Repression of LKB1 by \textit{miR-17\textasciitilde92} Sensitizes \textit{MYC}-Dependent Lymphoma to Biguanide Treatment

Highlights

- IM156 is a newly developed biguanide with more potency than phenformin
- IM156 inhibits mitochondrial respiration to cause bioenergetic stress in lymphoma
- \textit{miR-17}-dependent silencing of LKB1 reduces adaptation to bioenergetic stress
- \textit{miR-17\textasciitilde92} expression correlates with biguanide sensitivity in human cancer cells

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In Brief

Izreig et al. show that \textit{miR-17\textasciitilde92} expression in cancer cells sensitizes them to biguanide treatment by disrupting bioenergetic stability. These data implicate \textit{miR-17\textasciitilde92} expression as a potential biomarker for biguanide sensitivity in hematological malignancies and possibly solid tumors.
Repression of LKB1 by miR-17-92 Sensitizes MYC-Dependent Lymphoma to Biguanide Treatment

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SUMMARY

Cancer cells display metabolic plasticity to survive stresses in the tumor microenvironment. Cellular adaptation to energetic stress is coordinated in part by signaling through the liver kinase B1 (LKB1)-AMP-activated protein kinase (AMPK) pathway. Here, we demonstrate that miRNA-mediated silencing of LKB1 confers sensitivity of lymphoma cells to mitochondrial inhibition by biguanides. Using both classic (phenformin) and newly developed (IM156) biguanides, we demonstrate that elevated miR-17-92 expression in Myc+ lymphoma cells promotes increased apoptosis to biguanide treatment in vitro and in vivo. This effect is driven by the miR-17-dependent silencing of LKB1, which reduces AMPK activation in response to complex I inhibition. Mechanistically, biguanide treatment induces metabolic stress in Myc+ lymphoma cells by inhibiting TCA cycle metabolism and mitochondrial respiration, exposing metabolic vulnerability. Finally, we demonstrate a direct correlation between miR-17-92 expression and biguanide sensitivity in human cancer cells. Our results identify miR-17-92 expression as a potential biomarker for biguanide sensitivity in malignancies.

INTRODUCTION

A major driver of metabolic reprogramming in cancer is the c-Myc proto-oncogene (Myc), a transcription factor that broadly regulates the expression of genes involved in anabolic metabolism and cellular bioenergetics.1 Oncogene activation or tumor suppressor inactivation promotes changes in cellular metabolism that facilitate transformation and tumor cell proliferation.2 However, the increased metabolic activity stimulated by transformation imposes additional stresses on tumor cells, such as nutrient depletion and redox imbalance, which must be countered for tumors to survive and grow. The liver kinase B1 (LKB1)-AMP-activated protein kinase (AMPK) pathway contributes to tumor cell survival by promoting the cellular sensing of and adaptation to such bioenergetic stress.3 Inactivation of LKB1 in cancer, conversely, promotes anabolic metabolic reprogramming at the expense of metabolic flexibility.4-7 In cells lacking LKB1, including non-small cell lung cancer (NSCLC), AMPK activation is reduced and cells are more sensitive to metabolic stresses induced by nutrient limitation or inhibitors of oxidative phosphorylation (OXPHOS).8,9 This raises the possibility of using OXPHOS inhibitors to target cancers with reduced bioenergetic capacity and/or defects in energy-sensing control systems. Potent OXPHOS inhibitors,10 putative inhibitors of mitochondrial protein synthesis,11,12 and enhancers of the degradation of respiratory chain proteins13,14 are under study as antineoplastic agents.

Biguanides represent another class of drugs proposed to target cellular bioenergetics in tumor cells. The most well-known biguanide is metformin, a drug commonly used to treat type 2 diabetes. Metformin reduces the activity of mitochondrial complex I, thereby limiting mitochondrial ATP production and imposing bioenergetic stress on cells.15,16 This leads to reduced hepatic gluconeogenesis and reduced hyperglycemia in type 2
diabetes. However, if adequate drug levels can be achieved in other cell types, lineage-specific consequences of energetic stress are observed, including cytostatic or cytotoxic effects on cancer cells. Older retrospective studies reported that diabetics treated with metformin had reduced cancer risk and better cancer prognosis relative to diabetic patients not taking metformin, generating interest in repurposing biguanides as anticancer agents. However, further pharmaco-epidemiologic studies failed to confirm these findings, and early randomized clinical trials of metformin in advanced cancer have shown no survival benefit in pancreatic cancer and only minor benefit in lung cancer. Pharmacokinetic factors, such as the requirement for the active transport of metformin by the organic cation (OCT) transporters, may account in part for the discrepancies between preclinical models and clinical trials with respect to the anticancer effects of metformin. Nevertheless, many in vivo cancer models demonstrate significant in vivo antineoplastic activity of biguanides, raising the possibility that biguanides with better bioavailability and toxicity profiles may have clinical utility.

Important in the clinical development of OXPHOS inhibitors as antineoplastic drugs is the selection of subsets of cancers that are particularly sensitive to metabolic stress. Preclinical work by Shackelford et al. demonstrated that biguanides, specifically phenformin, could be effective as single agents for LKB1-deficient KRAS mutant NSCLC, in keeping with the role of LKB1 in adaptation to energetic stress. While the mutation of LKB1 is found in ~20%-30% of NSCLCs, we hypothesized that biguanide-sensitive cancers can be extended to those with increased expression of MYC, which we have previously reported to be a more potent inhibitor of mitochondrial respiration than phenformin.

