hrs at 37°C. Alamar Blue was then added to plates which were incubated at 37°C according to the methods provided by the supplier and a colorimetric change measured the reduction of resazurin as an indicator of overall mitochondrial function which correlated with cell number.

ATP measurements. Cellular ATP levels were measured using the Invitrogen ATP Determination Kit A22066, (Invitrogen, Burlington, ON, Canada). Cells were treated in 1% FBS RPMI in the absence or presence of KU-55933 or metformin, for 72 hrs. The kit was used as per the manufacturer's instructions, with $3x10^5$ cells per well. Measurements were done in triplicate.

Measurements of glucose consumption. Cells were cultured in complete medium with 10% FBS. After 24 h, the complete medium was replaced with test medium in the absence or presence of KU-55933 or metformin. Cells were incubated for 72 hrs and the culture medium was then collected and analyzed for measurement of glucose and lactate concentrations using colorimetric kits according to manufacturer's instructions. Glucose levels were determined using a Glucose assay kit (Eton Bioscience, Inc., Cambridge, MA). Glucose consumption was determined from the difference in glucose concentration compared to control.

Lactate production assay. Lactate levels were determined in 10µl culture medium collected from treated cells and results were standardised with the number of cells. Lactate was calculated using a Lactate Kit (BioVision, Inc., San Francisco, CA).

Flow cytometry for apoptosis induction and cell death analysis. After 72 hrs treatment adherent cells were briefly trypsinized, detached, combined with floating cells from the original growth medium, centrifuged, and washed twice with Phosphate-Buffered Saline (PBS). Approximately 10^6 cells (for each condition) were stained for 30 min with annexinV–FITC and propidium iodide (PI) using the AnnexinV–FITC kit (Invitrogen). Analysis was conducted on a FACSCalibur flow cytometer (BD Biosciences, Burlington, MA) with CellQuest software (BD Biosciences Immunocytometry Systems, Franklin Lakes, NJ). All apoptosis tests

were conducted in triplicate and results shown are representative of 3 independent experiments.

Mitochondrial membrane potential. To determine mitochondrial membrane potential JC-1 non-toxic fluorescence probe was dissolved in tissue culture grade dimethyl sulfoxide (DMSO) at a concentration of 1 mg/ml. After treatments, cells were probed with JC-1 and the mitochondrial membrane polarization changes were measured as described (47). Cells treated with rotenone, an inhibitor of complex I, or carbonyl cyanide m-chlorophenylhydrazone (FCCP), an uncoupler of oxidative phosphorylation that abolishes the mitochondrial membrane proton gradient, were dissolved in DMSO and the solutions were added to culture medium to final concentrations as described in each experiment.

Cell transfection. MCF-7 cells were transfected with small interfering RNA (siRNA) targeting the ATM , LKB1, and AMPKα or a negative control siRNA using Pipette-type electroporator (MicroPorator MP-100, Digital Bio Technology Co., Ltd., Seoul, Korea) as described by the manufacturer's instructions. Cells cultures were incubated for 24 hours with various concentrations of siRNA prior to KU-55933 or metformin treatment.

Protein extraction and western blot analysis. Cells were washed three times with ice-cold phosphate-buffered saline (PBS) and lysed in 100-400µl lysis buffer (20 mM Tris HCl (pH 7.5)), 150 mM NaCL, 2.5 mM sodium pyrophosphate, 1 mM ß-

glycerol phosphate, 1 mM Na_3VO_4, 1 mM EGTA, 1% Triton, and Complete Protease Inhibitor Cocktail Tablet from Roche Diagnostic (Laval, QC, Canada). Cell debris was removed by centrifugation at 14,000X rpm for 20 minutes at 4 ºC. Following the assay for total protein (Bio-Rad, Mississauga, ON, Canada), clarified protein lysates from each experimental condition (40-50 μg) were boiled for 5 minutes and subjected to electrophoresis in denaturing 8% SDS-polyacrylamide gel for ATM, 12% for SCO2, or 10% SDS-PAGE for other proteins. Separated proteins were transferred to a nitrocellulose membrane and after blocking, the membranes were probed with antibodies of interest. In some cases, developed blots were stripped in stripping buffer (62mM Tris HCL (pH 6.8), 100mM ß-mercaptoethanol, 2% SDS) to confirm equal protein loading. Horseradish peroxidase-conjugated anti-rabbit IgG and anti-mouse IgG were used as secondary antibodies. The position of proteins was visualized using the enhanced chemiluminescene reagent ECL.

Mitochondrial extraction. Mitochondrial isolation was achieved by using the Mitochondrial Isolation protocol (48). Mitochondrial pellets were lysed and protein concentration was determined with the Pierce® BCA Protein Assay Kit (Thermo Scientific), with bovine serum albumin (BSA) as a standard.

Cellular respiration assay. Cells were rinsed, trypsinized, and spun twice at 1200 rpm for 5 min and resuspended in assay medium (PBS, sodium pyruvate (1mM), glucose (25 mM), BSA 2% (w/v)). Cell viability was determined using trypan blue exclusions. Respiration in whole cells was measured using 1 million cells/ml

(suspended in assay medium at 37°C), which were placed into the chamber of a Clark-type oxygen electrode (Rank Brothers, Cambridge, UK). Total respiration was assessed in the absence of inhibitors; while proton leak was measured using the ATP synthase inhibitor oligomycin (2.5 μg/ 1 x 10^6 cells) and non-mitochondrial respiration using myxothiazol (12.2 μg/ 1 x 10^6 cells).

Cell growth for NMR. MCF-7 Cells were plated at 1x10^6 per *petri* in 10 fold and incubated in medium containing 10% FBS. After 24 hrs, the complete medium was replaced with test medium containing vehicle control or metformin or KU-55933 at 37°C. Three MCF-7 plates were not extracted but used for cell counting and protein analysis. The average cell counts were 3.37 million per plate for the control cells, 1.9 million per plate for the KU-55933 treated cells and 2.1 million per plate for the metformin treated cells. These counts were used to normalize the NMR and GC/MS metabolite quantitation.

NMR Sample Preparation. MCF-7 cells were extracted as described previously (49). Briefly, tissue culture plates were removed from the incubator and 2 mL of the spent media was collected and placed in an eppendorf tube. The remaining media was aspirated off the plate to waste. The plated cells were washed three times with ice cold isotonic saline solution. Volumes of 500 μL 80% methanol (prechilled to -20 ^{o}C) were added to the plates on ice. The cells were scraped off the plates and deposited in eppendorf tubes. The plates were rinsed with second 500 μL aliquots of cold 80% methanol and added to the cells. The cells were then lysed by 5 minutes of

sonication, 30 seconds on 30 seconds off, on ice using Bioruptor UCD-200TM-EX Sonicator (Diagenode, Denville, NJ, USA). The homogenates were then spun down in a 4 ^{o}C micro centrifuge for 10 minutes at 13,000g and the supernatants were removed to new eppendorf tubes. The extracts were dried in a pre-cooled vacuum centrifuge (Labconco Corp. Kansas City, MO, USA) operating at -4 ^{o}C and stored at -80 ^{o}C until the day of NMR analysis.

For NMR analysis, cell extracts were re-suspended in 220 µL $^{2}H_2O$ containing 0.2mM DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid), the chemical shift and concentration standard 0.1mM DFTMP (difluorotrimethylsilanylphosphonic acid) an internal pH standard (50) and 0.01 mM sodium azide. The pH of each sample was manually adjusted to an uncorrected pH of 6.8 +/- 0.1 with HCl or KOH as needed.

Medium samples were ultra-filtered using pre-rinsed 3 kDa cut off filters (Nanosep ultra filter, Pall Corp. Port Washington, NY USA). A volume of 195 µL was removed to a separate tube containing 22 µL of a 2 mM DSS and 1mM DFTMP solution in $^{2}H_2O$. The pH of each medium sample was manually adjusted to a pH of 6.8 +/- 0.1 as before. The samples were then transferred to 3 mm NMR tubes (Wilmad, Buena, NJ, USA) for analysis.

NMR Data Acquisition & Analysis. NMR data collection was performed on a 500 MHz Inova NMR system (Agilent Technologies, Palo Alto, CA, USA) equipped with an HCN cryogenically cooled probe operating at 25 K. One-dimensional NMR

spectra of samples were collected using the first increment of the standard NOESY experiment supplied with the instrument. All spectra were recorded at 25 ^{o}C with a mixing time of 100 ms, 256 transients for cell extracts and 32 transients for media extracts were recorded with 8 equilibration pre-scans, a spectral window of 12 ppm centered on the residual water which was suppressed by a low power pre-saturation pulse during both the mixing time and 2 second relaxation delay. The acquisition time was set for 3 seconds for a total scan recycle time of 5 seconds. The same pre-saturation strength and gain were used for all data acquisition (a slightly higher power pre-saturation pulse was used for all media samples) while the 90^{o} pulse length was calibrated for each sample. Metabolite chemical shift assignments were confirmed by two-dimensional 75 ms mixing time total correlation spectroscopy (Z-filtered dipsi-Tocsy (51)) and by comparison to the Madison Metabolomics Consortium (52) and Human Metabolome data bases (53).

The one-dimensional data were processed with 128 k zero filling and exponential line broadening of 0.33 Hz before Fourier transformation. Targeted profiling of metabolites were achieved using a 500 MHz metabolite library from Chenomx NMR Suite 7.0 (Chenomx, Inc, Edmonton, AB, Canada), where area fit for the metabolite peaks were compared to that of the internal concentration standard (DSS) resulting in a concentration based on the Chenomx library compounds as described previously (54). The amount of each reported metabolite was normalized to the number of cells per plate (nanomoles per million cells).

Statistical Analysis. Prior to statistical analysis, data were square-root transformed to normalize the distribution and to obtain variance homogeneity. All experiments were performed at least in triplicate, and results are expressed as mean ± S.E.M. Statistical significance was evaluated using GLM Procedure, and least-squares means *post hoc* for multiple unpaired comparisons of means (LSMEANS statement with Bonferroni correction) was applied. All statistical analyses were performed using Statistical Analysis System software, version 9.2 (SAS Institute, Cary, NC). *P* values < 0.05 were considered significant.

Results

Effects of KU-55933 and/or metformin on cancer cell growth

Data shown in Figure 1A-B confirm that KU-55933 has antiproliferative effects on MCF-7, HepG2 and HeLa cell lines, as assessed by Alamar blue dye reduction. While this method is often used to estimate cell number, it actually is a measure of oxidative phosphorylation (16), so artefacts are possible if one is studying effects of an agent that influences cellular energy metabolism. Therefore, we confirmed an antiproliferative effect using cell number as an endpoint (Figure 1C). We also provide evidence in Figure 1C that an off-target effect of KU-55933 is unlikely, as an antiproliferative effect was also seen with ATM knockdown by siRNA. Western blot analysis confirmed reduced expression of ATM by siRNA but not by KU-55933 (Figure 1D).

Effects of KU-55933 and metformin on metabolism in MCF-7 cells

Figure 2A-C shows effects of KU-55933 and metformin on cell number, lactate production, and glucose consumption for MCF-7 cells. As expected, metformin decreased cell number, increased glucose consumption, and increased lactate production. These findings are consistent with previously reported actions of metformin as a growth inhibitor (17) with a mechanism related to partial inhibition of oxidative phosphorylation by an incompletely characterized action at respiratory complex I (19-21). We observed that KU-55933 has previously unrecognized effects on each of these measurements similar to those of metformin.

Furthermore, as shown in Figure 2 (D-F), KU-55933 and metformin reduced ATP levels, mitochondrial membrane potential, and oxygen consumption, indicating inhibition of oxidative phosphorylation. Sequellae of exposure to either KU-55933 or metformin included both increased necrosis, as assessed by propidium iodide (PI) and increased apoptosis, as assessed by annexinV–FITC (Figures 2G and 2H). Measurement of the percentage of cellular respiration uncoupled from ATP production (uncoupled respiration) (Figure 2F) revealed that metformin, apart from its previously partially characterized action on respiratory complex I, also increases the fraction of mitochondrial respiration devoted to uncoupled respiration, an action which would be expected to contribute to the decrease in ATP production caused by exposure to this agent. Unexpectedly, KU-55933 also increased the percentage of uncoupled respiration. Most importantly, our data allow us to conclude a significant

inhibition in total cellular respiration devoted to ATP production by both metformin and KU-55933.

