Metformin Targets Central Carbon Metabolism and Reveals Mitochondrial Requirements in Human Cancers

Graphical Abstract

Highlights

- Metformin accumulates in human tumor biopsies at micromolar concentrations
- The response to metformin in humans can be modeled in nutrient-limited environments
- Mitochondrial substrate utilization underlies metformin sensitivity
- Restoration of specific mitochondrial outputs causes resistance to metformin

Authors
Xiaojing Liu, Iris L. Romero, Lacey M. Litchfield, Ernst Lengyel, Jason W. Locasale

Correspondence
jason.locasale@duke.edu

In Brief
Liu et al. carry out an integrative metabolomics analysis of metformin action in ovarian cancer. Using patient samples (including those from a “super-responder”), animal tumors, and cell culture models, they find that the predominant mechanism of action by metformin in cancer is to target tumor-cell-intrinsic mitochondrial metabolism.
Metformin Targets Central Carbon Metabolism and Reveals Mitochondrial Requirements in Human Cancers

Xiaojing Liu,1 Iris L. Romero,2 Lacey M. Litchfield,2 Ernst Lengyel,2 and Jason W. Locasale1,3,*

1Department of Pharmacology and Cancer Biology, Duke Cancer Institute, Duke Molecular Physiology Institute, Duke University School of Medicine, Durham, NC 27710, USA
2Section of Gynecologic Oncology, Department of Obstetrics and Gynecology, Center for Integrative Science, University of Chicago, Chicago, IL 60637, USA
3Lead Contact
*Correspondence: jason.locasale@duke.edu
http://dx.doi.org/10.1016/j.cmet.2016.09.005

SUMMARY

Repurposing metformin for cancer therapy is attractive due to its safety profile, epidemiological evidence, and encouraging data from human clinical trials. Although it is known to systemically affect glucose metabolism in liver, muscle, gut, and other tissues, the molecular determinants that predict a patient response in cancer remain unknown. Here, we carry out an integrative metabolomics analysis of metformin action in ovarian cancer. Metformin accumulated in patient biopsies, and pathways involving nucleotide metabolism, redox, and energy status, all related to mitochondrial metabolism, were affected in treated tumors. Strikingly, a metabolic signature obtained from a patient with an exceptional clinical outcome mirrored that of a responsive animal tumor. Mechanistically, we demonstrate with stable isotope tracing that these metabolic signatures are due to an inability to adapt nutrient utilization in the mitochondria. This analysis provides new insights into mitochondrial metabolism and may lead to more precise indications of metformin in cancer.

INTRODUCTION

Metformin, a biguanide, is a commonly prescribed agent for the management of type II diabetes. It is widely used clinically because of its desirable safety profile and reproducible actions on systemic glucose homeostasis, which lead to the reduction of glucose levels during hyperglycemia (Knowler et al., 2002). Its effects are attributed to suppression of hepatic gluconeogenesis, reduction of glucose absorption in the intestine, alterations in the composition of the gut microbiota, and direct control of glucose metabolism in muscle, subcutaneous, and visceral adipose tissue (Forslund et al., 2015; Fullerton et al., 2013; Madiraju et al., 2014; Zhou et al., 2001). The precise mechanism of action in diabetes, however, remains controversial (Pernicova and Korbonits, 2014). In epidemiological studies, metformin has been associated with reduced incidence in cancers such as breast, prostate, colorectal, endometrial, and ovarian (Decensi et al., 2010; Evans et al., 2005; Jiralerspong et al., 2009; Romero et al., 2012). Furthermore, a prospective clinical trial in non-diabetic patients showed its efficacy in patients presenting with adenomatous polyps when administered after surgical resection (Higurashi et al., 2016). Moreover, multiple clinical trials using metformin as a treatment in non-diabetic cancer patients are ongoing (Camacho et al., 2015). Thus, there is significant interest in using metformin as a cancer therapeutic, especially in cancers with limited treatment options, such as ovarian cancer (OvCa) (Bowtell et al., 2015; Febbraro et al., 2014). The mechanism of its anti-cancer properties has also been controversial, and it remains poorly understood whether metformin acts by altering host metabolism or by direct action on tumor cells (Chandel et al., 2016; Dowling et al., 2016). Further clinical advances are limited by controversies surrounding the biology of metformin and the poorly understood determinants of a response that lead to biomarkers that can be used to predict which patients may benefit.