**RESULTS**

**IM156 Is a Newly Developed Biguanide That Inhibits Mitochondrial Respiration**

The limited bioavailability of metformin and its dependence on OCT1 for cellular uptake potentially limit its applicability in the treatment of cancer. We investigated the biological properties of phenformin and the newly developed biguanide IM156, which are more hydrophobic and therefore potentially more bioavailable to cells than metformin. To test the impact of these biguanides on tumor cell respiration, we acutely treated mouse lymphoma cells (Eµ-Myc cells) with either metformin, phenformin, or IM156 and assessed changes in the oxygen consumption rate (OCR) using the Seahorse XF96 extracellular flux analyzer. Across a range of concentrations, phenformin and IM156 decreased OCR, with IM156 exhibiting greater potency than phenformin and metformin at equal concentrations. IM156 was more effective than phenformin at reducing cellular ATP production at equal concentrations, correlating with the effect of IM156 on oxidative phosphorylation (Figure 1C). These data are consistent with IM156 functioning as a more potent inhibitor of mitochondrial respiration than phenformin.

To confirm that the effect of IM156 on mitochondrial respiration was through complex I inhibition, we treated purified bovine mitochondrial membranes with IM156. IM156 treatment decreased complex I-dependent NADH oxidation in a significant, dose-dependent manner, with a lower half-maximal inhibitory concentration (IC50) of 2.2 μM than previously reported for phenformin (340 μM), while succinate oxidation (via complex II) was minimally affected at equivalent doses of IM156 (Figure 1D). This minimal decrease in succinate oxidation could be an effect of the drug on the coupled assay enzymes fumarase and malic enzyme. IM156 also reduced NADH oxidation in purified complex I in a dose-dependent manner (Figure S1A). To further confirm the specificity of IM156 for complex I, we assessed the IM156-mediated inhibition of OCR in cells expressing ND1, a yeast NADH dehydrogenase that is resistant to biguanides. ND1 expression rescued the effects of IM156 on cellular respiration, promoting an ~100-fold shift in the IC50 of IM156 (Figure 1E). These data support that IM156 blocks OXPHOS through the inhibition of complex I and does so more potently than phenformin.

We next determined whether phenformin and IM156 similarly activate the energy sensor AMPK, as depletion of cellular ATP by biguanide treatment is a known trigger for AMPK activation. Given the difference in potency of phenformin and IM156, we used doses of 100 and 10 μM, respectively, that produced an equivalent ~50% decrease in OCR from baseline after a 2-h treatment (Figures S1B and S1C). A 2-h treatment with these concentrations of phenformin or IM156 yielded similar levels of activating AMPK phosphorylation at T-172 (Figure 1F). Lastly, we treated Eµ-Myc lymphoma cells with a range of concentrations of either phenformin or IM156. Based on cell viability measurements, IM156 exhibited higher potency and induced lymphoma cell death at lower concentrations than phenformin (half-maximal effective concentration [EC50] of 12 μM for IM156 compared to 62 μM for phenformin; Figure 1G).

**miR-17–92 Sensitizes Lymphoma Cells to Apoptosis by Biguanides**

Previously, we demonstrated that the oncogenic miRNA cluster miR-17–92 is required for Myc-dependent metabolic reprogramming in lymphoma and does so in part through the translational suppression of LKB1. We examined whether the expression of miR-17–92 alters the sensitivity of lymphoma cells to biguanide treatment. We used Eµ-Myc B cell lymphoma cells harboring floxed miR-17–92 alleles, which allowed us to study the effect of the conditional deletion of miR-17–92 in the presence of constitutive Myc expression. Eµ-Myc lymphoma cells deleted for miR-17–92 (ΔΔ) were more resistant to phenformin treatment than their isogenic counterparts expressing miR-17–92 (ll/ll) (Figure 2A). Phenformin treatment actively induced apoptosis in miR-17–92-expressing Eµ-Myc lymphoma cells...
as shown by the presence of active (cleaved) caspase-3 (Figure 2B). Levels of caspase-3 cleavage were markedly reduced in Eμ-Myc lymphoma cells lacking miR-17–92 (Figure 2B).

Since miR-17–92 is recurrently amplified in lymphoma, we next tested whether an increased copy number of miR-17–92 was sufficient to increase the sensitivity of lymphoma cells to biguanides. To test this, we generated Eμ-Myc lymphoma cells and Raji lymphoma cells, a human Burkitt’s lymphoma cell line known to display low MYC levels, with ectopic expression of the entire miR-17–92 polycistron (hereafter denoted as +17–92). Eμ-Myc lymphoma cells overexpressing miR-17–92 were significantly more sensitive than control cells when treated with either phenformin or IM156 (Figures 2C and S1B). miR-17–92 overexpression led to a 10-fold shift in the EC50 of Eμ-Myc cells to IM156 treatment (2 μM versus 24 μM). Similar results were observed in Raji cells engineered to express higher levels of miR-17–92 (Figures 2D, 2E, and S1C). Although Raji cells were less sensitive to biguanide treatment, with EC50 values ranging from 90 μM to 3.5 mM as compared to Eμ-Myc+ EC50 values ranging from 2 to 62 μM (Figures S1B and S1C), the overexpression of miR-17–92 still conferred a ~3-fold increase in sensitivity to biguanides compared to control cells. These data indicate that elevated miR-17–92 enhances the sensitivity of lymphoma cells to biguanide treatments and that Myc and miR-17–92 synergize to increase sensitivity to biguanide treatment.

We next asked whether the enhanced sensitivity to biguanides observed in vitro extended to in vivo models. Nude mice harboring either control or miR-17–92-overexpressing Eμ-Myc cells were administered phenformin or IM156 in their drinking water ad libitum, and survival was tracked compared to tumor-bearing mice administered regular drinking water. While biguanide treatment of mice bearing control lymphoma cells produced no discernible survival benefit (Figure 3A), both phenformin and IM156 significantly prolonged the lifespan of mice bearing aggressive miR-17–92-overexpressing tumors (Figure 3B).
observations indicate that phenformin and IM156 can act as single agents to extend the survival of mice bearing tumors with elevated miR-17–92 expression, with elevated miR-17–92 expression functioning as a predictor of sensitivity to biguanides.