Inhibition of ATM by KU-55933 decreases SCO2 levels in MCF-7 cells

As ATM activates p53 (23) and p53 upregulates oxidative phosphorylation by increasing SCO2 (24), we considered the possibility that ATM inhibition may act to decrease p53 activation and therefore decrease SCO2 levels, which would be expected to decrease oxidative phosphorylation, as observed. This potential mechanism was appealing in view of a recent report (25) showing that in muscle, ATM inhibition reduces cytochrome c oxidase activity (by an unspecified mechanism), an action that is the expected consequence of SCO2 reduction, and which would result in the reduced mitochondrial function. As shown in Figure 3, KU-55933 had a major time-dependent effect in reducing SCO2 level in MCF-7 cells, consistent with this hypothesis. Figure 3 also demonstrates the expected effects of KU-55933 as an activator of AMPK secondary to energy stress, accompanied by a decline in S6 phosphorylation, in keeping with the previously described inhibitory effects of AMPK on mTOR by metformin (17).

Effects of KU-55933 and metformin on metabolism and SCO2 levels

Figure 4A shows effects of KU-55933 and metformin on cell number, lactate production, and glucose consumption in HepG2 cells. Similar to the effects observed in the MCF-7 cell-line, we also see an increase in glucose consumption, an increase in lactate production, as well as a decrease in cell number in the HepG2 cell line.

Further studies did not support the view that this mechanism is universal. As shown in Figure 4B the KU-55933-induced decline in SCO2 levels was cell line specific, and HepG2 cells provide an example of growth inhibition, increasing glucose consumption, and lactate production induced by the ATM inhibitor, in the absence of a significant change in SCO2 level. We also found that in response to treatment with KU-55933, the LKB1 deficient cancer cell line, HeLa, exhibited AMPK-α phosphorylation. This indicates the existence of an LKB1-independent AMPK phosphorylation pathway.

Effects of KU-55933 on HCT116 p53$^{+/+}$ and HCT116 p53$^{-/-}$ cells

We examined the effects of KU-55933 on mitochondrial function as assessed by reduction of resazurin in isogenic p53 wild type and p53 loss of function HCT116 cells, and observed that even in the absence of p53, the kinase inhibitor reduced mitochondrial function, demonstrating that inhibition of ATM dependent p53 activation with subsequent p53-mediated dependent SCO2 activation cannot account for the effect of KU-55933 on mitochondrial function (Figure 5). The ability of KU-55933 to inhibit oxidative phosphorylation in p53 null cells also argues against a mediating role of TIGAR, a p53-dependent mitochondrial regulator (26).

Effects of KU-55933 and metformin on TCA metabolites

In order to better understand the consequences of KU-55933 and metformin on cellular energy metabolism, we measured levels of the metabolites indicated in Figure 6-1 and Supplemental 3 Table. Interestingly, both compounds increased

intracellular lactate and glucose, consistent with data in Figure 2 concerning glucose absorption and lactate excretion, and with increased glycolysis. Both KU-55933 and metformin significantly reduced the concentrations of the TCA cycle intermediates fumarate, malate, citrate, and alpha ketoglutarate. The compounds differed with their effects on succinate level, which was increased more than 5-fold by KU-55933, but reduced by metformin. NAD^+ levels were significantly reduced only by metformin. The underlying mechanisms require further study, but these data suggest that in the case of metformin, effects on respiratory complex I are important, and that the compound reduces generation of NAD^+ by complex I. Thus, metformin may not only reduce oxidative phosphorylation, but also inhibit the TCA cycle via its effect on redox status, given that the TCA enzymes isocitrate dehydrogenase and alpha ketoglutarate dehydrogenase require NAD^+. Although the effects of KU-55933 lead to many derangements similar to those seen with metformin, the lack of a significant effect on NAD^+ and the greater than 5-fold increase in succinate levels seen with KU-55933 exposure raise the possibility of an effect on respiratory complex II (Figure 6-2). Complex II oxidizes succinate to fumarate and reduces ubiquinone to ubiquinol. The former reaction is part of the TCA cycle, while the latter forms part of the respiratory chain of oxidative phosphorylation. It is conceivable that KU-55933 may directly or indirectly cause complex II dysfunction in a manner that reduces oxidative phosphorylation as well as conversion of succinate to fumarate, leading to accumulation of succinate and inhibition of the Krebs cycle.

Subcellular localization of ATM in MCF-7 cells

Our observations raise the possibility of a direct role for ATM in the mitochondria. While traditionally considered a nuclear protein, there is prior evidence (27) for cytoplasmic localization of ATM, but ATM has not previously been localized to mitochondria. We prepared a subcellular fraction highly enriched for mitochondria, and detected immunoreactivity to a mitochondrial marker (VDAC) and to ATM, but neither to the cytoplasmic marker tubulin nor the nuclear marker Ki67 (Figure 7).

Figure legends

Fig. 1. Growth inhibition by the ATM inhibitor KU-55933 and metformin. (A) MCF-7 ($LKB^{+/+}$) and HeLa ($LKB^{-/-}$) cancer cells in exponential stages of growth were seeded into 96-well plates with 10% FBS and after 24 hrs exposed to increasing concentrations of KU-55933 (ATM inhibitor) in media containing 1% FBS for 72 hrs. Cell growth was estimated by Alamar Blue dye reduction (resazurin (3 μM)).

Data are presented as mean ± S.E.M. from 3 independent experiments. In each experiment triplicates were used for each dose combination. **(B)** MCF-7 HepG2 , and HeLa cells were growth inhibited by KU-55933 and metformin. Cells were seeded into 96-well plates in the presence of 1% FBS and after 24 hrs treated with KU-55933 (10μM) or metformin (5mM). Cell growth was estimated by Alamar Blue dye reduction. Data are presented as mean ± S.E.M. from 4 independent experiments done in triplicate. * indicates a result significantly different from that obtained in the absence or presence of KU-55933 or metformin as determined by 2-way ANOVA (P<0.0001). **(C)** MCF-7 cells were transfected with 50 nM ATM-siRNA or with control siRNA. Twenty-four hours after transfection, cells were treated with KU-55933 (10μM) or metformin (5 mM) and incubated for 48 h in RPMI containing 1% FBS. Cell growth in each well was measured by counting cells using Trypan blue. Results using cell number or Alamar blue as endpoints yielded the same conclusions. Columns, mean of 3 independent experiments carried out in triplicate (n = 9); bars, S.E.M.

(D) After transfecting MCF-7 cells with 50 nM ATM-siRNA or with control siRNA, cells were lysed and prepared for immunoblot analyses using antibodies against ATM. ß-actin is shown as a loading control.

Fig. 1

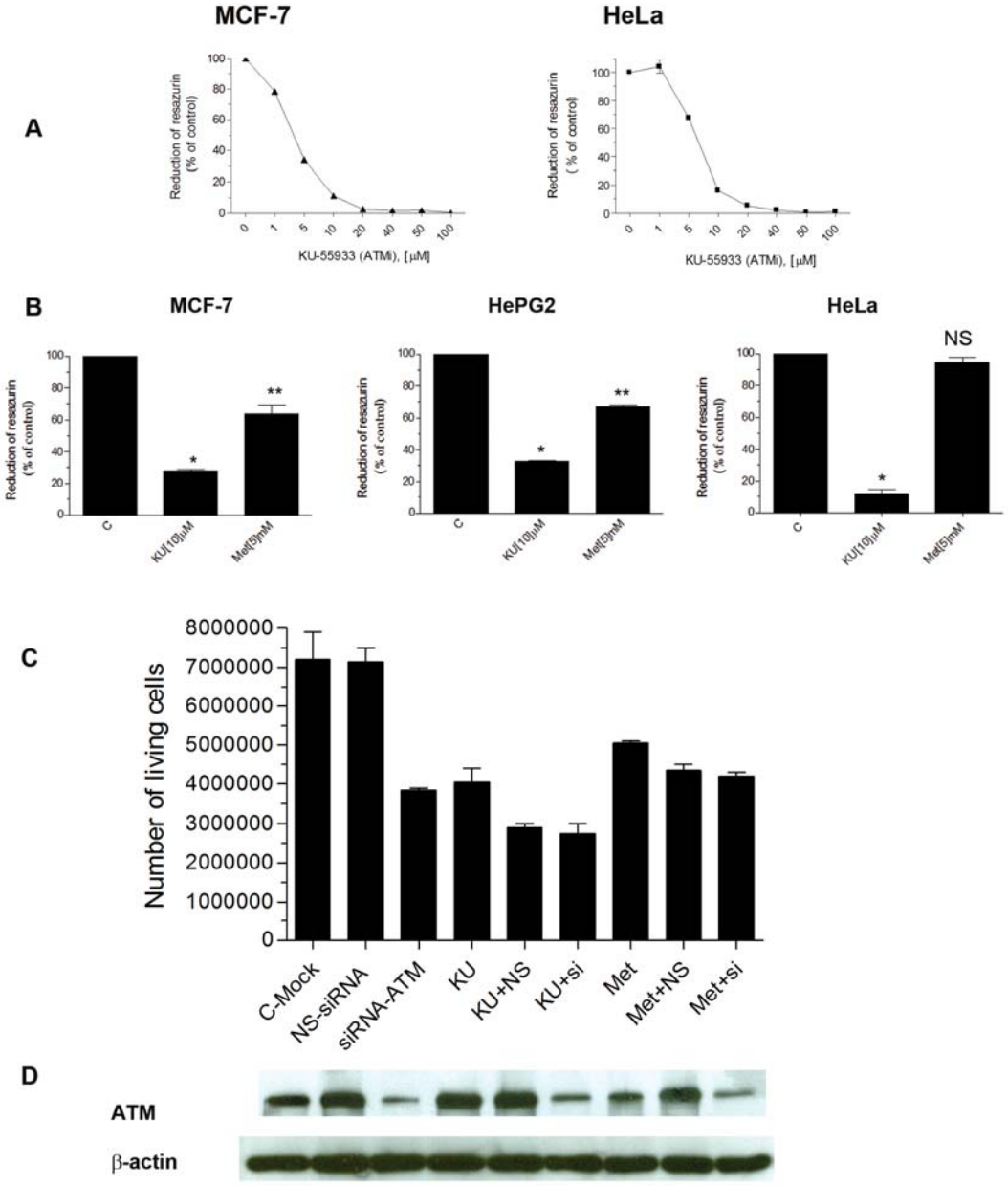

A
MCF-7
HeLa
Reduction of resazurin (% of control)
KU-55933 (ATMi), [μM]
B
MCF-7
HePG2
HeLa
NS
C
KU[10]μM
Met[5]nM
C
Number of living cells
C-Mock
NS-siRNA
siRNA-ATM
KU
KU+NS
KU+si
Met
Met+NS
Met+si
D
ATM
β-actin

Fig. 2. Effects of KU-55933 and metformin on metabolism in MCF-7 cells. Cells were exposed to KU (10μM) or metformin (5mM) for 72 hrs. **(A)** Effect of KU-55933 or metformin on cell number, obtained by counting cells able to exclude Trypan blue. Cell number was significantly reduced by KU-55933 (**P*=0.0042) and by metformin (***P*=0.0011). Effect of KU-55933 or metformin on lactate production. KU-55933 and metformin stimulated lactate production. Lactate production was significantly increased in cells treated with KU-55933 (**P*=0.0218) or metformin (***P*=0.0012). Effect of KU-55933 or metformin on glucose consumption. Glucose consumption was increased with exposure to either KU-55933 (**P*=0.0463) or metformin (***P*=0.0058) treated cells. **(B)** Effect of KU-5593 or metformin on cellular ATP levels. Cellular ATP levels decreased following KU-55933 or metformin exposure. We observed that both KU-55933 and metformin decreased ATP levels in MCF-7 cells. Results are the mean ± S.E (*n* = 4). (KU-55933 compared to control **P*=0.0015 and metformin compared to control ** *P*=0.0005). Effect of KU-55933 and metformin on membrane potential of mitochondria MCF-7 cells. Cells were incubated with JC-1 (2 μM) alone, or mitochondrial membrane potential-disruptors, rotenone (1 μM) or FCCP (1 μM). Mitochondrial membrane potential was probed with JC-1 and visualized via flow cytometry measurements. Loss of mitochondrial membrane potential (ΔΨ) is indicated by a decrease in FL2/FL1 fluorescence intensity ratio **(see supplemental figure 1 for flow cytometry data set)**. Results are expressed as mean ± S.E.M. (*n* = 4). KU-55933 (**P*=0.0003) and metformin (** *P*<0.0001) both significantly decreased ΔΨ. **(C)** Effect of KU- 55933 or metformin on cellular respiration.