At the cellular level, metformin is believed to disrupt mitochondrial function by partially inhibiting nicotinamide adenine dinucleotide (reduced form) (NADH) dehydrogenase (Wheaton et al., 2014) in general or by inhibiting glycerol phosphate dehydrogenase in liver cells, which also results in alterations to the electron transport chain (ETC) (Madiraju et al., 2014). As a result, electrons contained in NADH and flavin adenine dinucleotide (reduced form) (FADH2) are not as effectively transported through the ETC. Because each of these processes is central to numerous aspects of cell physiology, the cellular effects of metformin are pleiotropic. For example, it has been documented to affect AMPK signaling, protein kinase A signaling, folate metabolism, and anabolic metabolism (Birsoy et al., 2014; Cabreiro et al., 2013; Griss et al., 2015; Janzer et al., 2014; Madiraju et al., 2014; Miller et al., 2013; Shaw et al., 2005; Wheaton et al., 2014; Zhou et al., 2001). Most of these mechanisms point to mitochondrial biology as its mechanistic target.

Mitochondria utilize glucose, amino acids, and fatty acids as substrates to allow for ATP production, redox balance, and biomass precursor production. Thus, metformin results in alterations to the tricarboxylic acid (TCA) cycle, the generation of
reactive oxygen species, alterations of the mitochondrial phosphate to oxygen ratio that affects ATP production, and other essential mitochondrial functions. For example, it has recently been reported that one such essential function is to provide aspartate, which is used for the synthesis of nucleotides and protein (Birsoy et al., 2015; Cardaci et al., 2015; Sullivan et al., 2015). Other studies have found that metformin alters lipid synthesis from the mitochondria by affecting reductive carboxylation (Fendt et al., 2013). Consistently, metformin has also been found to suppress nucleotide levels (Janzer et al., 2014). Each of these mechanisms occurs through changes in the capacity of the ETC.

In light of this extensive body of literature, there is a lack of analysis of metabolism in physiological environments, where clinically relevant metabolic signatures of metformin-associated cytotoxicity can be observed. This limits many of these findings, and it is thus not understood how mitochondrial metabolism is altered to produce these changes and what compensatory pathways induced by disruption of the ETC must be overcome for metformin-induced cytotoxicity. There is, furthermore, a lack of direct study of metformin action on tumor metabolism in patients and comparisons to mechanisms observed in cell culture and in animal models. With the advent of recent metabolomics approaches, multiple aspects of tumor cell metabolism can be profiled in patient samples (Liu et al., 2014). We therefore hypothesized that such experiments could shed light on the anticancer mechanisms of metformin when integrated with studies on experimental models.

RESULTS

Altered Mitochondrial Metabolism in Human Tumors from Patients Taking Metformin

There has been a paucity of data surrounding the concentration of metformin that can be achieved in human tumors. We first developed a sensitive and quantitative method using liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS) to quantify metformin concentrations in tissue and serum. With an authentic reference compound and fragmentation pattern from tandem mass spectrometry (Figure 1A), we were able to unequivocally identify and quantify metformin in the sera (Figure 1B) and tumors (Figure 1C) of patients taking metformin prior to surgery and found concentrations reaching micromolar values. Interestingly, no differences in the insulin
levels in these patients were observed (Figure 1D). We then
generated a metabolite profile (Liu et al., 2014) of the tumors
from ten patients taking metformin presenting with stage III
and/or IV high grade papillary serous OvCa (Figure S1A, avail-
able online) compared to ten patients not taking metformin
with similar diagnoses, ages, and medication profiles. An inspec-
tion of the profile (Figure 1E) showed that most of the more than
300 metabolites measured did not show a difference indicating
extensive heterogeneity as expected (Hensley et al., 2016). Inter-
estingly, TCA intermediates and short chain acyl carnitines (suc-
cinyl carnitine and branched chain acyl carnitines), both related
to mitochondrial metabolism (Koves et al., 2008; Liu et al.,
2015), were the classes of compounds most frequently sup-
ppressed in the tumors of metformin-treated patients. Three of
the known functions of mitochondrial metabolism are to maintain
nucleotide levels, energy status, and oxidation status (Shadel
and Horvath, 2015; Weinberg and Chandel, 2015; Zong et al.,
2016). In each case, and as is expected since cancer is a
heterogeneous disease, there was large variation in metabolites
involved in each of these processes, with some patients exhibit-
ing alterations in the metabolites involved in each of these func-
tions (Figures 1E and S1B–S1D). Long chain acyl carnitines,
markers of fatty acid oxidation (Koves et al., 2008), were variable
in the patient tumors, indicating that a defect in fatty acid
oxidation, although possibly present in certain tumors, was not
commonly observed (Figure S1E). Together, these results indi-
cate that metformin accumulates in human tumors and affects
known mitochondrial biology.