**miR-17–92 Confers Sensitivity to Biguanides through the Suppression of LKB1**

We next explored the mechanism by which elevated miR-17–92 expression confers biguanide sensitivity in lymphoma cells. NSCLC cells that have lost LKB1 display increased sensitivity to phenformin. We previously identified LKB1 as a target of NSCLC cells that have lost LKB1 display increased sensitivity to phenformin.8 We previously identified LKB1 as a target of NSCLC cells that have lost LKB1 display increased sensitivity to phenformin.8 We previously identified LKB1 as a target of NSCLC cells that have lost LKB1 display increased sensitivity to phenformin.8 We previously identified LKB1 as a target of NSCLC cells that have lost LKB1 display increased sensitivity to phenformin.8 We previously identified LKB1 as a target of NSCLC cells that have lost LKB1 display increased sensitivity to phenformin.8

Gene silencing by 17 miR-17–92 expression resulted in reduced LKB1 protein levels but lacking expression of LKB1-AMPK axis to metabolic stress (Figure 4G).

Given that an increased copy number of miR-17–92 leads to LKB1 repression in lymphoma cells (Figure 4B), we tested whether AMPK activation was differentially engaged downstream of either phenformin or IM156 treatment. Lymphoma cells overexpressing miR-17–92 displayed reduced AMPK phosphorylation (Figure 4B), which downregulated Ser-792 phosphorylation of Raptor (Figure 4C) and thus enhanced mammalian target of rapamycin complex 1 (mTORC1) signaling, as evidenced by the increased phosphorylation of pS6 and 4EBP phosphorylation in miR-17–92-expressing cells (Figure 4C). The deletion of miR-17–92 in Eμ-Myc cells (Δ/Δ cells) was sufficient to confer a survival advantage in response to IM156 treatment (Figure 4D). Reducing LKB1 expression in Δ/Δ cells using small hairpin RNAs (shRNAs) targeting LKB130 restored biguanide sensitivity in Δ/Δ cells to match that observed in lymphoma cells expressing miR-17–92 (Figure 4D). Similarly, lymphoma cells expressing miR-17–92 but lacking expression of miR-17 and -20 were resistant to IM156 treatment (Figure 4E).

**Biguanide Treatment Affects Central Carbon Metabolism in miR-17–92-Expressing Myc+ Lymphoma Cells**

We next characterized the effect of IM156 treatment on lymphoma cell bioenergetics using a Seahorse XF96 extracellular flux analyzer. Lymphoma cells overexpressing miR-17–92 exhibit higher basal metabolic rates, both in terms of glycolytic rate (extracellular acidification rate [ECAR]) and respiration (OCR), than control cells (Figure 5A). This corresponded to higher overall rates of ATP production by miR-17–92-expressing lymphoma cells compared to controls, which was due largely to increased glycolytic ATP production (Figure 5B).
lymphoma cells also displayed a larger bioenergetic scope than control cells (Figure 5C). The bioenergetic profile of both cell types was significantly altered by IM156 treatment, characterized by a decrease in mitochondrial ATP production ($\text{U}_{\text{mito}}$-ATP ox) and an increase in glycolytic ATP production ($\text{U}_{\text{gly}}$-ATP) (Figure 5C). IM156 reduced OXPHOS-derived ATP production in both cell types by $\geq 50\%$ (Figure 5C).

We next examined the impact of biguanide treatment on metabolite dynamics in lymphoma cells. Consistent with their increased bioenergetic profile (Figures 5A–5C), miR-17–92-expressing lymphoma cells displayed an increased abundance of metabolites involved in glycolysis (e.g., pyruvate, lactate), the tricarboxylic acid (TCA) cycle (e.g., citrate, succinate, fumarate, malate), and amino acid metabolism (Figure 5D). Biguanide treatment had a minimal effect on amino acid levels in either cell type (Figure 5D). However, levels of TCA cycle intermediates were markedly reduced in miR-17–92-expressing cells upon treatment with phenformin or IM156 (Figure 5D). This may be the result of the stalled processing of TCA cycle intermediates due to NADH buildup following complex I inhibition, as previously suggested. The lack of increased intracellular $^{13}$C-glucose-derived lactate in phenformin- or IM156-treated cells may be due to increased export into the culture medium, as suggested by the shift in glycolytic ATP production in IM156-treated cells (Figure 5C). Biguanide treatment blunted $^{13}$C-glucose and $^{13}$C-glutamine entry into the TCA cycle, as evidenced by the reduced conversion of glucose and glutamine to citrate (Figure 5G). $^{13}$C-glutamine-derived production of aspartate, a key intermediate for protein and nucleotide biosynthesis and tumor cell growth, was reduced by 60%–65% following a 2-h administration of phenformin or IM156 (Figure 5H). These data suggest that although miR-17–92 enhances central carbon metabolism and bioenergetic potential in lymphoma cells, these pathways are uniquely sensitive to biguanide treatment.