Cellular respiration in MCF-7 cells treated with KU-55933 or metformin was compared with untreated cells. Results are the mean ± S.E.M. (KU-55933 compared to control *P=0.0045, and metformin compared to control ** P=0.0496). Uncoupled respiration was determined in the presence of oligomycin. The percentage of uncoupled respiration was calculated as: (uncoupled respiration/total mitochondrial respiration) X 100. **(D)** Effect of KU-55933 or metformin on cell death. KU-55933 or metformin treatment increased cell death (**see supplemental figure 2 for flow cytometry data set)**. Bars represent percentage of necrotic cells. Results are expressed as the mean ± S.E.M. (n = 3) in duplicate (KU-55933 compared to control *P=0.0005, and metformin compared to control **P=0.0299). Effect of KU-55933 or metformin on apoptosis. KU-55933 or metformin treatment resulted in increased apoptosis **(see supplemental figure 2 for flow cytometry data set).** Bars represent percentage of apoptotic cells. Results are expressed as the mean ± S.E.M. (n=3) in duplicate (KU-55933 compared to control *P<0.0001, and metformin compared to control **P=0.0458).

Fig.2

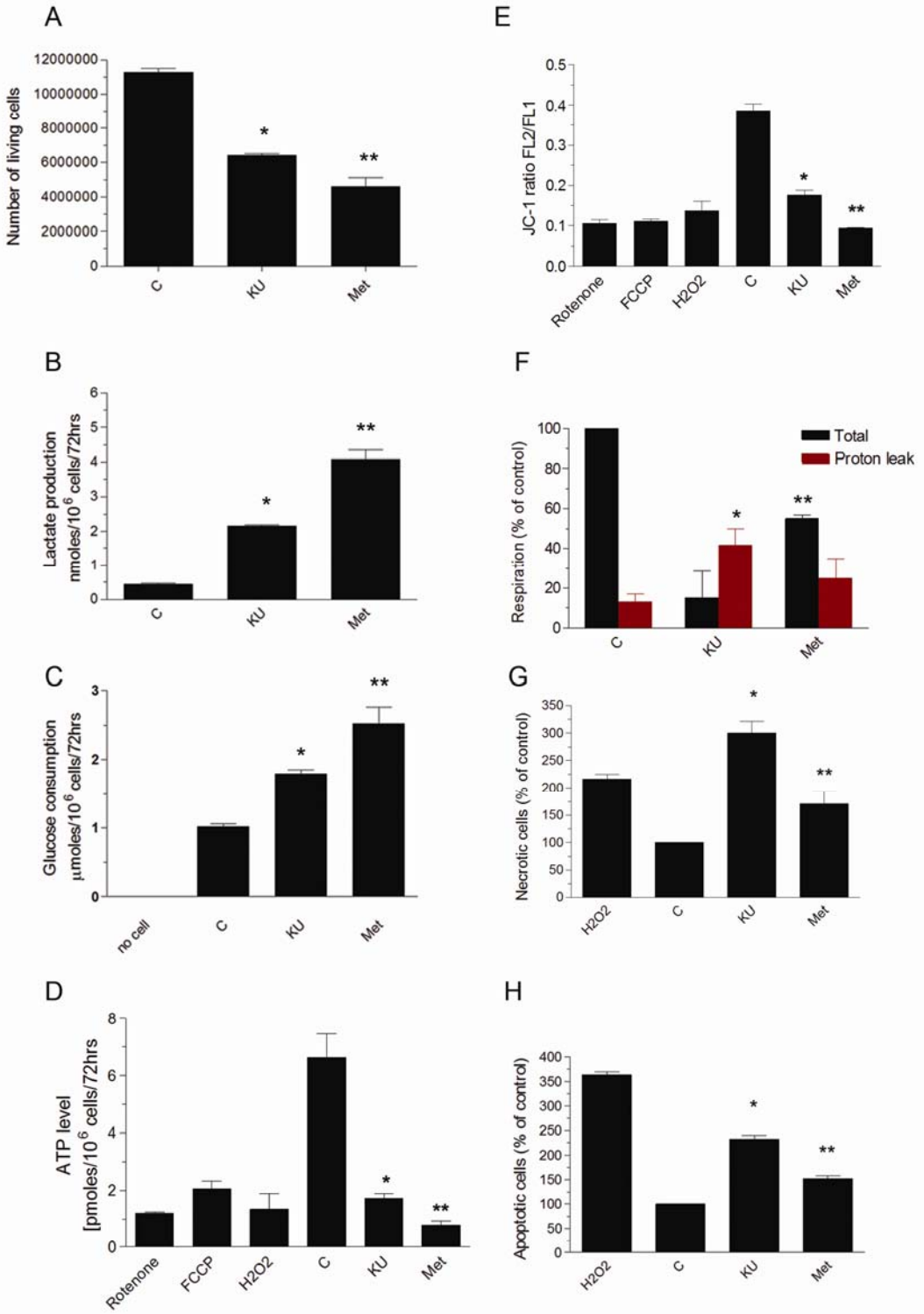

A
Number of living cells
12000000
10000000
8000000
6000000
4000000
2000000
0
C
KU
Met
E
JC-1 ratio FL2/FL1
0.5
0.4
0.3
0.2
0.1
0.0
Rotenone
FCCP
H2O2
B
Lactate production nmoles/10^6 cells/72hrs
F
Respiration (% of control)
Total
Proton leak
C
Glucose consumption µmoles/10^6 cells/72hrs
no cell
G
Necrotic cells (% of control)
D
ATP level [pmoles/10^6 cells/72hrs
H
Apoptotic cells (% of control)

Fig. 3. Inhibition of ATM by KU-55933 decreases SCO2 expression in MCF-7 cells. MCF-7 cells were exposed to KU-55933 (10μM) for the indicated time. After harvesting, cells were lysed and prepared for immunoblot analyses using antibodies against SCO2, phospho-ATM (Ser^{1981}), phosphorylated p53 (Ser^{15}), phospho-S6 ($Ser^{235/236}$), and phospho-AMPK (Thr^{172}). ß-actin is shown as a loading control. The results are representative of three individual experiments.

Fig. 3

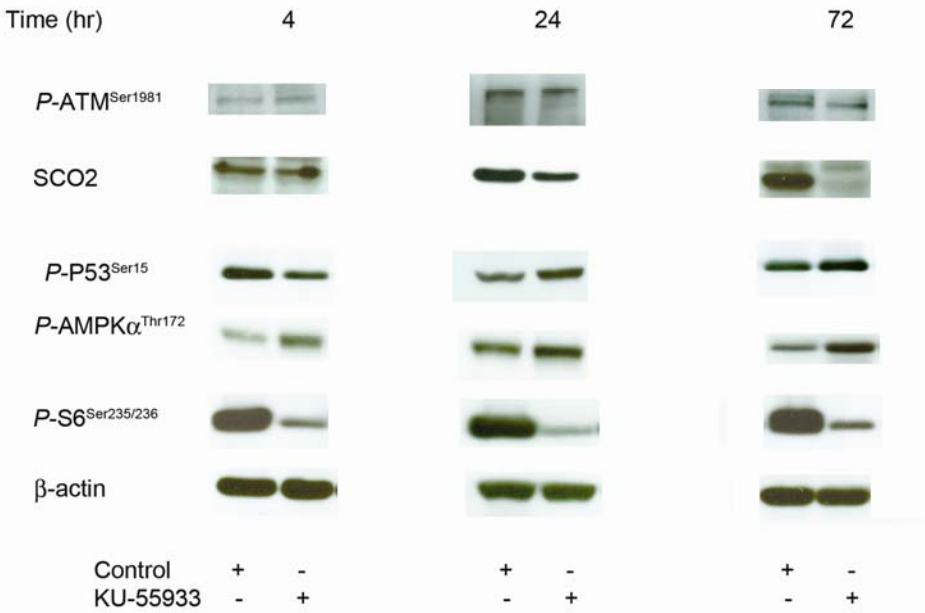
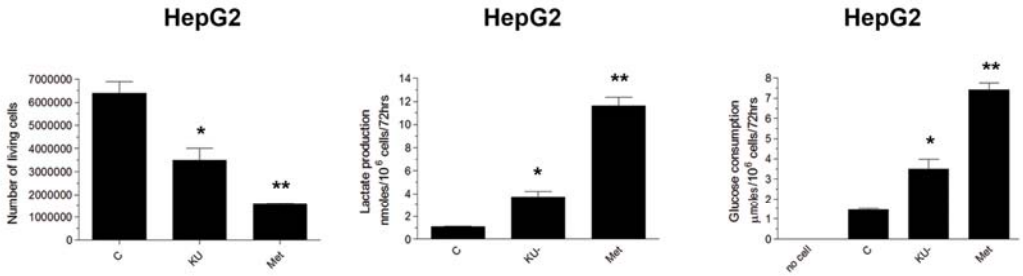

Time (hr)
4
24
72
P-ATMSer1981
SCO2
P-P53Ser15
P-AMPKαThr172
P-S6Ser235/236
β-actin
Control + - + - + -
KU-55933 - + - + - +

Fig. 4. Effects of KU-55933 and metformin on metabolism and SCO2 levels in different cancer cell lines. Cells were exposed to KU (10μM) or metformin (5mM) for 72 hrs. **(A)** Effect of KU-55933 or metformin on cell number obtained by counting cells able to exclude Trypan blue. Cell number was significantly reduced by KU-55933 (**P*=0.0394) and by metformin (***P*=0.0058). Effect of KU-55933 or metformin on lactate production. KU-55933 and metformin stimulated lactate production. Lactate production was significantly increased in cells treated with KU-55933 (**P*=0.0012) or metformin (***P*=0.0222). Effect of KU-5593 or metformin on glucose consumption. Glucose consumption was increased with exposure to either KU-55933 (**P*=0.0034) or metformin (***P*=0.0385) treated cells. **(B)** MCF-7, HeLa and HepG2 cells were exposed to KU-55933 (10μM) or metformin (5mM) for the indicated time. After harvesting, cells were lysed and prepared for immunoblot analyses using antibodies against SCO2, phospho-AMPK (Thr^{172}). ß-actin is shown as a loading control. The results are representative of three individual experiments.

Fig-4

A


HepG2
HepG2
HepG2
Number of living cells
Lactate production nmoles/10^6 cells/72hrs
Glucose consumption µmoles/10^6 cells/72hrs
C
KU
Met
no cell

B

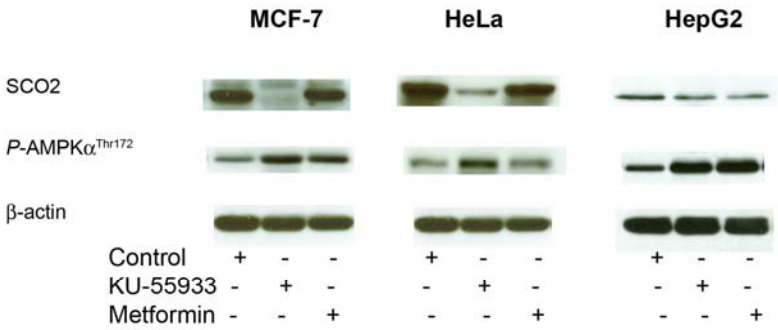

MCF-7
HeLa
HepG2
SCO2
P-AMPKα Thr172
β-actin
Control
KU-55933
Metformin

Fig. 5. Effects of KU-55933 on HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cells. Cells were seeded into 96-well plates with 10% FBS and after 24 hrs. exposed to KU-55933 (10μM) or metformin (5mM) in DMEM containing 1% FBS for 72 hrs. **(A)** Cell growth was estimated by Alamar Blue dye reduction. Results are presented as mean ± S.E.M. from 3 independent experiments in triplicate. HCT116 $p53^{+/+}$ cell growth was significantly inhibited by both KU-55933 (*P<0.0001) and metformin (** *P*=0.0013). For HCT116 $p53^{-/-}$ cells, KU-55933 significantly inhibited growth (**P*=0.0002) however this effect was not seen with metformin exposure (*P*=0.223). (**B**) Under the above conditions, after harvesting, cells were lysed and prepared for immunoblot analyses using antibodies against phosphorylated p53 (Ser^{15}), and SCO2. ß-actin is shown as a loading control.