Therapeutic Doses of Metformin Directly Suppress
Tumor Mitochondrial Metabolism

The metabolomics of human tumors (Figure 1) appear consistent
with the conclusion that metformin affects cell intrinsic mito-
chondrial metabolism. Nevertheless, these studies involved
a limited set of patient samples. We therefore considered labora-
tory models of ovarian cancer. HeyA8 cells were injected in the
intraperitoneal cavity of female mice and tumors formed in the
omentum, resembling a serous-papillary cystadenocarcinoma
(Lengyel et al., 2014) (Figure 2A). Treatment of mice 2 weeks after
initiation of OvCa showed that metformin effectively reduced tu-
ror weight (p = 0.01, one-tailed Student’s t test) (Figure 2B) and
the concentration of metformin reached micromolar concentra-
tions in serum (Figure 2C) and tumor (Figure 2D). Both serum
and intratumoral levels of metformin correlated (p = 0.07,
p = 0.03, respectively, Spearman’s correlation coefficient) with
treatment response. As observed in the patients, insulin levels
in mouse serum were unaffected (Figure 2E). Metabolite profiles
from metformin- and vehicle-treated animal tumors were gener-
ated using LC-HRMS (Figure 2F) and first analyzed by a principle
component (PC) analysis (Figure 2G), revealing that the largest
source variation in the cohort was treatment. Metabolites en-
riched in the pyrimidine and/or purine metabolism pathways
and the glutathione pathway (Figure 2H) contributed to the first
PC. Metabolites involved in nucleotide synthesis (Figure 2I),
redox status indicated by increased oxidized glutathione
(GSSG) levels (Figure 2J), and energy status (Figure 2K) were
generally reduced. Together, these findings indicate that metfor-
min affects several features related to mitochondrial metabolism
in animal tumors.

A Metformin-Treated Patient with an Exceptional
Outcome Shares the Metabolic Profile of the Animal
Tumors Responding to Metformin

While the metabolic profile of the metformin response in human
tumors was variable, metformin treatment in controlled settings
using laboratory animals appeared reproducible. Of note, the
time to recurrence was also variable in the patients taking
metformin, and one patient exhibited an exceptionally long
disease-free interval (long-term survivor) with no recurrence to
date (Figure 3A). Although single patient responses have long
been considered merely anecdotal in nature and lacking statisti-
cal rigor, there have recently been considerable advances made
by using these observations to understand the mechanism un-
derlying these exceptional outcomes (Grisham et al., 2015;
Marx, 2015; Wagle et al., 2014). Utilizing this long-term survivor,
we sought to compare the metabolic features of this patient, the
cohort of patients, and the mouse responding to metformin.
Overall, a small amount of overlap in the profiles of the animal
tumors and the patients was observed. Remarkably, when
comparing this long-term survivor, a substantial amount of the
metabolome overlapped with the signature in the mouse ovarian
tumor responding to metformin (Figure 3B). An analysis of
overlapping features (Figure 3C) revealed decreases in acyl-
carnitines, nucleotides, and glutathione-related metabolites
and increases in nucleotide intermediates, suggesting a buildup
in these pathways. These intriguing results indicate a possible
cell-intrinsic human signature of a response to metformin in
ovarian cancer.