**miR-17 and -20 Expression Correlates with Biguanide Sensitivity in Human Lymphoma Cells**

Having defined the downstream effects of biguanides on the metabolism and viability of miR-17–92-expressing Myc* lymphoma cells, we expanded our analysis to test biguanide sensitivity in a panel of human lymphoma cell lines. Ten verified Myc* lymphoma cell lines were used for this analysis: SU-DHL-4, OCI-Ly7, Jeko-1, Rec-1, Karpas 1718, OCI-Ly-1, OCI-Ly-2, OCI-Ly-3, OCI-Ly-8, and OCI-Ly-18. For each cell line, we measured levels of both the primary (pri-miR) and mature (miR) forms of miR-17 and miR-20 and determined the EC$_{50}$ for cell viability to phenformin or IM156. From these data, we observed a negative correlation between the relative level of miR-17 and -20 expression in cells and the dose of biguanide required to achieve EC$_{50}$ (Figures 6A, 6B, and S2; Table S1), with cells displaying the highest miR-17 and -20 expression showing the greatest sensitivity to biguanide treatment. Levels of pri-miR-17 were a
Figure 5. Biguanide Treatment Affects Central Carbon Metabolism in miR-17-92-Expressing Lymphoma Cells

(A) ECAR and OCR of control (Ctrl) or miR-17-92 expressing (+17-92) Eμ-Myc+ lymphoma cells. Data represent mean ± SD for biological replicates (n = 12 per group).

(B) ATP production rates (J_ATP) for Eμ-Myc+ lymphoma cells expressing control (Ctrl) or miR-17-92 (+17-92) expression vectors. Seahorse experiments were performed under standard cell culture media conditions (Glc, 25 mM; Gln, 2 mM). J_ATP total is the sum of the glycolytic (J_ATP gly) and OXPHOS (J_ATP ox) ATP production rates. Data represent mean ± SDs for biological replicates (n = 12 per group) (Ctrl, n = 8; Ctrl+IM156, n = 8; +17-92, n = 11; +17-92+IM156, n = 12).

(C) Bioenergetic capacity plot of Eμ-Myc+ lymphoma cells expressing control (Ctrl) or miR-17-92 overexpression (+17-92) vectors following a 2-h treatment with vehicle (gray) or IM156 (purple). Rectangles define the maximum bioenergetic space of each cell type.

(legend continued on next page)
**DISCUSSION**

Myc is abnormally expressed in many human cancers; however, the development of small molecule Myc inhibitors has been a persistent challenge,37 prompting consideration of alternative approaches to Myc targeting. Here, we report that a downstream target of Myc-driven metabolism, miR-17–92, while increasing bioenergetic capacity of tumor cells also increases sensitivity to apoptosis induced by biguanides. Using both classic (phenformin) and newly developed (IM156) biguanides, we demonstrated that human and mouse lymphoma cells with elevated miR-17–92 expression were highly sensitized to biguanide treatment. As single agents, both phenformin and IM156 were effective at extending the survival of mice bearing miR-17–92-expressing lymphomas. In addition, we observed a statistical correlation between miR-17 levels in human lymphoma cells and sensitivity to biguanide treatment. Our data provide rationale for the clinical investigation of miR-17–92 expression level as a potential biomarker for biguanide sensitivity in cancer.

Enhanced Myc activity leads to the overexpression of miR-17–92, which in turn lowers LKB1 expression. This leads to enhanced tumor growth associated with increased glycolysis and oxidative phosphorylation, but it also reduces the capacity of lymphoma cells to activate the LKB1-AMPK system to overcome the energetic stress imposed by biguanides. These cells are then susceptible to apoptosis upon biguanide treatment, as both phenformin and IM156 inhibit oxidative phosphorylation, and the LKB1-AMPK system cannot be activated to respond to this inhibition. Another important function of LKB1-AMPK signaling is the surveillance of mitochondrial integrity.38 The potential impact of miR-17–92 on mitochondrial homeostasis and changes in response to biguanide treatment remains to be determined.

Our results address gaps in knowledge related to the mechanism by which LKB1 loss induces biguanide sensitivity. Previous work indicates that phenformin treatment increases reactive oxygen species (ROS) levels in cells with reduced LKB1 expression,3 leading to oxidative damage and preferential cell death of LKB1 null tumor cells. We show here that glucose- and glutamine-dependent fueling of the TCA cycle was inhibited in miR-17–92-expressing lymphoma cells responding to biguanide treatment. This fueling is a key node for the production of reducing equivalents for ATP production and biosynthetic intermediates for growth (e.g., citrate, aspartate). Our data are consistent with observations that reducing mitochondrial aspartate production in tumor cells can impair cell viability, due in part to diminished purine and pyrimidine biosynthesis.39,40 Reduced biosynthetic metabolism, combined with reduced mitochondrial NADH production and an inability to activate AMPK signaling, appear to sensitize miR-17–92-expressing (+17 + Myc) lymphoma cells responding to biguanide treatment.

Our results provide a rationale for the clinical investigation of miR-17–92 expression level as a potential biomarker for biguanide sensitivity in cancer.

**Figure 6. miR-17 and -20 Expression Correlates with Biguanide Sensitivity in Human Lymphoma Cells**

(A and B) EC50 for phenformin (Phen, left) and IM156 (right) correlated to the relative levels of pri-miR-17 (A) and mature miR-17 (B) transcripts for 10 human lymphoma cell lines. Linear regression (dotted line) is shown for both drugs with shaded 95% confidence interval.

*p < 0.05, **p < 0.01, and ***p < 0.001.
expressing lymphoma cells to metabolic stress. Consistent with this, biguanide treatment, particularly IM156, slowed lymphoma cell growth in vitro and extended the lifespan of tumor-bearing mice.

There has been increased interest in the clinical use of biguanides for cancer therapy. Encouraging preclinical data\(^8,47\) have led to >100 clinical trials of metformin for indications in oncology,\(^21\) but the results of randomized trials have been disappointing. The lack of clinical benefit of metformin in trials reported to date does not necessarily imply that biguanides as a class have no activity. Pharmacokinetic factors may account at least in part for the discrepancies between preclinical models and clinical trials with respect to the antineoplastic activity of metformin. The hydrophilic nature of metformin dictates that cellular uptake is dependent on OCT1 transporters before gaining access to mitochondria.\(^21\) This inherently limits the action of metformin to those cells expressing OCT1, which can vary between tumors.\(^41\) The biguanide phenformin displays greater bioavailability and antineoplastic activity than metformin,\(^21,41,42\) but its clinical use has been limited due to the risk of lactic acidosis, especially in patients with compromised renal or liver function.\(^43\) These results justify the investigation of novel biguanides with better pharmacokinetic and pharmacodynamic properties than metformin in neoplastic tissue as a strategy for OXPHOS inhibition for cancer treatment.

