Dual Inhibition of Tumor Energy Pathway by 2-Deoxyglucose and Metformin Is Effective against a Broad Spectrum of Preclinical Cancer Models

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Abstract

Tumor cell proliferation requires both growth signals and sufficient cellular bioenergetics. The AMP-activated protein kinase (AMPK) pathway seems dominant over the oncogenic signaling pathway suppressing cell proliferation. This study investigated the preclinical efficacy of targeting the tumor bioenergetic pathway using a glycolysis inhibitor 2-deoxyglucose (2DG) and AMPK agonists, AICAR and metformin. We evaluated the in vitro antitumor activity of 2DG, metformin or AICAR alone, and 2DG in combination either with metformin or AICAR. We examined in vivo efficacy using xenograft mouse models. 2DG alone was not sufficient to promote tumor cell death, reflecting the limited efficacy showed in clinical trials. A combined use of 2DG and AICAR also failed to induce cell death. However, 2DG and metformin led to significant cell death associated with decrease in cellular ATP, prolonged activation of AMPK, and sustained autophagy. Gene expression analysis and functional assays revealed that the selective AMPK agonist AICAR augments mitochondrial energy transduction (OXPHOS) whereas metformin compromises OXPHOS. Importantly, forced energy restoration with methyl pyruvate reversed the cell death induced by 2DG and metformin, suggesting a critical role of energetic deprivation in the underlying mechanism of cell death. The combination of 2DG and metformin inhibited tumor growth in mouse xenograft models. Deprivation of tumor bioenergetics by dual inhibition of energy pathways might be an effective novel therapeutic approach for a broad spectrum of human tumors. Mol Cancer Ther; 10(12); 2350–62. ©2011 AACR.

Introduction

Proliferation of cancer cells requires oncogenic growth signals as well as sufficient metabolic energy for biogenesis of cellular constituents. The “Warburg effect” (1), a metabolic derangement in cancer cells resulting in increased glucose uptake and glycolysis, provides a selective advantage to rapidly proliferating tumor cells by supplying cellular bioenergetics required to support tumor progression. Clinically, tumors with high glucose uptake detected by the 2[18F]fluoro-2-deoxy-D-glucose–positron emission tomography (FDG–PET) scan show a worsened outcome (2–4), underscoring the therapeutic potential of inhibition of glycolysis.

The mammalian target of rapamycin complex 1 (mTORC1) is a key downstream effector of the phosphoinositide 3-kinase (PI3K)-AKT signaling pathway (5) that regulates and promotes cell proliferation, growth, and survival in many cancer lineages (6–8). The mTORC1 complex integrates oncogenic growth signaling with the glycolytic switch (9). The growth-promoting effects of mTORC1 including protein synthesis and cell-cycle progression are highly energy consuming (10, 11), suggesting that mTORC1 activity would be dependent on cellular energy status. Indeed, mTORC1 is directly inhibited by phosphorylation of raptor as a consequence of activation of the AMP-activated protein kinase (AMPK), a key cellular energy sensor that is activated by bioenergetic stress (12–15).

Remarkably, the energy sensing AMPK pathway seems dominant over the growth-promoting effects of the PI3K-AKT pathway determining cellular functional outcomes.
This ensures that proliferation does not occur in the absence of adequate nutrients, energy and cellular building blocks, providing a novel "bioenergetics checkpoint." Therefore, manipulation of the AMPK signaling pathway, thus mimicking energy stress, could represent a therapeutic target potentially overriding the oncogenic effects of the PI3K-AKT-mTORC1 pathway.

Herein, we describe the functional outcomes of glycolysis inhibition by 2-deoxyglucose (2DG) in combination with AMPK agonists, AICAR and metformin. Unexpectedly, we found that combined use of 2DG and metformin, but not 2DG and AICAR, exhibits significant antitumor effects in vitro and in vivo. Gene expression analysis and subsequent functional assays suggest that AMPK activation protects tumor cells in energetically stressed conditions by augmenting mitochondrial respiration. Metformin, unlike AMP-mimetic AICAR, inhibits mitochondrial energy generation thereby explaining the observed antitumor effect in combination with 2DG. Finally, the in vivo efficacy in mouse xenograft models provides the rationale for the clinical evaluation of this novel strategy for the treatment of cancer patients.