Fig. 5

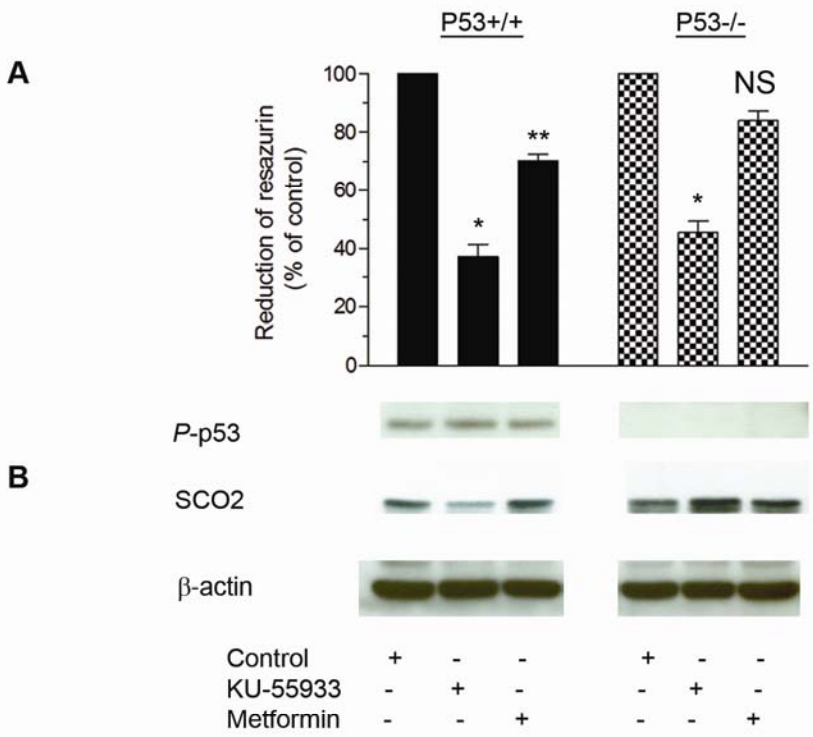

A
P53+/+
P53-/-
Reduction of resazurin (% of control)
100
80
60
40
20
0
*
**
*
NS
B
P-p53
SCO2
β-actin
Control + - - + - -
KU-55933 - + - - + -
Metformin - - + - - +

Fig. 6. Effects of KU-55933 and metformin on TCA metabolites. (6-1) TCA metabolites were measured by NMR. Changes in metabolite levels with KU and metformin compared to control are depicted. *P* values for the various TCA metabolites are shown in supplemental 3 Table **(6-2)** Schema of metabolites effected based on our findings, **(A)** ATM is hypothesized to have a role in oxidative phosphorylation, effecting respiratory complex II. Therefore the ATM inhibitor KU55933 leads not only to reduced ATP production, but also to accumulation of succinate. **(B)** KU-55933 also may in some cells reduce oxidative phosphorylation by a mechanism involving SCO2, as discussed in the text. **(C)** Metformin also acts to inhibit oxidative phosphorylation, but prior evidence together with our findings of decreased NAD^+ suggest a site of action involving respiratory complex I. **(D)** Both KU-55933 and metformin exposure lead to increased glucose uptake and lactate production, consistent with a compensatory increase in glycolysis following decreased oxidative phosphorylation. **(E)** Our observations provide evidence for reduced concentrations of TCA cycle intermediates with exposure to either KU-55933 or metformin, but we postulate different reasons for this: metformin may reduce TCA cycles activity because of a reduction in supply of complex I-generated NAD^+, while KU-55933 may act to inhibit conversion of succinate to fumarate.

ATM, Ataxia Telangiectasia Mutated protein; SCO2, Synthesis of Cytochrome C Oxidase 2; AMPK, AMP-activated protein kinase; TSC1/TSC2, Tuberous Sclerosis 1/Tuberous Sclerosis 2; mTOR, Mammalian Target of Rapamycin complex 1; rpS6, ribosomal protein S6.

Fig. 6-1

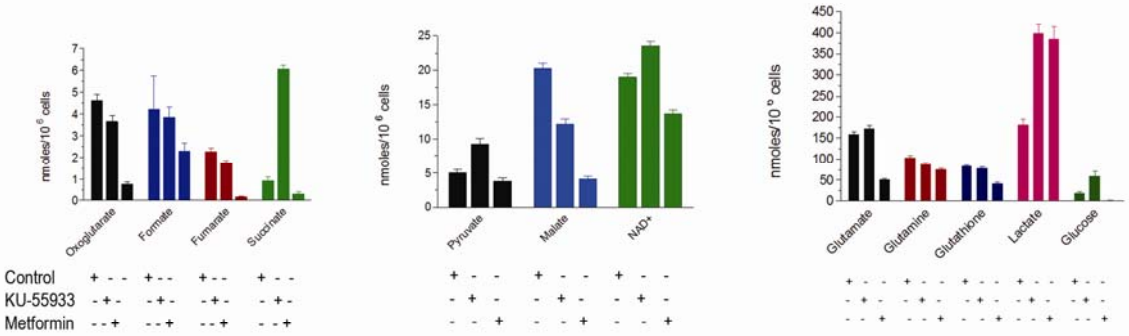

nmoles/10 6 cells
Oxoglutarate
Formate
Fumarate
Succinate
Control
KU-55933
Metformin
nmoles/10 6 cells
Pyruvate
Malate
NAD+
nmoles/10 5 cells
Glutamate
Glutamine
Glutathione
Lactate
Glucose

Fig. 6-2

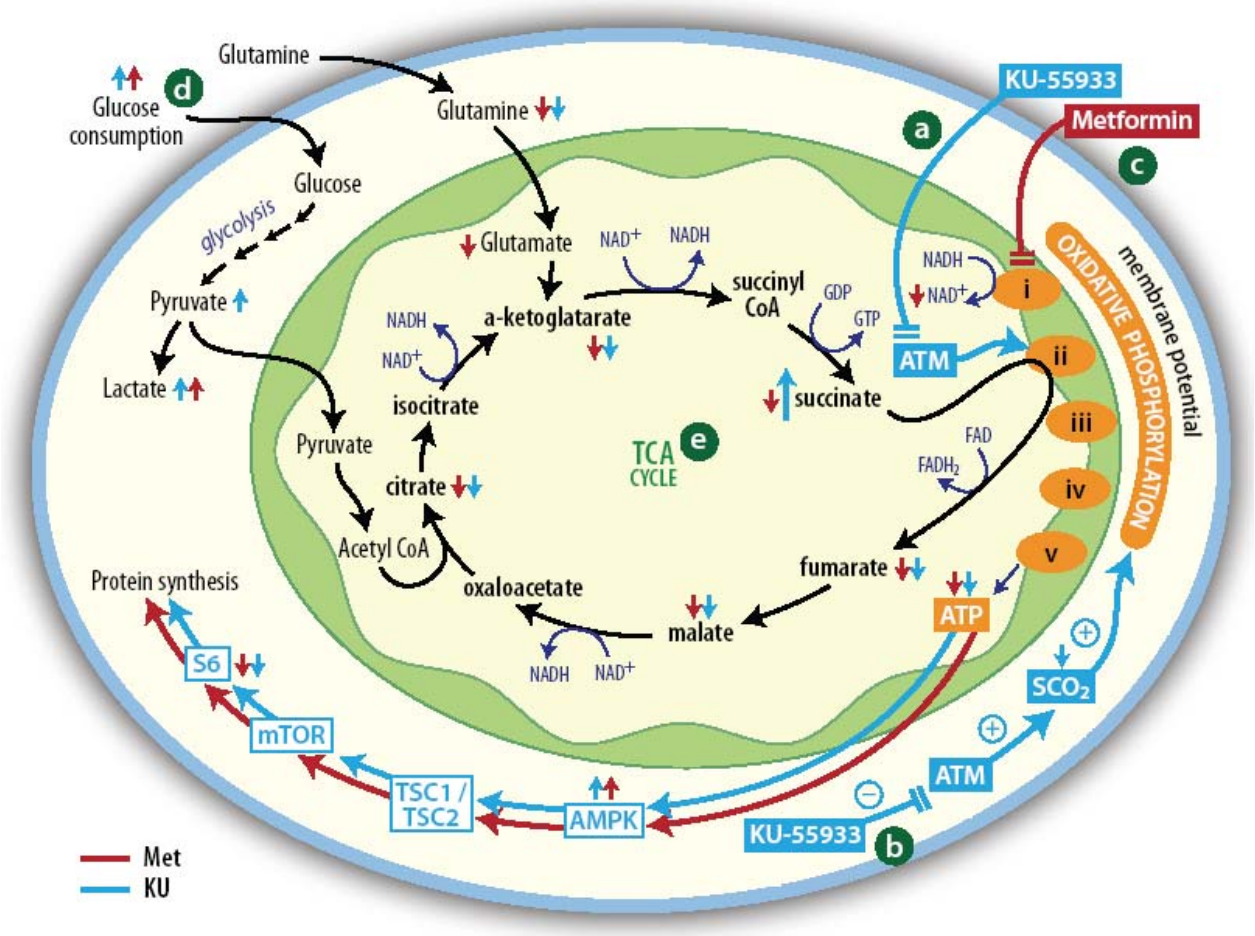
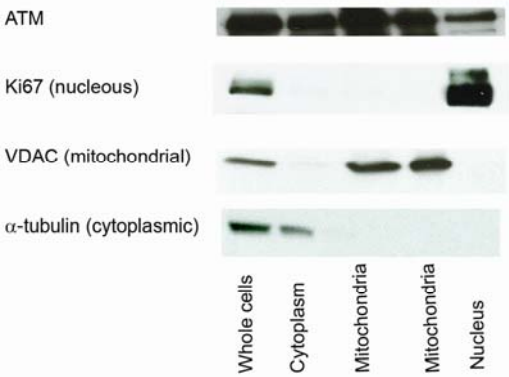

Glutamine
Glucose consumption
d
Glucose
glycolysis
Pyruvate
Lactate
Glutamine
Glutamate
NAD+
NADH
succinyl CoA
GDP
GTP
a-ketoglatarate
NADH
NAD+
isocitrate
Pyruvate
citrate
TCA CYCLE
e
succinate
FAD
FADH2
Acetyl CoA
oxaloacetate
fumarate
malate
NADH
NAD+
Protein synthesis
S6
mTOR
TSC1 / TSC2
AMPK
KU-55933
b
ATM
SCO2
ATP
KU-55933
Metformin
a
c
ATM
NADH
NAD+
i
ii
iii
iv
v
OXIDATIVE PHOSPHORYLATION
membrane potential
Met
KU

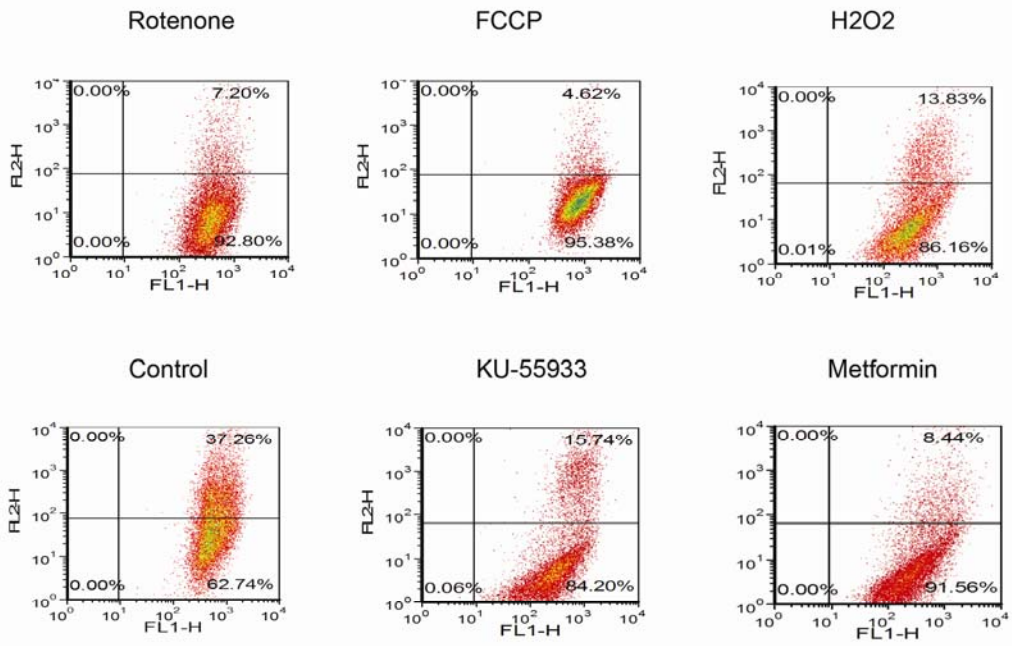
Fig. 7. Subcellular localization of ATM. Total MCF-7 cell lysate and MCF-7 cells fractionated into cytoplasmic, nuclear and mitochondrial extracts were immunoblotted with ATM antibody, α-Tubulin (cytoplasmic marker), Ki67 (nuclear marker) and VDAC (mitochondrial marker). The results indicate ATM immunoreactivity in mitochondrial extracts that are negative for cytoplasmic and nuclear markers.