We identified IM156 from a library of >1,000 biguanides as a potential anticancer drug candidate.\(^43\) Our data indicate that IM156 is a more potent OXPHOS inhibitor than metformin or phenformin. IM156 may have advantages over metformin as it is a more potent, hydrophilic biguanide that we hypothesize is more bioavailable in neoplastic tissues. IM156 is presently being evaluated in a Phase I clinical trial (NCT 032 72256) in patients with advanced solid tumors and lymphoma. Our work here explored the use of IM156 as a monotherapy for cells with reduced LKB1-AMPK signaling, but combination therapy with other metabolic targets may increase efficacy with this agent. Recent reports have demonstrated synthetic lethality in tumor cells when metformin is combined with the pharmacological inhibition of monocarboxylate transporters (MCTs),\(^45,46\) due in part to ATP depletion following the loss of NAD\(^+\) regeneration when lactate cannot be exported by MCT1 or MCT4. Inhibition of aspartate synthesis by the malate-aspartate shuttle enzyme Got1 also synergizes with biguanides to enhance cancer cell death.\(^40\)

Our research adds to prior evidence\(^8,47\) that clinical trials of biguanides or other OXPHOS inhibitors are likely to show clinical activity if evaluated in patients with cancers that are particularly sensitive to energetic stress, such as those with elevated expression of MYC or miR-17–92 and/or decreased function of LKB1. Although LKB1 inactivation is relatively common in NSCLC,\(^7\) it is not common among other malignancies. In contrast, MYC is among the most commonly dysregulated oncogenes in both hematologic malignancies and solid tumors. Because of the paucity of viable treatments targeting MYC-driven cancers, linking the downstream MYC target miR-17–92 to the inhibition of LKB1 may identify a cohort of patients without LKB1 mutations who are particularly sensitive to biguanide treatment, opening avenues for the treatment of these cancers.

### STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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### SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.xcrm.2020.100014.

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### AUTHOR CONTRIBUTIONS

S.I., A.G., I.K., H.R.B., A.O.D., L.M.D., S.M.K.-G., G.B., T.F.D., M.N.P., and R.G.J. created the experimental design and executed it. R.C.L., M.D.M., N.A.J., and S.Y. provided the essential reagents. S.I., R.D.S., and R.G.J. conducted the bioinformatics and data analysis. S.I., A.G., A.O.D., G.B., D.A., R.D.S., T.F.D., I.K., H.R.B., and R.G.J. performed the data interpretation. S.I., K.S.W., M.S.R., S.Y., M.N.P., and R.G.J. wrote and edited the manuscript.

### DECLARATION OF INTERESTS

R.G.J. and M.N.P. serve on the Scientific Advisory Board of ImmunoMet Therapeutics. M.S.R. is a consultant to ImmunoMet Therapeutics. S.Y. is an employee of ImmunoMet Therapeutics. R.G.J. and ImmunoMet Therapeutics have financial interests in ImmunoMet Therapeutics.
hold a provisional patent for the use of IM156 for treatment of malignancy based on miR-17–92, Myc, or LKB1 expression.

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### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Rabbit polyclonal anti-human/mouse beta-actin (1:2000 dilution) | Cell Signaling | Cat # 4967 |
| Rabbit polyclonal anti-human/mouse 4E-BP (1:1000 dilution) | Cell Signaling | Cat # 9452 |
| Rabbit polyclonal anti-human/mouse 4E-BP (phospho-T36/47) (1:1000 dilution) | Cell Signaling | Cat # 9459 |
| Rabbit polyclonal anti-human/mouse rS6 (pS235/236) (1:1000 dilution) | Cell Signaling | Cat # 2211 |
| Mouse monoclonal anti-human/mouse rS6 (54D2) (1:1000 dilution) | Cell Signaling | Cat # 2317 |
| Rabbit monoclonal anti-human/mouse Raptor (24C12) (1:1000 dilution) | Cell Signaling | Cat # 2280 |
| Rabbit polyclonal anti-human/mouse Raptor (pS792) (1:1000 dilution) | Cell Signaling | Cat # 2083 |
| Rabbit polyclonal anti-human/mouse AMPKα (1:1000 dilution) | Cell Signaling | Cat # 2532 |
| Rabbit monoclonal anti-human/mouse AMPKα (pt172) (4OH9) (1:1000 dilution) | Cell Signaling | Cat # 2535 |
| Mouse monoclonal primary anti-human/mouse/rat LKB1 (Ley 37D/G6) (1:2500 dilution) | Santa Cruz Biotechnology | Cat # sc-32245 |
| Rabbit polyclonal anti-human/mouse Caspase3 (1:1000 dilution) | Cell Signaling | Cat # 9662 |
| **Biological Samples** |        |            |
| *Bos taurus* (bovine) heart | C. Humphries & Sons | N/A |
| **Chemicals, Peptides, and Recombinant Proteins** |        |            |
| Metformin hydrochloride | Toronto Research Chemicals | Cat # M258815 |
| phenformin | Toronto Research Chemicals | Cat # P296900 |
| sucralose | Sigma | Cat # 69293-100G |
| 4-OHT | Sigma | Cat # H7904-5MG |
| XF Base Medium Minimal DMEM without Phenol Red | Agilent | Cat # 103335-100 |
| IMDM GlutaMAX | Thermo Fisher | Cat # 3198-022 |
| Fetal bovine serum | Wisent-Bioproducts | Cat # 080-450 |
| Penicillin-streptomycin | Wisent-Bioproducts | Cat # 450-201-EL |
| L-Glutamine | Biochrom AG | Cat # M11-004 |
| 2-mercaptoethanol | GibCO | Cat # 21985-023 |
| RPMI | Wisent | Cat # 350-000 |
| puromycin | BioShop Canada | Cat # PUR333.25 |
| butanol | Sigma-Aldrich | Cat # B7906-500ML |
| hydrochloric acid, 6N | Fisher Scientific | Cat # SA56-4 |
| sodium dicyanamide | Sigma-Aldrich | Cat # 178322-25G |
| phenylamine | Sigma-Aldrich | Cat # 242284-500ML |
| trypan blue | LifeTech (Biobar) | Cat # 15250-061 |
| Fixable Viability Dye eFluor 780 | eBioscience | Cat # 65-0865-14 |
| [U-13C]-glucose | Cambridge Isotopes | Cat # CLM-1396 |