In Vivo Metabolite Profiles of Metformin Response Can
Be Modeled in a Glucose-Limited Environment

We next questioned the specific biochemical events and envi-
ronmental conditions that directly give rise to these correllative
signatures seen in human and mouse xenograft tumors.
Although serum levels of glucose in the mice and humans were
in the range of 5–10 mM (Figure 4A), the values measured in
human and mouse tumors were markedly lower, ranging from
0–1 mM (Figure 4B). We thus reasoned that the metformin re-
sponses observed in vivo may require limiting mitochondrial
substrate availability in the glucose-deprived tumor microen-
vironment. To test this hypothesis, we generated a metabolite
profile in HeyA8 cells treated with metformin or vehicle in
glucose-limited (1 mM) conditions that mimic the tumor microen-
vironment (Figure 4C). Notably, intracellular metformin concen-
tration could reach values comparable to that of the culture
media (Figure 2C). An increase in NADH, indicating disruption
of ETC (Figure S2B), was observed with no changes in glucose
uptake and lactate secretion fluxes (Figures S2C and S2D).
Moreover, decreases in glutathione-related metabolites were also
observed (Figure 4C) in addition to depletions in the TCA cy-
cle (Figure 4D) and nucleotide levels (Figure 4E). An additional
feature associated with ETC inhibition, involving a decrease in
short chain acyl carnitines metabolites, was observed (Fig-
ure S2E), which was consistent with observations in patient
(Figure 1E) and mouse tumors (Figure S2F). Notably, metformin
also induced an increase of NADH in glucose-rich medium (Fig-
ure S2G), indicating metformin also targets on the ETC when
there is sufficient glucose. However, no changes in glutathione
(GSH)-related metabolites (Figure S2F), TCA intermediates
nucleotides were observed (Figure S2I), indicating that global metabolic reprogramming is not induced upon metformin treatment in high glucose conditions. Thus, most of the metabolic alterations found in metformin-responsive mouse and human tumor tissues can be reproduced in glucose-limited conditions, but not in high-glucose conditions, observed in typical cell culture media.

**Metabolite Profiles of Metformin Are the Result of Altered Substrate Utilization in the Mitochondria**

Although metabolite levels are direct reflections of physiological status, their interpretation is confounded by the dynamic nature of metabolism that involves the flow of nutrients or flux (Zamboni et al., 2015). Therefore, the utilization of each key macronutrient that is catabolized in the mitochondria was investigated. Stable isotopes of carbohydrate (U-\(^{13}\)C glucose), lipid (U-\(^{13}\)C palmitate), and amino acid (U-\(^{13}\)C glutamine) were used to trace the fate of mitochondrial substrate utilization in response to metformin treatment (Figure 5A). Glucose entry into the mitochondria, as measured by the mass isotopologue patterns of citrate (Figure 5B), glutamine (Figure 5C), and palmitate (Figure 5D) in the TCA cycle were each affected by metformin treatment. In addition glucose-derived (Figure 5E), glutamine-derived (Figure 5F), and palmitate-derived (Figure 5G) aspartate, an intermediate for pyrimidine synthesis, were decreased, as were corresponding labeling patterns of glucose-derived (Figure 5H) and glutamine-derived (Figure 5I) pyrimidine that originate from the TCA cycle. Moreover, glutathione biosynthesis, which uses glutamate generated from the TCA cycle, was reduced (Figures S3A–S3D). However, addition of the antioxidant N-acetylcysteine (NAC) didn’t increase GSH levels in the absence of metformin (Figure S3E), but it decreased glutathione disulfide (GSSG) levels.
Metformin Response and Resistance Are Determined by the Availability of Specific Nutrients