Fig. 7


ATM
Ki67 (nucleous)
VDAC (mitochondrial)
α-tubulin (cytoplasmic)
Whole cells
Cytoplasm
Mitochondria
Mitochondria
Nucleus

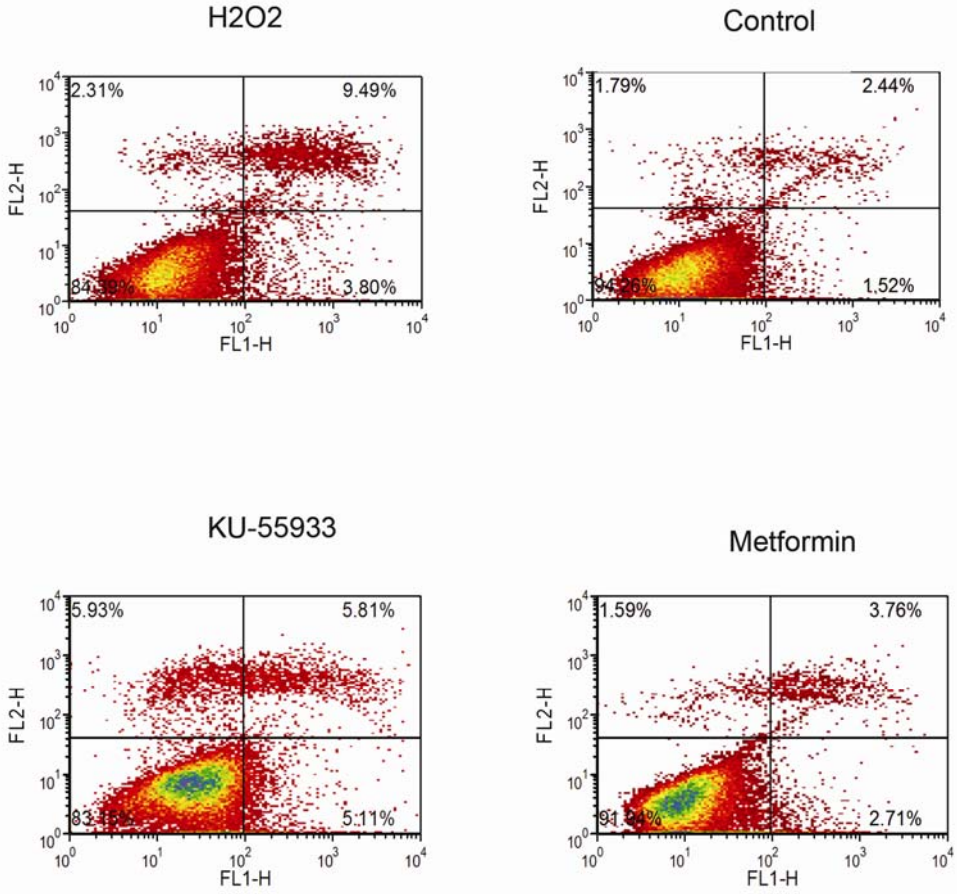
Supplemental 1. Loss of mitochondrial membrane potential ($\Delta\Psi$) as indicated by flow cytometry is seen by a decrease in FL2/FL1 fluorescence intensity ratio. Results indicate that treatment with KU-55933 or metformin decreases mitochondrial membrane potential of MCF-7 cells.

Supplemental 1



Supplemental 2. Cytograms of PI uptake (ordinate) vs. annexin V binding (abscissa). Apoptotic (annexin V^+/ PI^-), vital (V^-/PI^-), and damaged (annexin V^-/PI^+) cells are shown. Cells displayed an increase in cell death and apoptosis when treated with KU-55933 (**P*<0.0001) or metformin (***P*= 0.0155).

Supplemental 2



Supplemental 3. Significance of differences in metabolites levels in the MCF-7 treated with KU-55933 or metformin. (n=9)

Metabolite / Treatment	Oxoglutarate	Formate	Fumarate	Succinate
C *vs* KU	***P* = 0.0200**	**NS**	***P* = 0.0078**	***P* < 0.0001**
C *vs* met	***P* < 0.0001**	**NS**	***P* < 0.0001**	***P* = 0.0275**
KU *vs* met	***P* < 0.0001**	**NS**	***P* < 0.0001**	***P* < 0.0001**

Metabolite / Treatment	Pyruvate	Malate	NAD+
C *vs* KU	***P* = 0.0002**	***P* < 0.0001**	***P* < 0.0001**
C *vs* met	***P* =NS**	***P* < 0.0001**	***P* < 0.0001**
KU *vs* met	***P* < 0.0001**	***P* < 0.0001**	***P* < 0.0001**

Metabolite / Treatment	Glutamate	Glutamine	Glutathione	Lactate	Glucose
C *vs* KU	***P* = NS**	***P* = 0.0105**	***P* = NS**	***P* < 0.0001**	***P* = 0.0009**
C *vs* met	***P* < 0.0001**	***P* < 0.0001**	***P* < 0.0001**	***P* < 0.0001**	***P* < 0.0001**
KU *vs* met	***P* < 0.0001**	***P* = NS**	***P* < 0.0001**	***P* = NS**	***P* = NS**

Discussion

ATM-related proteins are ancient in evolutionary terms (28), and our findings add to recent evidence suggesting that these kinases have important functions in addition to those initially described that are related to DNA repair [(2)]. A prior study of fibroblasts obtained from a patient with the ataxia telangiectasia syndrome (16) provided early evidence that ATM deficiency is associated with abnormalities in mitochondrial function that could not be accounted for by DNA repair deficits. Our studies extend this work by showing that pharmacologic inhibition of ATM with KU-55933 results in reduced mitochondrial membrane potential, reduced coupled respiration, and reduced ATP levels, while increasing glucose uptake and lactic acid production. These actions are similar to those of metformin, a compound known to partially inhibit respiratory complex I. We speculate that the increased glucose uptake and lactic acid production are a consequence of increased glycolysis that partially compensates for the decrease in mitochondrial ATP production in the setting of loss of function of ATM, suggesting that neoplasms involving loss of function of ATM will exhibit a "Warburg" metabolic phenotype.

Although inhibition of ATM by KU-55933 decreased expression of SCO2 (a protein required for cytochrome c oxidase assembly) in a p53 dependent fashion, the compound retained antiproliferative activity in p53 null cells, indicating that the actions of p53 are dispensable for the effects of KU-55933 on metabolism and proliferation. ATM has other substrates than p53 (29,30), including Sp1 (31), that may alter nuclear gene expression patterns in ways that influence metabolism.

Alternatively, our finding that ATM is present in mitochondria raises the possibility that it may play a more proximal role in regulating oxidative phosphorylation. The significant increase in succinate concentration associated with exposure to the ATM inhibitor allows speculation that the drug may have a direct or indirect effect that compromises the activity of respiratory complex II (succinate:ubiquinone oxidoreductase) (Figure 6-2). Little is known about potential regulation of the activity of this complex by phosphorylation (32), but we did not detect a consensus sequence for the kinase activity of ATM against any of the subunits, arguing against a direct effect of ATM on this complex, despite the evidence for a requirement of ATM for optimum mitochondrial function.

In keeping with the fact that mitochondrial toxins are commonly found in nature, metformin is a respiratory complex I inhibitor derived from plant guanidines (33), and the plant toxin 3-nitropropionic acid (34) as well as the atpenin antibiotics (35) are complex II inhibitors. While complete inhibition of oxidative phosphorylation by agents such as cyanide is obviously lethal (36), and crude attempts to inhibit oxidative phosphorylation in cancer patients with cyanogenic molecules such as amygdalin derivatives are discredited (37), attention is being given to the possibility that reduction of oxidative phosphorylation by biguanides such as metformin may be useful in cancer treatment (21,38).

Despite the fact that metformin has credentials as a complex I inhibitor, it is known to have a favorable safety profile in the treatment of type II diabetes (21,38).

It has both AMPK dependent (17,39) and AMPK independent (40,41) antiproliferative actions. The safety and efficacy of inhibitors of oxidative phosphorylation will relate in part to their cellular and whole organism pharmacokinetic profiles. It remains to be determined if the effects of KU and metformin we observed on levels of TCA cycle intermediates, uncoupled respiration, and oxidative phosphorylation are achievable *in vivo*.

The basis for the surprising observation that polymorphisms in the ATM locus influence efficacy of metformin in diabetes treatment (42) remains obscure. It has been pointed out (14) that laboratory evidence used to support the genetic results for this funding is open to question, as the ATM inhibitor used in the experiments may inhibit metformin influx into cells (43). More importantly in the context of our results, however, is the fact that KU-55933 was noted (14) to enhance the phosphorylation of AMPK, finding which was unexplained by the authors (14) but is consistent with our results.

There are recent precedents for regulation of metabolism by oncogenes and tumor suppressor genes (44,45). Our results add to the evidence that ATM is a regulatory kinase with relevance to cellular energy metabolism. While the classic tumor suppressor properties of ATM are related to a requirement for the protein for normal DNA repair, our results provide evidence that the antiproliferative consequences of ATM inhibition arise as a consequence of a novel role for ATM in mitochondrial function.

Acknowledgments

We thank Drs Ernesto Schiffrin for sharing laboratory resources and Orval Mamer for sharing his extensive knowledge of GC/MS, Marie-José Blouin for reviewing the manuscript prior to submission and Ms. L. Lui for valuable assistance in manuscript preparation.

NMR experiments were recorded at the Québec/Eastern Canada High Field NMR Facility, supported by the Natural Sciences and Engineering Research Council of Canada, the Canada Foundation for Innovation, the Québec ministère de la recherche en science et technologie, and McGill University.

Reference List

1. Lavin MF. (2008) Ataxia-telangiectasia: from a rare disorder to a paradigm for cell signalling and cancer. Nat Rev Mol Cell Biol 9: 759-769.

2. Savitsky K, Bar-Shira A, Gilad S, Rotman G, Ziv Y, et al. (1995) A single ataxia telangiectasia gene with a product similar to PI-3 kinase. Science 268: 1749-1753.

3. Helleday T, Petermann E, Lundin C, Hodgson B, Sharma RA. (2008) DNA repair pathways as targets for cancer therapy. Nat Rev Cancer 8: 193-204.

4. Hickson I, Zhao Y, Richardson CJ, Green SJ, Martin NM, et al. (2004) Identification and characterization of a novel and specific inhibitor of the ataxia-telangiectasia mutated kinase ATM. Cancer Res 64: 9152-9159.

5. Li Y, Yang DQ. (2010) The ATM inhibitor KU-55933 suppresses cell proliferation and induces apoptosis by blocking Akt in cancer cells with overactivated Akt. Mol Cancer Ther 9: 113-125.

6. Ditch S, Paull TT. (2012) The ATM protein kinase and cellular redox signaling: beyond the DNA damage response. Trends Biochem Sci 37: 15-22.

7. Yang DQ, Kastan MB. (2000) Participation of ATM in insulin signalling through phosphorylation of eIF-4E-binding protein 1. Nat Cell Biol 2: 893-898.

8. Shackelford RE, Innes CL, Sieber SO, Heinloth AN, Leadon SA, et al. (2001) The Ataxia telangiectasia gene product is required for oxidative stress-induced G1 and G2 checkpoint function in human fibroblasts. J Biol Chem 276: 21951-21959.

9. Guo Z, Kozlov S, Lavin MF, Person MD, Paull TT. (2010) ATM activation by oxidative stress. Science 330: 517-521.

10. Alexander A, Cai SL, Kim J, Nanez A, Sahin M, et al. (2010) ATM signals to TSC2 in the cytoplasm to regulate mTORC1 in response to ROS. Proc Natl Acad Sci U S A 107: 4153-4158.

11. Eaton JS, Lin ZP, Sartorelli AC, Bonawitz ND, Shadel GS. (2007) Ataxia-telangiectasia mutated kinase regulates ribonucleotide reductase and mitochondrial homeostasis. J Clin Invest 117: 2723-2734.

12. Kruger A, Ralser M. (2011) ATM is a redox sensor linking genome stability and carbon metabolism. Sci Signal 4: e17.

13. Cosentino C, Grieco D, Costanzo V. (2011) ATM activates the pentose phosphate pathway promoting anti-oxidant defence and DNA repair. EMBO J 30: 546-555.

14. Yee SW, Chen L, Giacomini KM. (2012) The role of ATM in response to metformin treatment and activation of AMPK. Nat Genet 44: 359-360.

15. Woods A, Leiper JM, Carling D. (2012) The role of ATM in response to metformin treatment and activation of AMPK. Nat Genet 44: 360-361.