(Continued on next page)
| REAGENT or RESOURCE SOURCE | IDENTIFIER |
|----------------------------|------------|
| FCCP                       | Cayman     | Cat # 15218 |
| Oligomycin                 | Sigma-Aldrich | Cat # 1404-19-9 |
| Rotenone                   | Cayman     | Cat # 13955 |
| Antimycin A                | Sigma Aldrich | Cat # A8674 |
| Poly-D-lysine hydrobromide | Sigma Aldrich | Cat # P6407 |
| Lipolectamine 3000         | Thermo Fisher | Cat # L3000001 |
| Trizma                     | Sigma Aldrich | Cat # T1503 |
| sucrose                    | Sigma Aldrich | Cat # S0389 |
| horse heart cytochrome c   | Sigma Aldrich | Cat # C2506 |
| NADH                       | Sigma Aldrich | CAS # 606-68-6 |
| Malic enzyme               | Jones and Hirst | N/A |
| Fumarase                   | Jones and Hirst | N/A |
| potassium sulfate (KSO₄)   | Sigma Aldrich | Cat # P0772 |
| magnesium sulfate (MgSO₄)  | VWR        | Cat # 2312982 |
| sodium succinate           | Sigma Aldrich | Cat # S2378 |
| NADP⁺                      | Sigma Aldrich | Cat # N0585 |
| 3-((3-cholamidopropyl) dimethylammonium)-1-propanesulfonate (CHAPS) | Santa Cruz Biotechnology | Cat # sc-29088A |
| soy bean asolectin         | Avanti Polar Liquids | Cat # 541601G |
| decylubiquinone (DQ)       | Santa Cruz Biotechnology | Cat # sc-358659 |
| DMSO                       | Fisher Bioreagents | Cat # BP231-100 |
| Glucose ≥ 99.5% D(+)       | Roth       | Cat # HN06.1 |
| [¹³C]-glutamine             | Cambridge Isotopes | Cat # CLM-3612-PK |
| [U-¹³C]-glucose            | Cambridge Isotopes | Cat # CLM-1396 |
| methanol                   | VWR        | CAMX0475-1 |
| D-myristic acid            | Sigma Aldrich | Cat # 366889-1G |
| methoxamine hydrochloride  | Sigma Aldrich | Cat # 226904-5G |
| pyridine                   | Sigma Aldrich | Cat # 270970-100ML |
| N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA) | Sigma Aldrich | Cat # 394882-5ML |
| CHAPS buffer (10 mM Tris-HCl, 1 mM MgCl₂, 1 mM EGTA, 0.5 mM CHAPS, 10% glycerol, and 5 mM NaF) | (Faubert et al., 2014) | N/A |
| AMPK lysis buffer, RIP buffer | Cell Signaling | Cat # 9806S |
| QiAzol lysis reagent       | QIAGEN     | Cat # 79306 |
| HBSS                       | Wisent     | Cat # 311-510-CL |

Critical Commercial Assays

| Seahorse XFe96 FluxPak     | Agilent Technologies | Cat # 102416-100 |
| miRNeasy Mini kit          | QIAGEN             | Cat # 217004 |
| miScript II RT kit         | QIAGEN             | Cat # 218160 |
| SensiFAST SYBR Lo-Rox Mix  | Bioline            | Cat # BIO-92005 |
| miScript primer assays     | QIAGEN             | Cat # 218300 |
| Dual-Glo Luciferase assay kit | Promega          | Cat # E2920 |

Experimental Models: Cell Lines

| Ej-Myc lymphoma cells      | Izreig et al. | N/A |
| Ej-Myc Cre-ERT2⁺; miR-17-92ΔΔ | Mu et al. | N/A |

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RESOURCE AVAILABILITY

Lead Contact
Additional information and request for resources and reagents should be directed to and will be made available by the Lead Contact, Russell G. Jones (russell.jones@vai.org).

Materials Availability
All unique/stable reagents generated in this study are available from the Lead Contact with a completed Materials Transfer Agreement. The biguanide IM156 is the intellectual property of ImmunoMet Therapeutics. Requests for ImmunoMet Therapeutics reagents can be made through the Lead Contact.