Next, we asked whether the nutrients utilized and metabolic outputs generated by mitochondria determine the cytotoxic effects of metformin. Consistent with previous observations (Birsoy et al., 2014; Litchfield et al., 2015; Wheaton et al., 2014), we found that HeyA8 OvCa cells are resistant to metformin in nutrient-rich conditions containing typical concentrations of glucose in culture media. When subjected to nutrient-limiting conditions, including glucose and amino acid deprivation, these cells become sensitive to metformin, implying that these environments impose sensitivity to metformin (Figure 6A). Annexin V and propidium iodide staining of HeyA8 cells in glucose-limited (vehicle) and glucose-complete media (+glucose) demonstrated that glucose availability affects metformin-induced apoptosis and cell death in this model (Figure 6B). Furthermore, oxidative stress involving sub-lethal concentrations (30 μM) of hydrogen peroxide (Figure 6C) sensitized cells to metformin-induced cell death. Interestingly, the tumor from the long-term survivor (Figure 6D) exhibited low levels of glucose and a higher oxidative state as measured by glutathione potential and other related metabolites. Similarly, increased oxidative stress was also observed in metformin-treated cells (Figure 6E). To understand the metabolic features that underlie this sensitivity, we attempted to rescue metformin cytotoxicity using a series of nutrients. Cell number was quantified using both a colorimetric tetrazolium-based assay and direct cell counting. We found that pyruvate, an esterified version of α-ketoglutarate (DMKG), acetate, and α-ketobutyrate (Sullivan et al., 2015), all immediate mitochondrial substrates, were able to rescue metformin-induced cytotoxicity. Furthermore, we investigated downstream pathways altered by metformin and observed that nucleosides, deoxynucleotide triphosphates (dNTPs) (Figure 6G), or N-acetyl cysteine (NAC) (Figure 6H) exhibited modest but significant effects on rescuing cytotoxicity. The combinations of NAC with nucleotides, nucleosides, acetate, or α-ketobutyrate could, to a larger extent, rescue cells from metformin-induced cell death (Figure 6I). Importantly, results from the tetrazolium colorimetric assay were consistent...
with those obtained both from cell counting and cell imaging (Figures S5A–S5E). The supply of aspartate has been proposed to be an essential role of the electron transfer chain (Birsoy et al., 2015; Cardaci et al., 2015; Sullivan et al., 2015), but aspartate alone failed to rescue metformin-induced cell death, while the combination with NAC exerted only a modest effect (Figure S5F). Taken together, these results demonstrate that, consistent with signatures of metformin response in mice and humans, metformin cytotoxicity is caused by limited mitochondrial substrate availability and that redox and nucleotide biosynthesis are the critical functions required for cell viability in the presence of metformin. In agreement, redox status was preserved with the addition of mitochondrial substrates or NAC (Figure 6J) and oxidative stress was diminished (Figure 6K). Furthermore, we found that metformin also decreased energy status (ATP/ADP and ATP/AMP), and this effect was partially recovered in all four conditions that rescued cell proliferation (Figure S6A). Increased NADH/ nicotinamide adenine dinucleotide (oxidized form) (NAD\(^+\)) due to NADH accumulation, however, was not rescued by these nutrients (Figure S6B). This finding is in contrast to previous observations, where pyruvate preserved the NAD\(^+\)/NADH ratio in the presence of electron chain transport inhibitors (Sullivan et al., 2015). The discrepancy highlights the heterogeneity of metabolic requirements and likely depends on other fluxes that metabolize NAD\(^+\). TCA intermediates were dramatically elevated by pyruvate acid (PYR) and DMKG, but not by NAC or nucleosides (Figure S6C). Similarly, nucleotide levels are also differentially regulated by these nutrients (Figure S6D). Therefore, these findings additionally confirm that redox, energy homeostasis, and nucleotide biosynthesis in combination appear to be determining factors to the cytotoxic response in these environments.

The Response to Metformin Depends on whether Substrate Utilization in the Mitochondria Is Flexible

Altered nutrient utilization in cells with defective mitochondria is well documented with numerous pathway fluxes involved (Fendt et al., 2013; Worth et al., 2014). These include pyruvate carboxylase, reductive glutaminolysis, and branched-chain amino acid

![Figure 4. In Vivo Metabolite Profiles of Metformin Treatment Can Be Modeled in a Glucose-Limited Environment](image)