Materials and Methods

Cell culture

Human gastric and esophageal cancer cell lines parental (p)-SK4 and OE33 were kindly provided in June 2006 by Dr. Julie Izzo (The University of Texas MD Anderson Cancer Center, Houston, TX) and cultured in Dulbecco’s Modified Eagle’s Medium (DMEM)/Ham’s F-12 50:50 supplemented with 10% FBS in a humidified incubator containing 5% CO₂ at 37°C. U2OS, MCF-7, MDA-MB-468, MDA-MB-231, and MCF10A were obtained in May 2007 from the American Type Culture Collection (ATCC) and cultured in Dulbecco’s Modified Eagle’s Medium (DMEM)/Ham’s F-12 50:50 supplemented with 10% FBS in a humidified incubator containing 5% CO₂ at 37°C. U2OS, MCF-7, MDA-MB-468, MDA-MB-231, and MCF10A were obtained in May 2007 from the American Type Culture Collection (ATCC) and grown in medium RPMI-1640 with 5% FBS. The identities of all cell lines were validated by short tandem repeat (STR) DNA fingerprinting using the AmpFISTR Identifier Kit according to manufacturer’s instructions (Applied Biosystems; catalogue no: 4322288) at Characterized Cell Line Core Facility (All the cells were last tested in October 2009). The STR profiles were compared with known ATCC fingerprints (http://atcc.org) and to the Cell Line Integrated Molecular Authentication database (CLIMA) version 0.1.200808 (http://bioinformatics.istge.it/clima/; Nucleic Acids Research 37:D925-D932 PMCID: PMC2686526). The STR profiles matched known DNA fingerprints or were unique.

Cell viability assay

Cell viability was determined by trypan blue dye exclusion. For the assay, 0.3 × 10⁶ cells were plated in 6-well plates and treated the next day. Methyl pyruvate (MP, 10 mmol/L) was added 2 hours before treatment, where indicated. Cells were trypsinized, resuspended, and mixed with 1:1 0.4% trypan blue. Percentage cell death = number of stained cells/(number of stained + unstained cells) × 100.

Reverse phase protein array

Reverse phase protein array (RPPA) was processed as previously described (16, 17). Briefly, serially diluted lysates were spotted on FAST slides (Schleicher & Schuell BioSciences) by a robotic GeneTAC arrayer (Genomic Solutions). After printing, slides were blotted sequentially with Re-Blot (Chemicon), 1-Block, and biotin blocking system (Dako), probed with primary antibodies and incubated with biotin-conjugated secondary antibodies. The signals were then amplified by a catalyzed signal amplification kit (DakoCytomation) according to the manufacturer’s instructions. The processed slides were scanned and quantified with MicroVigene software (VigeneTech Inc.).

Measurement of intracellular ATP levels and mitochondrial transmembrane potential (ΔΨm)

Intracellular ATP was measured by a luciferin/luciferase-based assay. Cells were grown under each experimental condition for indicated times, harvested, and counted. Aliquots containing equal number of cells were processed following manufacturer’s guidelines (Roche).

Rhodamine-123, a cationic voltage-sensitive mitochondrial probe, was used to detect changes in mitochondrial transmembrane potential (ΔΨm). Cells were incubated as indicated and labeled with 1 μmol/L rhodamine-123 at 37°C for 30 minutes. After washing, the samples were analyzed by flow cytometry.

Immunoblotting

Cell lysis and immunoblotting were carried out as previously described (18). A total of 50 μg protein was used for the immunoblotting, unless otherwise indicated. β-Actin and glyceraldehyde-3-phosphate dehydrogenase were used as loading controls. Anti-LC3 antibody was a gift from Dr. S. Kondo. All other antibodies were purchased from Cell Signaling.