Data and Code Availability
All data associated with this study are available in the main text or the supplementary materials. A spreadsheet to assist in converting OCAR and ECAR Seahorse data into oxidative and glycolytic ATP production rates is available at https://russelljoneslab.vai.org/tools. This study did not generate any code.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice
CD-1 nude mice were purchased from Charles River Laboratories (Wilmington, MA). All animals had ad libitum access to food and water and were maintained at 22–24°C on a 12-hour light/12-hour dark cycle with 5 mice per cage. Mice were bred and maintained under specific pathogen-free conditions at McGill University under approved protocols. For tumor xenograft assays, water bottles carrying 1.2% sucralose, 0.9 mg/mL phenformin +1.2% sucralose, or 0.8 mg/mL IM156 + 1.2% sucralose were provided for ad libitum consumption. Mice were observed daily for health status until clinical displays of disease, such as weight loss and poverty of movement, met IACUC criteria for euthanasia at which point mice were euthanized. Experiments were performed on female mice between 8 and 20 weeks of age.

Cell lines
HEK293 cells were obtained from ATCC. Expression constructs for NDI1 were a generous gift of Dr. Navdeep Chandel. The generation of Em-Myc Cre-ERT2; mir17−/−92/fl/fl lymphoma cells has been described previously. Deletion of mir17−/−92 was achieved by culturing Em-Myc Cre-ERT2; mir17−/−92/fl/fl cells with 250 nM 4-OHT for four days, followed by subcloning 4-OHT-treated cells to isolate cells deficient for mir17−/−92. Em-Myc cells were cultured on a layer of irradiated Ink4anull MEF feeder cells in DMEM and
IMDM medium (50:50 mix) supplemented with 10% fetal bovine serum (FBS), 20000 U/mL penicillin, 7 mM streptomycin, 2 mM glutamine, and β-mercaptoethanol. Raji cells were cultured in RPMI medium supplemented with 10% FBS, 20000 U/mL penicillin, 7 mM streptomycin, and 2 mM glutamine. Cells were grown at 37 °C in a humidified atmosphere supplemented with 5% (v/v) CO2. Retroviral-mediated gene transfer into lymphoma cells was conducted as previously described. Briefly, lymphoma cells were transduced via spin infection, followed by culture in 4 μg/mL puromycin for four days, and subsequent subcloning by limiting dilution. miR-17-5p constructs have been previously described. Knockdown of Stk11 via shRNA (sequence: 5’-AGGTCAAGATCCTCAAGAAGAA-3’) has been described.

**METHOD DETAILS**

**Synthesis of IM156**

IM156 was synthesized as previously reported. In brief, pyrrolidine was dissolved in butanol before concentrated hydrochloric acid was added and stirred for 30 min at 0 °C. Sodium dicyanamide was then added and stirred for 24 h under reflux. After completion of the reaction was confirmed, the adduct—N1-pyrrolidine cyanoguanidine—was purified. Next, phenylamine was dissolved in butanol before concentrated hydrochloric acid was added and stirred for 30 min at room temperature. The N1-pyrrolidine cyanoguanidine prepared above was then added and stirred for 6 h under reflux. This mixture was concentrated under reduced pressure then dissolved in a 6 N hydrochloric acid/methanol solution before adding ethyl acetate. The precipitate—IM156 HCl—was filtered and dried under reduced pressure. IM156.HCl can be converted to free form by adding sodium hydroxide. The free form can be converted to the acetate salt form—IM156A—by adding acetic acid.

**Cell proliferation and viability assays**

Cells were seeded at a density of 1 × 10⁶ cells/mL in 3.5 cm dishes, and cell counts determined via trypan blue exclusion using a TC20 Automated Cell Counter (Biorad). For viability measurements, cells were stained with Fixable Viability Dye eFluor 780 (eBioscience) and analyzed using a Gallios flow cytometer (Beckman Coulter, Fullerton, CA) and FlowJo software (Tree Star, Ashland, OR).

**Seahorse XF96 Respirometry and metabolic assays**

Cellular oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were determined using an XF96 Extracellular Flux Analyzer (Seahorse Bioscience) using established protocols. In brief, lymphoma cells (7.5 × 10⁶ per well) were plated in poly-D-lysine-coated XF96 Seahorse plates in 140 μL of unbuffered DMEM containing 25 mM glucose and 2 mM glutamine, followed by centrifugation at 500xg for five minutes. XF assays consisted of sequential mix (3 min), pause (3 min), and measurement (5 min) cycles, allowing for determination of OCR and ECAR every 8 min. Following four baseline measurements, 20 μL of untreated media, phenformin, or IM156 were injected into respective wells, and OCR and ECAR tracked over time. We converted ECAR and OCR measurements into glycolytic and oxidative ATP production rates based on protocols developed by Mookerjee and Brand. This method allows us to directly compare the contribution of glycolysis and OXPHOS to ATP production while correcting for the contribution of glycolysis and TCA cycle metabolism to ECAR. Tools to complete this analysis are available for download at https://russelljoneslab.vai.org/tools.

HEK293 cells were transfected with vectors expressing empty vector (Ctrl) or yeast NADH dehydrogenase NDI1 using Lipofectamine 3000 according to manufacturer’s instructions. Transfected HEK293 cells were seeded at a density of 30,000 cells/well in poly-D-Lysine–coated Seahorse plates. Oxygen consumption rates were recorded by using Seahorse XF96 bioanalyzer in the presence of serial dilution of IM156 as described above.