16. Ambrose M, Goldstine JV, Gatti RA. (2007) Intrinsic mitochondrial dysfunction in ATM-deficient lymphoblastoid cells. Hum Mol Genet 16: 2154-2164.

17. Zakikhani M, Dowling R, Fantus IG, Sonenberg N, Pollak M. (2006) Metformin is an AMP kinase-dependent growth inhibitor for breast cancer cells. Cancer Res 66: 10269-10273.

18. Zakikhani M, Dowling RJ, Sonenberg N, Pollak MN. (2008) The effects of adiponectin and metformin on prostate and colon neoplasia involve activation of AMP-activated protein kinase. Cancer Prev Res (Phila Pa) 1: 369-375.

19. Owen MR, Doran E, Halestrap AP. (2000) Evidence that metformin exerts its anti-diabetic effects through inhibition of complex 1 of the mitochondrial respiratory chain. Biochem J 348 Pt 3: 607-614.

20. Schafer G. (1969) Site-specific uncoupling and inhibition of oxidative phosphorylation by biguanides. II. Biochim Biophys Acta 172: 334-337.

21. Viollet B, Guigas B, Sanz GN, Leclerc J, Foretz M, et al. (2012) Cellular and molecular mechanisms of metformin: an overview. Clin Sci (Lond) 122: 253-270.

22. Turner N, Li JY, Gosby A, To SW, Cheng Z, et al. (2008) Berberine and its more biologically available derivative, dihydroberberine, inhibit mitochondrial respiratory complex I: a mechanism for the action of berberine to activate AMP-activated protein kinase and improve insulin action. Diabetes 57: 1414-1418.

23. Canman CE, Lim DS, Cimprich KA, Taya Y, Tamai K, et al. (1998) Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. Science 281: 1677-1679.

24. Matoba S, Kang JG, Patino WD, Wragg A, Boehm M, et al. (2006) p53 regulates mitochondrial respiration. Science 312: 1650-1653.

25. Patel AY, McDonald TM, Spears LD, Ching JK, Fisher JS. (2011) Ataxia telangiectasia mutated influences cytochrome c oxidase activity. Biochem Biophys Res Commun 405: 599-603.

26. Bensaad K, Tsuruta A, Selak MA, Vidal MN, Nakano K, et al. (2006) TIGAR, a p53-inducible regulator of glycolysis and apoptosis. Cell 126: 107-120.

27. Barlow C, Ribaut-Barassin C, Zwingman TA, Pope AJ, Brown KD, et al. (2000) ATM is a cytoplasmic protein in mouse brain required to prevent lysosomal accumulation. Proc Natl Acad Sci U S A 97: 871-876.

28. Morrow DM, Tagle DA, Shiloh Y, Collins FS, Hieter P. (1995) TEL1, an S. cerevisiae homolog of the human gene mutated in ataxia telangiectasia, is functionally related to the yeast checkpoint gene MEC1. Cell 82: 831-840.

29. Linding R, Jensen LJ, Ostheimer GJ, van Vugt MA, Jorgensen C, et al. (2007) Systematic discovery of in vivo phosphorylation networks. Cell 129: 1415-1426.

30. Matsuoka S, Ballif BA, Smogorzewska A, McDonald ER, III, Hurov KE, et al. (2007) ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. Science 316: 1160-1166.

31. Olofsson BA, Kelly CM, Kim J, Hornsby SM, Azizkhan-Clifford J. (2007) Phosphorylation of Sp1 in response to DNA damage by ataxia telangiectasia-mutated kinase. Mol Cancer Res 5: 1319-1330.

32. Tomitsuka E, Kita K, Esumi H. (2009) Regulation of succinate-ubiquinone reductase and fumarate reductase activities in human complex II by phosphorylation of its flavoprotein subunit. Proc Jpn Acad Ser B Phys Biol Sci 85: 258-265.

33. Witters LA. (2001) The blooming of the French lilac. J Clin Invest 108: 1105-1107.

34. Sun F, Huo X, Zhai Y, Wang A, Xu J, et al. (2005) Crystal structure of mitochondrial respiratory membrane protein complex II. Cell 121: 1043-1057.

35. Miyadera H, Shiomi K, Ui H, Yamaguchi Y, Masuma R, et al. (2003) Atpenins, potent and specific inhibitors of mitochondrial complex II (succinate-ubiquinone oxidoreductase). Proc Natl Acad Sci U S A 100: 473-477.

36. Way JL. (1984) Cyanide intoxication and its mechanism of antagonism. Annu Rev Pharmacol Toxicol 24: 451-481.

37. Milazzo S, Ernst E, Lejeune S, Boehm K, Horneber M. (2011) Laetrile treatment for cancer. Cochrane Database Syst Rev 11: CD005476.

38. Pollak M. (2010) Metformin and other biguanides in oncology: advancing the research agenda. Cancer Prev Res (Phila) 3: 1060-1065.

39. Shaw RJ, Lamia KA, Vasquez D, Koo SH, Bardeesy N, et al. (2005) The kinase LKB1 mediates glucose homeostasis in liver and therapeutic effects of metformin. Science 310: 1642-1646.

40. Foretz M, Hebrard S, Leclerc J, Zarrinpashneh E, Soty M, et al. (2010) Metformin inhibits hepatic gluconeogenesis in mice independently of the LKB1/AMPK pathway via a decrease in hepatic energy state. J Clin Invest 120: 2355-2369.

41. Kalender A, Selvaraj A, Kim SY, Gulati P, Brule S, et al. (2010) Metformin, independent of AMPK, inhibits mTORC1 in a rag GTPase-dependent manner. Cell Metab 11: 390-401.

42. Zhou K, Bellenguez C, Spencer CC, Bennett AJ, Coleman RL, et al. (2011) Common variants near ATM are associated with glycemic response to metformin in type 2 diabetes. Nat Genet 43: 117-120.

43. Minematsu T, Giacomini KM. (2011) Interactions of tyrosine kinase inhibitors with organic cation transporters and multidrug and toxic compound extrusion proteins. Mol Cancer Ther 10: 531-539.

44. Vander Heiden MG, Cantley LC, Thompson CB. (2009) Understanding the Warburg effect: the metabolic requirements of cell proliferation. Science 324: 1029-1033.

45. Lemarie A, Grimm S. (2011) Mitochondrial respiratory chain complexes: apoptosis sensors mutated in cancer? Oncogene 30: 3985-4003.

46. Bunz F, Dutriaux A, Lengauer C, Waldman T, Zhou S, et al. (1998) Requirement for p53 and p21 to sustain G2 arrest after DNA damage. Science 282: 1497-1501.

47. Troiano L, Ferraresi R, Lugli E, Nemes E, Roat E, et al. (2007) Multiparametric analysis of cells with different mitochondrial membrane potential during apoptosis by polychromatic flow cytometry. Nat Protoc 2: 2719-2727.

48. Frezza C, Cipolat S, Scorrano L. (2007) Organelle isolation: functional mitochondria from mouse liver, muscle and cultured fibroblasts. Nat Protoc 2: 287-295.

49. Xu Q, Vu H, Liu L, Wang TC, Schaefer WH. (2011) Metabolic profiles show specific mitochondrial toxicities in vitro in myotube cells. J Biomol NMR 49: 207-219.

50. Reily MD, Robosky LC, Manning ML, Butler A, Baker JD, et al. (2006) DFTMP, an NMR reagent for assessing the near-neutral pH of biological samples. J Am Chem Soc 128: 12360-12361.

51. Shaka AJ, Lee CJ, Pines A. (1988) Iterative schemes for bilinear operators; application to spin decoupling. Journal of Magnetic Resonance 77: 274-293.

52. Cui Q, Lewis IA, Hegeman AD, Anderson ME, Li J, et al. (2008) Metabolite identification via the Madison Metabolomics Consortium Database. Nat Biotechnol 26: 162-164.

53. Wishart DS, Knox C, Guo AC, Eisner R, Young N, et al. (2009) HMDB: a knowledgebase for the human metabolome. Nucleic Acids Res 37: D603-D610.

54. Weljie AM, Newton J, Mercier P, Carlson E, Slupsky CM. (2006) Targeted profiling: quantitative analysis of 1H NMR metabolomics data. Anal Chem 78: 4430-4442.

Chapter IV

Conclusions and Future Work

Conclusions and Future Work

The possible role of biguanides in cancer prevention and/or treatment has been receiving much attention in recent years. While the effects of metformin on systemic metabolic functions have been elucidated in diabetes, there is a gap in knowledge regarding direct influences at the level of the tumor. Our work demonstrates that while metformin increases glycolysis and reduces oxidative phosphorylation, which are phenotypes of the "Warburg" phenomenon, it also leads to a decline in proliferation. This effect varies with carbon source as cells supplied with only glutamine are significantly inhibited by metformin, while cells grown on glucose only are not. Our results suggest that glutamine utilization sensitizes cells to metformin. This may yield be relevant to design of clinical trials of biguianides for neoplastic disease.

In order to further validate our hypothesis that cells using predominantly glutamine as a carbon source have an increased sensitivity to metformin, we varied cells in accordance to glutamine dependence. As prior evidence has showed that glutamine influx and metabolism are increased by *myc* we decided to use an experimental system in which *myc* levels were varied. Our results revealed that overexpression of this oncogene was associated to sensitization to the antiproliferative effects of metformin. Further signaling end-points involving glutamine metabolism such as the enzyme glutaminase (GLS) responsible for the conversion of glutamine into glutamate and genes involved in glutamine transport

such as ASCT2 and SLC7A1 will need to be examined in our to extend our understanding of the inhibition by metformin in glutamine addicted cells. In vivo Myc tumor models will be useful in exploring the anti-neoplastic effects of metformin. Since Myc activation/amplification is one of the most common oncogenic events observed in a wide variety of cancers such as human lymphomas, neuroblastomas, and small cell lung cancers, perhaps the use of metformin could be targeted to these types of tumors.

We also compared the actions of metformin to that of the ATM inhibitor. We demonstrated that pharmacologic inhibition of ATM with KU-55933 resulted in reduced mitochondrial membrane potential, reduced coupled respiration, and reduced ATP levels while increasing glucose uptake and lactic acid production. These metabolic changes were similar to those of metformin. These results provide evidence that the antiproliferative consequences of ATM inhibition may be due to the role of ATM in mitochondrial function. In addition, further experiments involving inhibition by KU-55933 as a function of carbon source will also be of interest.

As both metformin and KU-55933 inhibited oxidative phosphorylation, it would be of interest to combine these agents with those inhibiting glycolysis such as 2-deoxyglucose to assess whether this would potentiate the antiproliferative effect of the drugs. However, as ATM has a classic role in DNA repair, further experiments

need to be done in order to establish the clinical efficacy and toxicity of ATM inhibitors in cancer.

Reference List

1. Algire C, Amrein L, Bazile M, David S, Zakikhani M, & Pollak M 2011 Diet and tumor LKB1 expression interact to determine sensitivity to anti-neoplastic effects of metformin in vivo. *Oncogene* **30** 1174-1182.

2. Algire C, Zakikhani M, Blouin M-J, Shuai JH, & Pollak M 2008 Metformin attenuates the stimulatory effect of a high energy diet on in vivo H59 carcinoma growth. *Endocr Relat Cancer* **15** 833-839.

3. Alimova IN, Liu B, Fan Z, Edgerton SM, Dillon T, Lind SE, & Thor AD 2009 Metformin inhibits breast cancer cell growth, colony formation and induces cell cycle arrest in vitro. *Cell Cycle* **8** 909-915.

4. Ambrose M, Goldstine JV, & Gatti RA 2007 Intrinsic mitochondrial dysfunction in ATM-deficient lymphoblastoid cells. *Hum Mol Genet* **16** 2154-2164.

5. Azoulay L, Dell'Aniello S, Gagnon B, Pollak M, & Suissa S 2011 Metformin and the incidence of prostate cancer in patients with type 2 diabetes. *Cancer Epidemiol Biomarkers Prev* **20** 337-344.

6. Bailey CJ & Day C 2004 Metformin: its botanical background. *Pract Diab Int* **21** 115-117.

7. Bailey CJ & Turner RC 1996 Metformin. *N Engl J Med* **334** 574-579.

8. Bakkenist CJ & Kastan MB 2003 DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. *Nature* **421** 499-506.

9. Barlow C, Ribaut-Barassin C, Zwingman TA, Pope AJ, Brown KD, Owens JW, Larson D, Harrington EA, Haeberle AM, Mariani J, Eckhaus M, Herrup K, Bailly Y, & Wynshaw-Boris A 2000 ATM is a cytoplasmic protein in mouse brain required to prevent lysosomal accumulation. *Proc Natl Acad Sci USA* **97** 871-876.