(A) Glucose concentrations in RPMI medium, patient, and mouse serum.
(B) Glucose concentration in RPMI medium, patient, and mouse ovarian tumors.
(C) Network of significant metabolic changes in ovarian cancer cells in response to metformin treatment. The node size represents the p value, and the color represents the fold change. The size of the node indicates the magnitude of the change. Red represents metabolites that are higher in metformin-treated cells, and green denotes metabolites that are lower in metformin-treated cells.
(D and E) Representative metabolites among TCA (D) and nucleotides (E). The relative level was calculated by dividing MS intensity of each metabolite by corresponding MS intensity in the vehicle. *p < 0.05, **p < 0.01. Error bars obtained from SEM of n = 3 independent measurements.
oxidation (BCAA), which are used as compensatory pathways when cells with functioning mitochondria are under mitochondrial stress (Figure 7A). In glucose-limited conditions, cells were unable to increase reductive glutaminolysis activity when oxidative glutamine metabolism was inhibited by metformin, while the presence of PYR, DMKG, NAC, or glucose caused up-regulation of the activity of this pathway upon treatment with metformin (Figure 7B). Moreover, the addition of excess glucose (11.1 mM) increased pyruvate carboxylase activity in response to metformin and maintained a stable carbon supply in the mitochondria (Figure 7C). In contrast, when glucose-limited, these cells were not able to use this pathway. Moreover, branched-chain amino acid oxidation (BCAA), also a pathway used as a fuel source, was significantly diminished by metformin treatment and recovered with addition of PYR, DMKG, or glucose (Figure 7D). Consistently, pyruvate was found to increase the concentration of TCA cycle intermediates (Figure S6C). Indeed, upon adding U-13C pyruvate for 6 hr, citrate (M+2) and malate (M+3), representing flux into the TCA cycle through citrate synthase and pyruvate carboxylase, were both increased (Figure 7E), as was alanine (M+3), indicating transamination and generation of α-ketoglutarate (Figure 7F). Thus, the inability to use these adaptive pathways in the TCA cycle in response to metformin treatment contributes to the sensitivity in glucose-limited conditions (Figure 7G).

DISCUSSION

Cell-Intrinsic Metabolic Reprogramming by Metformin in Tumors

Epidemiological data have strongly associated metformin usage with improved cancer outcomes in multiple cohorts in numerous different cancers, including OvCa (Decensi et al., 2010; Evans et al., 2005; Ezewuiro et al., 2016; Jiralerspong et al., 2009; Libby et al., 2009; Romero et al., 2012; Wright and Stanford, 2009). However, understanding how metformin might act to protect against or treat cancer has been complicated by controversies concerning its mechanism of action and the organ site on which it acts. By quantifying metformin concentrations in patient and mouse tumors, we were able to show it accumulates in tumors at concentrations that are sufficient to induce metabolic alterations consistent with changes in the activity of the electron transport chain. Importantly, the extent of the anti-tumor effect correlated with the amount of the compound that was measured.
in the tumor. Accordingly, critical aspects of mitochondrial metabolism were altered after metformin treatment, such as the levels of TCA intermediates, the accumulation of NADH, and the increase of reactive oxygen species (ROS). The accumulation of ROS could be attributed either to decreased glutathione (GSH) biosynthesis or electron leakage from electron transport chain, whose efficiency is inhibited by metformin. Aspartate biosynthesis was recently identified as the essential function of the respiratory chain (Birsoy et al., 2015; Cardaci et al., 2015; Sullivan et al., 2015). Although metformin inhibited nucleotide synthesis in several settings, aspartate, surprisingly, did not appear to be limiting in these conditions.

Because mitochondria function as a centralized hub in the metabolic network, we observed global metabolic reprogramming in response to metformin treatment in human, mouse, and cell culture. Nevertheless, most of these metabolic alterations can be rationalized from a perturbation in mitochondrial metabolism, and rescue of metformin-induced cell death was achieved either by supplementing cells with mitochondrial substrates or combining nutrients that produce metabolites involved in downstream processes, such as nucleotides and antioxidants.