**Complex I and mitochondrial membrane kinetic measurements**

Mitochondrial membranes and purified complex I were prepared from Bos taurus (bovine) heart. All assays were performed at 32 °C in a SpectraMax 96-well plate reader and maximal rates determined by linear regression. NADH:O₂ oxidoreduction by membranes was measured using 5 μg/mL membranes in 10 mM Tris-HCl (pH 7.4), 250 mM sucrose, and 0.15 μM horse heart cytochrome c (Sigma-Aldrich Ltd.) with 200 μM NADH and monitored using ε₃₄₀–₃₈₀[NADH] = 4.81 mM⁻¹ cm⁻¹. Succinate:O₂ oxidoreduction was measured using a coupled enzyme assay using 300 μg/mL Malic enzyme and 60 μg/mL Fumarase both prepared as described. Activity was measured in the same buffer as above with the addition of 1 mM KSO₄ and 2 mM MgSO₄ using 5 μg/mL membranes with 5 mM succinate and 2 mM NADP⁺. This was monitored using ε₃₄₀–₃₈₀[NADPH] = 4.81 mM⁻¹ cm⁻¹ as described previously. NADH:decylubiquinone oxidoreduction by purified complex I was measured using 0.5 μg/mL complex I in 20 mM Tris-HCl (pH 7.2), 0.15% 3-(3-cholamidopropyl)dimethylammonium)-1-propanesulfonate (CHAPS, Merck Chemicals Ltd), and 0.15% soy bean asolectin (Avanti Polar Lipids), with 200 μM decylubiquinone (DQ, Santa Cruz Biotechnology) and 200 μM NADH, and monitored as above. IM156A was added from a DMSO stock and control experiments included DMSO.

**GC-MS analysis of ¹³C-labeled metabolites**

Cellular metabolites were extracted and analyzed by GC-MS using previously described protocols. E64-Eucalyptus cells (3-5 × 10⁶ per 3.5 cm dish) were incubated for 2 hours in untreated, 100 μM phenformin, or 10 μL IM156 medium containing 10% dialyzed FBS and U-[¹³C]-glucose or U-[¹³C]-glutamine (Cambridge Isotope Laboratories). Cells were washed twice with normal saline, then lysed in ice-cold 80% methanol and sonicated. For GC-MS analysis, D-myristic acid (750 ng/sample) was added to metabolite extracts as an internal standard. The samples were then extracted with an equal volume of hexane. The extracts were derivatized with N-methyl-N-(trimethylsilyl)trifluoroacetamide, and the resulting silylated products were analyzed by GC-MS. The data were processed using the ChromaTOF software (Shimadzu).
internal standard prior to drying samples by vacuum centrifugation with sample temperature controlled at –4°C (LabConco). Dried extracts were dissolved in 30 µL methoxyamine hydrochloride (10 mg/ml) in pyridine and derivatized as tert-butyldimethylsilyl (TBDMS) esters using 70 µL N-(tert-butyldimethylsilyl)-N-methyl trifluoroacetamide (MTBSTFA). An Agilent 5975C GC-MS equipped with a DB-5MS+DG (30 m x 250 µm x 0.25 µm) capillary column (Agilent J&W, Santa Clara, CA, USA) was used for all GC-MS experiments, and data collected by electron impact set at 70 eV. A total of 1 µL of derivatized sample was injected per run in splitless mode with inlet temperature set to 280°C, using helium as a carrier gas with a flow rate of 1.5512 mL/min (rate at which D27-myristic acid elutes at 17.94 min). The quadrupole was set at 150°C and the GC/MS interface at 285°C. The oven program for all metabolite analyses started at 60°C held for 1 min, then increasing at a rate of 10°C/min until 320°C. Bake-out was at 320°C for 10 min. Sample data were acquired in scan mode (1–600 m/z). Mass isotopomer distribution for TCA cycle intermediates was determined using a custom algorithm developed at McGill University54. After correction for natural 13C abundances, a comparison was made between non-labeled (12C) and 13C-labeled abundances for each metabolite. Metabolite abundance was expressed relative to the internal standard (D-myristic acid) and normalized to cell number.

**Immunoblotting and Quantitative Real-Time PCR**

Lymphoma cell lines were subjected to SDS-PAGE and immunoblotting using CHAPS and AMPK lysis buffers as previously described.55 Primary antibodies against β-actin, 4EBP (total, phospho-T36/47), rS6 (total and p S235/236), Raptor (total and pS792), AMPKα (total and phospho-T172), and Caspase-3 were obtained from Cell Signaling Technology (Danvers, MA). Primary antibody against LKB1 (Ley 37D/G6) was obtained from Santa Cruz Biotechnology (Dallas, TX, USA). HRP-conjugated secondary antibodies from Cell Signaling Technologies were used to detect primary antibodies, and immobilized proteins detected by chemiluminescence using Pierce ECL Substrate (ThermoFisher Scientific). For qPCR quantification of mature miRNAs, Qiazol was used to isolate RNA, miRNEasy Mini kit was used to purify miRNAs and total mRNA, and cDNA was synthesized using the miScript II RT kit (QIAGEN). Quantitative PCR was performed using the SensiFAST SYBR Hi-ROX kit (Bioline) and an AriAXM Real Time PCR system (Agilent Technologies). miScript primer assays (QIAGEN) were used to detect mature miRNAs of the miR-17-92 cluster, with miRNA expression normalized relative to U6 RNA levels.

**Tumor xenograft assays**

Lymphoma cells were resuspended in HBSS at a concentration of 5 × 10⁶ cells/mL, and 10⁶ cells/200 µL were injected intravenously into CD-1 nude mice (Charles River). Water bottles carrying 1.2% sucralose, 0.9 mg/mL phenformin +1.2% sucralose, or 0.8 mg/mL IM156 + 1.2% sucralose were provided for ad lib consumption.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Statistics were determined using paired Student’s t test, ANOVA, or Log-rank (Mantel–Cox) using Prism software (GraphPad) unless otherwise stated. Data are calculated as the mean ± SEM for biological triplicates and the mean ± SD for technical replicates unless otherwise stated. Statistical significance is represented in figures by: *, p < 0.05; **, p < 0.01; ***, p < 0.001.

**ADDITIONAL RESOURCES**

Spreadsheet (to assist in converting OCAR and ECAR Seahorse data into glycolytic and oxidative ATP production rates based on protocols developed by Mookerjee and Brand56): https://russelljoneslab.vai.org/tools.