Transmission electron microscopy

Samples were fixed with a solution containing 3% glutaraldehyde plus 2% paraformaldehyde in 0.1 mol/L cacodylate buffer, pH 7.3, for 1 hour. After fixation, samples were washed and treated with 0.1% Millipore-filtered cacodylate-buffered tannic acid, postfixed with 1% buffered osmium tetroxide for 30 minutes, and stained en bloc with 1% Millipore-filtered uranyl acetate. Samples were dehydrated in increasing concentrations of ethanol, infiltrated, and embedded in LX-112 medium. Samples were polymerized in a 70°C oven for 2 days. Ultrathin sections were cut in a Leica Ultracut microtome (Leica), stained with uranyl acetate and lead citrate in a Leica EM stainer, and examined in a JEM 1010 transmission electron microscope (JEOL USA, Inc.) at an accelerating voltage of 80 kV. Digital images were obtained by the AMT Imaging System (Advanced Microscopy Techniques Corporation).
Animal protocol

Athymic female nude mice (CD-1 nu/nu from the NIH National Cancer Institute repository) were used for in vivo tumor growth studies. All of the in vivo studies were carried out under approved institutional experimental animal care and use protocols. For the breast tumor orthotopic model, MDA-MD-231 cells were resuspended at 6.5 × 10^6 cells per 200 μL in PBS and injected into the seventh mammary gland fat pad of 4-week-old athymic nude mice. When tumors were approximately 50 mm^3 in size (14 days), the animals were randomly allocated into 4 groups. Following randomization, vehicle, 0.2 mL of 2DG (500 mg/kg), metformin (250 mg/kg, equivalent to a human dose of 20 mg/kg by normalization to surface area), or both were delivered via intraperitoneal administration daily for the duration of the experiment. For the esophageal cancer xenograft model, nude mice received subcutaneous implantation with 200 μL of the s-SK4 (selected from p-SK4 cells) at 4 × 10^6 cells per 200 μL in PBS. When tumors were approximately 100 mm^3 in size (one week later), the animals were randomized. A total of 0.2 mL of PBS or 2DG (500 mg/kg) + metformin (250 mg/kg) were delivered via intraperitoneal administration, daily for the duration of the experiment. MRI was conducted, and tumor volumes in vivo were assessed.

Tumor size was measured with digital calipers 2 to 3 times weekly. Tumor measurements were converted to tumor volume using the formula as follows: \( V = \frac{L \times S^2}{2} \) (where \( L \) is the longest diameter; \( S \) is the shortest diameter). Mice were killed when either \( L \) or \( S \) exceeded 15 mm in control group. At sacrifice, tumors were excised and assessed histologically for verification of tumor growth. Statistical significance was determined by the Student t test.

Microarray experiment and data analysis

Total RNA was isolated from cells harvested after each treatment by the mirVana miRNA Isolation Kit (Ambion Inc.) according to manufacturer’s protocol. Biotin-labeled cRNA samples were prepared by using the Illumina TotalPrep RNA Amplification Kit (Ambion Inc.). A total of 500 ng of RNA was used for the synthesis of cDNA and then followed by amplification and biotin labeling as recommended by the manufacturer. A total of 1.5 μg of biotinylated cRNA per sample was hybridized to Illumina Human-6 BeadChip v2 microarray, and signals were detected using GenePix scanner (BeadStation 500GXDW; Illumina Inc.).

Statistical analysis was conducted with the R 2.3.0 and BRB-ArrayTools Version 3.5 (http://linus.nci.nih.gov/BRB-ArrayTools.html). To minimize the effect of variation from nonbiological factors, the values of each sample were normalized by a quantile normalization method. Random variance t test was applied for the calculation of significance of each gene in the comparison of 2 classes, and one-way ANOVA was applied for the evaluation of significance in multigroup comparison. Cluster analysis was conducted with Cluster and Treeview (http://rana.lbl.gov/EisenSoftware.htm). For cluster analysis, log-transformed data were centered to mean values of each gene expression. Gene set enrichment analysis was conducted against Gene Ontology (GO) of biological process, and Kolmogorov–Smirnov statistic was applied for the evaluation of statistical significance of each GO category.

Results

Inhibition of glycolysis by 2DG is not sufficient to activate AMPK or promote cell death

To investigate the consequences of metabolic stress on key signaling pathways, a panel of cancer cell lines of multiple lineages was subjected to glucose deprivation and combinations of 2DG and known AMPK activators (AICAR and metformin). The cell lysates were subject to multiplexed RPPA (refs. 16, 17) to evaluate multiple protein expression levels simultaneously (Fig. 1A and B, Supplementary Fig. S1).