**Metformin and Nutrient Limitations in the Mitochondria**

Glucose concentrations were found to be less than 1 mM in tumors from OvCa patients. Consistently, cytotoxicity at physiologically meaningful doses of metformin is achieved when glucose availability is limited (Birsoy et al., 2014; Litchfield et al., 2015; Birsoy et al., 2015; Cardaci et al., 2015; Sullivan et al., 2015). Although metformin inhibited nucleotide synthesis in several settings, aspartate, surprisingly, did not appear to be limiting in these conditions.
Wheaton et al., 2014). Interestingly, in conditions with sufficient nutrient availability, such as standard tissue culture conditions, cells readily resist metformin treatment by bringing other nutrients into the mitochondria to compensate for partially impaired ETC activity. Accordingly, metformin treatment enhanced pyruvate carboxylase activity and increased the reverse exchange flux in the TCA cycle, which resulted in increased production of malate, fumarate, and oxaloacetate and decreased flux along the TCA cycle. Such effects may also result in suppression of hepatic gluconeogenesis, which is thought to be a determinant of its mechanism of action in diabetes. Each of these features is thought to confer requirements for tumor growth in different contexts (Davidson et al., 2016; Hensley et al., 2016). However, in contrast to recent findings on biguanides and aspartate synthesis (Birsoy et al., 2015; Cardaci et al., 2015; Sullivan et al., 2015), our data show that, under limiting conditions of nutrient availability, inhibition of the respiratory chain by metformin can actually cause secretion of aspartate as a waste product (Figure S4B). This finding exemplifies the heterogeneity of tumor metabolism and may extend to other cases where cells have certain respiratory chain defects, such as the case of succinate dehydrogenase mutations that increase dependence on pyruvate carboxylase (Cardaci et al., 2015). Thus, inhibition of the respiratory chain in proliferating cells does not always cause a defect in biosynthesis derived from aspartate metabolism.

We have reported that OvCa cells obtain fatty acids from adjacent adipocytes in the tumor microenvironment (Nieman et al., 2011). Here, we observed that catabolism of lipids by fatty acid

Figure 7. The Response to Metformin Depends on whether Substrate Utilization in the Mitochondria Is Flexible
(A) Schematic of substrate utilization. The adaptive pathways under mitochondrial stress are highlighted in green. OAA, oxaloacetate; keto acids, ketoleucine/ketoisoleucine.
(B) Reductive glutaminolysis activity as represented by the fraction of 13C enriched citrate isotopologue (M+5) from cells treated with 13C glutamine in different medium conditions.
(C) Pyruvate carboxylase activity as estimated by the fraction of malate (M+3) from cells treated with 13C glucose in different medium conditions.
(D) Ketoleucine/ketoisoleucine levels in different media conditions, representing BCAA oxidation.
(E) Representative metabolite enrichment pattern from U-13C pyruvate-treated cells.
(F) Scheme of coupled transamination reactions demonstrating stoichiometric α-ketoglutarate generation from alanine labeling.
(G) Model of resistant and sensitive response to metformin treatment. *p < 0.05, **p < 0.01. Error bars obtained from SEM of n = 3 independent measurements.
oxidation (FAO) could be used to fuel the TCA cycle in environments of limited glucose availability. However, when increased consumption of fatty acid was observed, cells were sensitive to metformin, likely due to the fact that FAO is much slower in fueling the TCA cycle relative to the rate of glucose or glutamine oxidation. Therefore, in glucose-limited conditions, adaptive pathways, such as FAO, that are upregulated and available in response to metformin are unable to fully compensate.

Finally, recent studies have suggested an enhanced dependency on mitochondrial metabolism during tumor dormancy or latency, a poorly characterized state of cancer cells that may underlie how tumors recur (Viale et al., 2014). Because nearly complete inhibition of the respiratory chain that can be achieved with compounds such as rotenone and cyanide is limited by toxicity, a speculative suggestion is that the safety profile of metformin may lend itself to a treatment for this cancer cell state of dormancy as a means to prevent recurrence.

Molecular Determinants of Metformin Action and a Long-Term Survivor

In addition to an overall signature pointing to alterations in mitochondrial metabolism across a set of human tumors, we further provide evidence, albeit an “n of 1,” that a late-stage ovarian cancer patient taking metformin has an exceptionally long disease-free interval (long-term survivor) after surgical resection and exhibits numerous intratumoral features of what defined a response to metformin in both a mouse model of OvCa and cultured cells. It is understood that this long-term survivor provides only anecdotal evidence of a patient response to metformin; nevertheless, examples of defining a mechanistic basis for an exceptionable patient response have led to numerous new and important insights (Grisham et al., 2015; Marx, 2015; Wagle et al., 2014). Typically, these responses are characterized by further validation of a predictive model for why a patient with a defined genetic lesion responded to a particular therapy. In this case, we show that a mechanistic basis of a long-term survivor can be understood through analysis of the specific metabolism occurring in the patient’s tumor. Thus, knowledge of the metabolic profile of the tumor can be predictive of therapeutic response independent of cancer genetics.