10. Bayraktar S, Hernadez-Aya LF, Lei X, Meric-Bernstam F, Litton JK, Hsu L, Hortobagyi GN, & Gonzalez-Angulo AM 2012 Effect of metformin on survival outcomes in diabetic patients with triple receptor-negative breast cancer. *Cancer* **118** 1202-1211.

11. Ben Sahra I, Laurent K, Loubat A, Giorgetti-Peraldi S, Colosetti P, Auberger P, Tanti JF, Marchand-Brustel Y, & Bost F 2008 The antidiabetic drug metformin exerts an antitumoral effect in vitro and in vivo through a decrease of cyclin D1 level. *Oncogene* **27** 3576-3586.

12. Ben S, I, Marchand-Brustel Y, Tanti JF, & Bost F 2010 Metformin in cancer therapy: a new perspective for an old antidiabetic drug? *Mol Cancer Ther* **9** 1092-1099.

13. Berg JM, Tymoczko JL, & Stryer L 2006 Biochemistry. Freeman **6th edition.**

14. Bonanni B, Puntoni M, Cazzaniga M, Pruneri G, Serrano D, Guerrieri-Gonzaga A, Gennari A, Stella TM, Galimberti V, Veronesi P, Johansson H, Aristarco V, Bassi F, Luini A, Lazzeroni M, Varricchio C, Viale G, Bruzzi P, & Decensi A 2012 Dual Effect of Metformin on Breast Cancer Proliferation in a Randomized Presurgical Trial. *J Clin Oncol, In press.*

15. Bowker SL, Majumdar SR, Veugelers P, & Johnson JA 2006 Increased cancer-related mortality for patients with type 2 diabetes who use sulfonylureas or insulin. *Diabetes Care* **29** 254-258.

16. Brand K 1985 Glutamine and glucose metabolism during thymocyte proliferation. Pathways of glutamine and glutamate metabolism. *Biochem J* **228** 353-361.

17. Brown KD, Ziv Y, Sadanandan SN, Chessa L, Collins FS, Shiloh Y, & Tagle DA 1997 The ataxia-telangiectasia gene product, a constitutively expressed nuclear protein that is not up-regulated following genome damage. *Proc Natl Acad Sci USA* **94** 1840-1845.

18. Bush A, Mateyak M, Dugan K, Obaya A, Adachi S, Sedivy J, & Cole M 1998 c-myc null cells misregulate cad and gadd45 but not other proposed c-Myc targets. *Genes and Development* **12** 3797-3802.

19. Buzzai M, Jones RG, Amaravadi RK, Lum JJ, DeBerardinis RJ, Zhao F, Viollet B, & Thompson CB 2007 Systemic treatment with the antidiabetic

drug metformin selectively impairs p53-deficient tumor cell growth. *Cancer Research* **67** 6745-6752.

20. Cantrell LA, Zhou C, Mendivil A, Malloy KM, Gehrig PA, & Bae-Jump VL 2010 Metformin is a potent inhibitor of endometrial cancer cell proliferation--implications for a novel treatment strategy. *Gynecol Oncol* **116** 92-98.

21. Carstensen B, Witte DR, & Friis S 2012 Cancer occurrence in Danish diabetic patients: duration and insulin effects. *Diabetologia* **55** 948-958.

22. Chenevix-Trench G, Spurdle AB, Gatei M, Kelly H, Marsh A, Chen X, Donn K, Cummings M, Nyholt D, Jenkins MA, Scott C, Pupo GM, Dork T, Bendix R, Kirk J, Tucker K, McCredie MR, Hopper JL, Sambrook J, Mann GJ, & Khanna KK 2002 Dominant negative ATM mutations in breast cancer families. *J Natl Cancer Inst* **94** 205-215.

23. Coquerelle TM, Weibezahn KF, & Lucke-Huhle C 1987 Rejoining of double strand breaks in normal human and ataxia-telangiectasia fibroblasts after exposure to 60Co gamma-rays, 241Am alpha-particles or bleomycin. *Int J Radiat Biol Relat Stud Phys Chem Med* **51** 209-218.

24. Cornforth MN & Bedford JS 1985 On the nature of a defect in cells from individuals with ataxia-telangiectasia. *Science* **227** 1589-1591.

25. Curi R, Newsholme P, & Newsholme EA 1988 Metabolism of pyruvate by isolated rat mesenteric lymphocytes, lymphocyte mitochondria and isolated mouse macrophages. *Biochem J* **250** 383-388.

26. Cusi K, Consoli A, & DeFronzo RA 1996 Metabolic effects of metformin on glucose and lactate metabolism in noninsulin-dependent diabetes mellitus. *J Clin Endocrinol Metab* **81** 4059-4067.

27. DeBerardinis RJ & Cheng T 2010 Q's next: the diverse functions of glutamine in metabolism, cell biology and cancer. *Oncogene* **29** 313-324.

28. DeBerardinis RJ, Mancuso A, Daikhin E, Nissim I, Yudkoff M, Wehrli S, & Thompson CB 2007 Beyond aerobic glycolysis: transformed cells can engage in glutamine metabolism that exceeds the requirement for protein and nucleotide synthesis. *Proc Natl Acad Sci USA* **104** 19345-19350.

29. Decensi A, Puntoni M, Goodwin P, Cazzaniga M, Gennari A, Bonanni B, & Gandini S 2010 Metformin and cancer risk in diabetic patients: a systematic review and meta-analysis. *Cancer Prev Res (Phila)* **3** 1451-1461.

30. Dowling RJ, Goodwin PJ, & Stambolic V 2011 Understanding the benefit of metformin use in cancer treatment. *BMC Med* **9** 33.

31. Dowling RJ, Zakikhani M, Fantus IG, Pollak M, & Sonenberg N 2007 Metformin inhibits mammalian target of rapamycin-dependent translation initiation in breast cancer cells. *Cancer Research* **67** 10804-10812.

32. EAGLE H 1955 Nutrition needs of mammalian cells in tissue culture. *Science* **122** 501-514.

33. Efremov RG & Sazanov LA 2011 Structure of the membrane domain of respiratory complex I. *Nature* **476** 414-420.

34. El Mir MY, Nogueira V, Fontaine E, Averet N, Rigoulet M, & Leverve X 2000 Dimethylbiguanide inhibits cell respiration via an indirect effect targeted on the respiratory chain complex I. *The Journal of Biological Chemistry* **275** 223-228.

35. Enoch T & Norbury C 1995 Cellular responses to DNA damage: cell-cycle checkpoints, apoptosis and the roles of p53 and ATM. *Trends in Biochemical Science3* **20** 426-430.

36. Evans JM, Donnelly LA, Emslie-Smith AM, Alessi DR, & Morris AD 2005 Metformin and reduced risk of cancer in diabetic patients. *BMJ* **330** 1304-1305.

37. FALCONE AB, MAO RL, & SHRAGO E 1962 A study of the action of hypoglycemia-producing biguanide and sulfonylurea compounds on oxidative phosphorylation. *J Biol Chem* **237** 904-909.

38. Fantin VR, St Pierre J, & Leder P 2006 Attenuation of LDH-A expression uncovers a link between glycolysis, mitochondrial physiology, and tumor maintenance. *Cancer Cell* **9** 425-434.

39. Foray N, Priestley A, Alsbeih G, Badie C, Capulas EP, Arlett CF, & Malaise EP 1997 Hypersensitivity of ataxia telangiectasia fibroblasts to ionizing

radiation is associated with a repair deficiency of DNA double-strand breaks. *Int J Radiat Biol* **72** 271-283.

40. Foretz M, Hebrard S, Leclerc J, Zarrinpashneh E, Soty M, Mithieux G, Sakamoto K, Andreelli F, & Viollet B 2010 Metformin inhibits hepatic gluconeogenesis in mice independently of the LKB1/AMPK pathway via a decrease in hepatic energy state. *J Clin Invest* **120** 2355-2369.

41. Fowler JS & Ido T 2002 Initial and subsequent approach for the synthesis of $^{18F\text{-}FDG}$. *Semin Nuc Med* **32** 6-12.

42. Gambhir SS 2002 Molecular imaging of cancer with positron emission tomography. *Nat Rev Cancer* **2** 683-693.

43. Gao P, Tchernyshyov I, Chang TC, Lee YS, Kita K, Ochi T, Zeller KI, De Marzo AM, Van Eyk JE, Mendell JT, & Dang CV 2009 c-Myc suppression of miR-23a/b enhances mitochondrial glutaminase expression and glutamine metabolism. *Nature* **458** 762-765.

44. Gibala MJ, Young ME, & Taegtmeyer H 2000 Anaplerosis of the citric acid cycle: role in energy metabolism of heart and skeletal muscle. *Acta Physiol Scand* **168** 657-665.

45. Goodwin PJ, Pritchard KI, Ennis M, Clemons M, Graham M, & Fantus IG 2008 Insulin-lowering effects of metformin in women with early breast cancer. *Clin Breast Cancer* **8** 501-505.

46. Gotlieb WH, Saumet J, Beauchamp MC, Gu J, Lau S, Pollak MN, & Bruchim I 2008 In vitro metformin anti-neoplastic activity in epithelial ovarian cancer. *Gynecol Oncol* **110** 246-250.

47. Guppy M, Greiner E, & Brand K 1993 The role of the Crabtree effect and an endogenous fuel in the energy metabolism of resting and proliferating thymocytes. *Eur J Biochem* **212** 95-99.

48. Hadad SM, Baker L, Quinlan PR, Robertson KE, Bray SE, Thomson G, Kellock D, Jordan LB, Purdie CA, Hardie DG, Fleming S, & Thompson AM 2009 Histological evaluation of AMPK signalling in primary breast cancer. *BMC Cancer* **9** 307.

49. Hadden DR 2005 Goat's rue - French lilac - Italian fitch - Spanish sainfoin: gallega officinalis and metformin: the Edinburgh connection. *J R Coll Physicians Edinb* **35** 258-260.

50. Hardie DG 2007 AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy. *Nat Rev.Mol.Cell Biol.* **8** 774-785.

51. Hardie DG, Hawley SA, & Scott JW 2006 AMP-activated protein kinase--development of the energy sensor concept. *J Physiol* **574** 7-15.

52. Hasan NM, Longacre MJ, Stoker SW, Boonsaen T, Jitrapakdee S, Kendrick MA, Wallace JC, & MacDonald MJ 2008 Impaired anaplerosis and insulin secretion in insulinoma cells caused by small interfering RNA-mediated suppression of pyruvate carboxylase. *J Biol.Chem.* **283** 28048-28059.

53. Hassel B 2000 Carboxylation and anaplerosis in neurons and glia. *Mol.Neurobiol.* **22** 21-40.

54. Hatzivassiliou G, Zhao F, Bauer DE, Andreadis C, Shaw AN, Dhanak D, Hingorani SR, Tuveson DA, & Thompson CB 2005 ATP citrate lyase inhibition can suppress tumor cell growth. *Cancer Cell* **8** 311-321.

55. Hedeskov CJ 1968 Early effects of phytohaemagglutinin on glucose metabolism of normal human lymphocytes. *Biochem.J* **110** 373-380.

56. Hickson I, Zhao Y, Richardson CJ, Green SJ, Martin NM, Orr AI, Reaper PM, Jackson SP, Curtin NJ, & Smith GC 2004 Identification and characterization of a novel and specific inhibitor of the ataxia-telangiectasia mutated kinase ATM. *Cancer Res* **64** 9152-9159.

57. Hirst J 2010 Towards the molecular mechanism of respiratory complex I. *Biochem.J* **425** 327-339.

58. Holley RW & Kiernan JA 1974 Control of the initiation of DNA synthesis in 3T3 cells: low-molecular weight nutrients. *Proc.Natl.Acad.Sci.U.S.A* **71** 2942-2945.

59. Hosono K, Endo H, Takahashi H, Sugiyama M, Sakai E, Uchiyama T, Suzuki K, Iida H, Sakamoto Y, Yoneda K, Koide T, Tokoro C, Abe Y, Inamori M, Nakagama H, & Nakajima A 2010 Metformin suppresses colorectal aberrant crypt foci in a short-term clinical trial. *Cancer Prev Res (Phila)* **3** 1077-1083.

60. Hume DA & Weidemann MJ 1979 Role and regulation of glucose metabolism in proliferating cells. *J Natl.Cancer Inst.* **62** 3-8.