Nevertheless, it is worth noting that there are several confounding factors in the patient study. The patients taking metformin had diabetes, the major clinical indication of its usage, and some patients were also taking other medications. Also, 2 other patients of the 20-patient total had not experienced recurrence at the time of this paper’s submission; albeit, these patients were much closer to the time of their surgical resection. Nevertheless, we found signatures of mitochondrial inhibition in certain human tumors that we studied more deeply with model systems, and our conclusions were drawn from the integration of three independent systems (human, mouse, and cell culture).

Conclusions

The data presented to our knowledge provide the most comprehensive metabolomics analysis of metformin action in ovarian cancer to date. By integrating data from metabolic features remnant in human patient tumors with idealized responses in pre-clinical models, we were able to provide insight into the nature of metformin’s effect on cancer. In doing so, we were also able to identify new aspects of metabolism that are controlled by mitochondria. With this deeper biochemical and systems-level understanding using a thorough metabolomics platform, we identified many of the pleiotropic features that determine an anti-cancer response to metformin. Hopefully these results will inform the design and patient selection for upcoming clinical investigations of this intriguing agent.
**Metabolite Extraction in Cell Culture**

HeyA8 cells were seeded at a density of 150,000 cells per well in six-well plates. After overnight incubation in full growth medium, the old medium was removed, and cells were washed with 1 mL PBS before the addition of 2 mL of treatment medium. Glucose consumption and lactate secretion measurement are described in **Supplemental Information**. For intracellular metabolite analysis, after an incubation of 20 hr, metabolites were extracted as described in a previous study (Liu et al., 2014). Detailed information on 13C tracing is provided in **Supplemental Information**.

**Insulin Measurement**

The concentration of insulin in serum was measured using Ultra Sensitive Mouse Insulin ELISA Kit (Crystal Chem) according to the manufacturer’s protocol. This insulin kit was kindly provided by Drs. Daniel Cooper and David Kirsch.

**Apoptotic Cell Death**

Phosphatidylserine on the external surface and propidium iodide uptake were measured using the Annexin V Alexa Fluor 488 and Propidium Iodide (PI) Dead Cell Apoptosis Kit (ThermoFisher Scientific) according to the manufacturer’s recommendations. A detailed experimental procedure is described in the **Supplemental Information**.

**Statistical Analysis and Bioinformatics**

The LC-HRMS data analysis method is described in the **Supplemental Information**. Pathway analysis of metabolites using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (http://www.genome.jp/kegg/) was carried out with Metaboanalyst software (http://www.metaboanalyst.ca). Principle component analysis (PCA) and statistical analysis were done using R programmer (https://www.r-project.org). The metabolic network was constructed based on the Human Metabolome Database (HMDB) (http://www.hmdb.ca) using open source software GAM (https://artymovilab.wustl.edu/shiny/gam/). All data are represented as mean ± SEM. All p values were obtained from two-tailed Student’s t test unless otherwise noted.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2016.09.005.

**AUTHOR CONTRIBUTIONS**

I.L.R. and E.L. initiated the studies by suggesting analysis of the patient and animal samples. J.W.L. and X.L. designed the metabolism experiments, performed all data analysis and interpretation, and wrote the paper with essential edits from I.L.R. and E.L. L.M.L. and I.L.R. performed the animal experiments. X.L. performed all other experiments. E.L. and I.L.R. contributed the human patient samples.

**ACKNOWLEDGMENTS**

We acknowledge support from NIH awards R01CA193256 and R00CA168997 (to J.W.L.), P50CA136393 (to E.L. and I.L.R.), and K12HD000849 (to I.L.R.). We thank Dr. Donald McDonnell, Dr. Deborah Muoio, members of the J.W.L. lab, and Dr. Abir Mukherjee for helpful discussions.

Received: May 10, 2016
Revised: July 26, 2016
Accepted: September 14, 2016
Published: October 13, 2016

**REFERENCES**