61. Hundal RS, Krssak M, Dufour S, Laurent D, Lebon V, Chandramouli V, Inzucchi SE, Schumann WC, Petersen KF, Landau BR, & Shulman GI 2000 Mechanism by which metformin reduces glucose production in type 2 diabetes. *Diabetes* **49** 2063-2069.

62. Inoki K, Zhu T, & Guan KL 2003 TSC2 mediates cellular energy response to control cell growth and survival. *Cell* **115** 577-590.

63. Isakovic A, Harhaji L, Stevanovic D, Markovic Z, Sumarac-Dumanovic M, Starcevic V, Micic D, & Trajkovic V 2007 Dual antiglioma action of metformin: cell cycle arrest and mitochondria-dependent apoptosis. *Cell Mol.Life Sci.* **64** 1290-1302.

64. Jiralerspong S, Palla SL, Giordano SH, Meric-Bernstam F, Liedtke C, Barnett CM, Hsu L, Hung MC, Hortobagyi GN, & Gonzalez-Angulo AM 2009 Metformin and Pathologic Complete Responses to Neoadjuvant Chemotherapy in Diabetic Patients With Breast Cancer. *J Clin Oncol* **27** 3297-3302.

65. Jones RG & Thompson CB 2009 Tumor suppressors and cell metabolism: a recipe for cancer growth. *Genes and Development* **23** 537-548.

66. Kamsler A, Daily D, Hochman A, Stern N, Shiloh Y, Rotman G, & Barzilai A 2001 Increased oxidative stress in ataxia telangiectasia evidenced by

alterations in redox state of brains from Atm-deficient mice. *Cancer Res* **61** 1849-1854.

67. Kovacevic Z 1971 The pathway of glutamine and glutamate oxidation in isolated mitochondria from mammalian cells. *Biochem.J* **125** 757-763.

68. Kozlov S, Gueven N, Keating K, Ramsay J, & Lavin MF 2003 ATP activates ataxia-telangiectasia mutated (ATM) in vitro. Importance of autophosphorylation. *J Biol.Chem.* **278** 9309-9317.

69. Lakin ND, Weber P, Stankovic T, Rottinghaus ST, Taylor AM, & Jackson SP 1996 Analysis of the ATM protein in wild-type and ataxia telangiectasia cells. *Oncogene* **13** 2707-2716.

70. Landman GW, Kleefstra N, van Hateren KJ, Groenier KH, Gans RO, & Bilo HJ 2010 Metformin associated with lower cancer mortality in type 2 diabetes: ZODIAC-16. *Diabetes Care* **33** 322-326.

71. Lavin MF 2008 Ataxia-telangiectasia: from a rare disorder to a paradigm for cell signalling and cancer. *Nat.Rev.Mol.Cell Biol.* **9** 759-769.

72. Lee JH & Paull TT 2005 ATM activation by DNA double-strand breaks through the Mre11-Rad50-Nbs1 complex. *Science* **308** 551-554.

73. Lehman DM, Lorenzo C, Hernandez J, & Wang CP 2012 Statin use as a moderator of metformin effect on risk for prostate cancer among type 2 diabetic patients. *Diabetes Care* **35** 1002-1007.

74. Lehninger AL, Nelson DL, & Cox MM 1993 Principles of biochemistry. **2**

75. Libby G, Donnelly LA, Donnan PT, Alessi DR, Morris AD, & Evans JM 2009 New users of metformin are at low risk of incident cancer: A cohort study among people with type 2 diabetes. *Diabetes Care* **32** 1620-1625.

76. Lim DS, Kirsch DG, Canman CE, Ahn JH, Ziv Y, Newman LS, Darnell RB, Shiloh Y, & Kastan MB 1998 ATM binds to beta-adaptin in cytoplasmic vesicles. *Proc.Natl.Acad.Sci.U.S.A* **95** 10146-10151.

77. Liu P, Cheng H, Roberts TM, & Zhao JJ 2009 Targeting the phosphoinositide 3-kinase pathway in cancer. *Nat Rev Drug Discov.* **8** 627-644.

78. Lunt SY & Vander Heiden MG 2011 Aerobic glycolysis: meeting the metabolic requirements of cell proliferation. *Annu.Rev Cell Dev.Biol.* **27** 441-464.

79. Maschek G, Savaraj N, Priebe W, Braunschweiger P, Hamilton K, Tidmarsh GF, De Young LR, & Lampidis TJ 2004 2-deoxy-D-glucose increases the efficacy of adriamycin and paclitaxel in human osteosarcoma and non-small cell lung cancers in vivo. *Cancer Res* **64** 31-34.

80. Miles PD, Treuner K, Latronica M, Olefsky JM, & Barlow C 2007 Impaired insulin secretion in a mouse model of ataxia telangiectasia. *Am.J Physiol Endocrinol.Metab* **293** E70-E74.

81. Monami M, Lamanna C, Balzi D, Marchionni N, & Mannucci E 2009 Sulphonylureas and cancer: a case-control study. *Acta Diabetol.* **46** 279-284.

82. Moreno-Sanchez R, Rodriguez-Enriquez S, Marin-Hernandez A, & Saavedra E 2007 Energy metabolism in tumor cells. *FEBS J* **274** 1393-1418.

83. Nicklin P, Bergman P, Zhang B, Triantafellow E, Wang H, Nyfeler B, Yang H, Hild M, Kung C, Wilson C, Myer VE, MacKeigan JP, Porter JA, Wang YK, Cantley LC, Finan PM, & Murphy LO 2009 Bidirectional transport of amino acids regulates mTOR and autophagy. *Cell* **136** 521-534.

84. Nies AT, Hofmann U, Resch C, Schaeffeler E, Rius M, & Schwab M 2011 Proton pump inhibitors inhibit metformin uptake by organic cation transporters (OCTs). *Plos One* **6** e22163.

85. Owen MR, Doran E, & Halestrap AP 2000 Evidence that metformin exerts its anti-diabetic effects through inhibition of complex 1 of the mitochondrial respiratory chain. *Biochem.J* **348 Pt 3** 607-614.

86. Owen OE, Kalhan SC, & Hanson RW 2002 The key role of anaplerosis and cataplerosis for citric acid cycle function. *J Biol.Chem.* **277** 30409-30412.

87. Pardee AB 1974 A restriction point for control of normal animal cell proliferation. *Proc.Natl.Acad.Sci.U.S.A* **71** 1286-1290.

88. Patel T, Hruby G, Badani K, Abate-Shen C, & McKiernan JM 2010 Clinical outcomes after radical prostatectomy in diabetic patients treated with metformin. *Urology* **76** 1240-1244.

89. Pfeiffer T, Schuster S, & Bonhoeffer S 2001 Cooperation and competition in the evolution of ATP-producing pathways. *Science* **292** 504-507.

90. Pollak M 2010 Metformin and other biguanides in oncology: advancing the research agenda. *Cancer Prev Res (Phila)* **3** 1060-1065.

91. Pollak M 2012 Metformin in cancer prevention and treatment: the end of the beginning. *Cancer Discov.* In press.

92. Ristow M & Cuezva JM 2009 Oxidative phosphorylation and cancer: the ongoing Warburg hypothesis. In: Apte S, Sarangarajan R, editors. Cellular Respiration and Carcinogenesis. New York: Springer, p. 1-18.

93. Segal ED, Yasmeen A, Beauchamp MC, Rosenblatt J, Pollak M, & Gotlieb WH 2011 Relevance of the OCT1 transporter to the antineoplastic effect of biguanides. *Biochem.Biophys.Res Commun.* **414** 694-699.

94. Shaw RJ, Kosmatka M, Bardeesy N, Hurley RL, Witters LA, Depinho RA, & Cantley LC 2004 The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress. *Proc.Natl.Acad.Sci.U.S.A* **101** 3329-3335.

95. Srivastava S & Moraes CT 2009 Cellular adaptations to oxidative phosphorylation defects in cancer. In: Apte S, Sarangarajan R, editors. Cellular Respiration and Carcinogenesis. New York: Springer, p. 55-72.

96. Turner N, Li JY, Gosby A, To SW, Cheng Z, Miyoshi H, Taketo MM, Cooney GJ, Kraegen EW, James DE, Hu LH, Li J, & Ye JM 2008 Berberine and its more biologically available derivative, dihydroberberine, inhibit mitochondrial respiratory complex I: a mechanism for the action of berberine to activate AMP-activated protein kinase and improve insulin action. *Diabetes* **57** 1414-1418.

97. Vander Heiden MG, Cantley LC, & Thompson CB 2009 Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science* **324** 1029-1033.

98. Wang T, Marquardt C, & Foker J 1976 Aerobic glycolysis during lymphocyte proliferation. *Nature* **261** 702-705.

99. WARBURG O 1956 On the origin of cancer cells. *Science* **123** 309-314.

100. Watters D, Khanna KK, Beamish H, Birrell G, Spring K, Kedar P, Gatei M, Stenzel D, Hobson K, Kozlov S, Zhang N, Farrell A, Ramsay J, Gatti R, & Lavin M 1997 Cellular localisation of the ataxia-telangiectasia (ATM) gene product and discrimination between mutated and normal forms. *Oncogene* **14** 1911-1921.

101. Wise DR, DeBerardinis RJ, Mancuso A, Sayed N, Zhang XY, Pfeiffer HK, Nissim I, Daikhin E, Yudkoff M, McMahon SB, & Thompson CB 2008 Myc regulates a transcriptional program that stimulates mitochondrial glutaminolysis and leads to glutamine addiction. *Proc.Natl.Acad.Sci.U.S.A* **105** 18782-18787.

102. Wise DR & Thompson CB 2010 Glutamine addiction: a new therapeutic target in cancer. *Trends in Biochemical Science3* **35** 427-433.

103. Wullschleger S, Loewith R, & Hall MN 2006 TOR signaling in growth and metabolism. *Cell* **124** 471-484.

104. Yuneva M, Zamboni N, Oefner P, Sachidanandam R, & Lazebnik Y 2007 Deficiency in glutamine but not glucose induces MYC-dependent apoptosis in human cells. *J Cell Biol.* **178** 93-105.

105. Zakikhani M, Dowling R, Fantus IG, Sonenberg N, & Pollak M 2006 Metformin is an AMP kinase-dependent growth inhibitor for breast cancer cells. *Cancer Research* **66** 10269-10273.

106. Zu XL & Guppy M 2004 Cancer metabolism: facts, fantasy, and fiction. *Biochem.Biophys.Res Commun.* **313** 459-465.

Appendix

Contribution of Authors-Manuscript A

Carbon source and *myc* expression influence the antiproliferative actions of metformin

Shiva Javeshghani[4], Mahvash Zakikhani[1,2], Shane Austin[3,5], Miguel Bazile[2], Marie-José Blouin[1,2], Julie St-Pierre[3,5], Michael N. Pollak[1,2,3,4*]

1. Shiva Javeshghani: Carried out and designed all *in vitro* studies including proliferation assays, lactate production assays, glucose consumption assays, NAD+/NADH assays, and ATP assays.
2. Mahvash Zakikhani: Assisted in immunoblot experiments and ATP assays. She also performed all statistical analysis of data.
3. Shane Austin: Carried out all oxygen consumption experiments.
4. Miguel Bazile: Provided assistance in the carrying out of lactate assay for MC38 cells.
5. Marie-José Blouin: Provided assistance in the carrying out of lactate assays.
6. Julie St-Pierre: Collaborated with our lab and provided oxygen consumption facility. Dr. St-Pierre is Shane Austin's supervisor.
7. Michael Pollak is my M.Sc. supervisor.

Contribution of Authors-Manuscript B

Alterations in Cellular Energy Metabolism Associated with the Antiproliferative Effects of the ATM Inhibitor KU-55933 and with Metformin

Mahvash Zakikhani, PhD[1,2], Miguel Bazile, BSc[2], Sina Hashemi[2], Shiva Javeshghani, BSc[4], Daina Avizonis, PhD[3], Julie St Pierre, PhD[3,5], Michael N. Pollak, MD[1,2,3,4*]

1. Mahvash Zakikhani: Designed all experiments involving treatment with KU-55933 and metformin
2. Miguel Bazile: Carried out immunoblot experiments, and assisted in the carrying out of lactate production and glucose consumption assays.
3. Sina Hashemi: Aided in the design of KU-55933 experiments
4. Shiva Javeshghani: Carried out ATP assays, assisted with oxygen consumption experiments and the preparation of TCA cycle metabolite measurement experiments. I also helped with the writing and preparation of the manuscript including figures and figure legends.
5. Daina Avizonis: Carried out experiments involving the measurement of TCA cycle metabolites.
6. Julie St Pierre, PhD: Provided oxygen consumption facility.
7. Michael Pollak is my M.Sc. supervisor.