Incubation in glucose-free medium consistently inactivated PI3K-AKT-mTORC1 downstream signals (p-p70S6K, p-S6, and cyclin D1) and reduced expression of cell-cycle regulators (cyclin D1, cyclin B1, and p-Rb; \( P < 0.01 \), Supplementary Fig. S1). p-S6 levels were markedly reduced by glucose deprivation representing the most sensitive biomarker (85% decrease, Fig. 1A). Interestingly, p-AMPK and p-ACC levels, markers of cellular bioenergetic stress, were essentially unchanged at 24 hours despite glucose deprivation or the addition of 2DG, suggesting that the cells were able to compensate for the loss of glucose-derived ATP production (Fig. 1B and C).

Of note, metformin, which is known to activate AMPK, did not induce AMPK activation or subsequent phosphorylation of acetyl-CoA carboxylase (ACC) and raptor when high concentrations of glucose (25 mmol/L) were present (Fig. 1C). In contrast, the combination of 2DG and metformin markedly activated AMPK and subsequently deactivated mTORC1 downstream signaling. These coordinated alterations were compatible with activation of the tuberous sclerosis protein 2 (TSC2) by AMPK resulting in decreased signaling through mTORC1 and downstream targets (19–22). The AMP-mimetic AICAR, however, markedly activated AMPK and inhibited mTORC1 downstream effectors even in the presence of 25 mmol/L glucose, and these effects were not augmented when combined with 2DG. These results suggest that the pharmacobiological outcomes between metformin and AICAR would be different when high level of glucose is present or glucose is deprived.

On the basis of these results, we predicted that inhibition of glycolysis alone would not be sufficient to promote bioenergetic stress. Indeed, clinically achievable concentrations of 3 to 4 mmol/L 2DG (23) did not substantially activate AMPK and affect cell signaling in the majority of the cell lines assessed (Fig. 1B). Furthermore, inhibition of...
glycolysis with 2DG at clinically achievable doses was not sufficient to induce a significant degree of cell death, even with prolonged incubation (Fig. 2A and B).

**Metformin but not AICAR induces cell death in combination with 2DG**

On the basis of the alterations in signaling pathways (Fig. 1C), we next compared the effects of each agent on tumor cell viability. We hypothesized that the AMP-mimetic AICAR, 2DG + metformin, and 2DG + AICAR should reduce cell viability based on the substantial changes in AMPK and mTORC1 downstream signals (Fig. 1C).

Surprisingly, neither AICAR nor 2DG + AICAR induced cell death, whereas the 2DG + Met significantly reduced cell viability in a time-dependent manner (Fig. 2A and C). Furthermore, the combination effect of 2DG and metformin resulted in a marked increase in cell death compared with either agent alone (Fig. 2A, B, and D).

Unexpectedly, AICAR as a single agent or in combination with 2DG failed to mimic the effects of metformin in combination with 2DG on cell death (Fig. 2A–D). This suggests that activation of AMPK was not sufficient to induce cancer cell death irrespective of the presence of 2DG. Instead, it suggests that enhanced cell death observed with metformin in combination with 2DG might be caused by AMPK-independent mechanisms or through mechanisms in addition to AMPK activation.

**Metformin and AICAR induce markedly different gene sets controlling mitochondrial energy transduction**

Although metformin and AICAR have been reported to activate AMPK (24–29), our data indicated that the administration of each alone or in combination with 2DG leads to markedly different biological outcomes. Furthermore, when glucose was sufficient, metformin did not substantially activate AMPK at least in the cell lines tested. To explore potential mechanisms underlying the ability of metformin, but not AICAR, to induce cell death when combined with 2DG in cancer cells, we conducted genome-wide transcriptional profile analysis. Gene expression patterns of each treatment group were dramatically different (Fig. 3A; 2,527 significant genes; $P < 10^{-6}$ one-way ANOVA). Furthermore, whereas 2DG and control groups clustered closely together, metformin and AICAR showed distinct and distant clusters independent of the presence of 2DG, indicative of markedly different mechanisms of action (Fig. 3A).

To estimate the functional and biological consequences of differences in gene expression between the treatments, we conducted gene set enrichment analysis (Kolmogorov–Smirnov permutation test, $P < 0.001$) against the biological process category of GO. Of note, metformin in the presence or absence of 2DG significantly downregulated expression of 12 components of the electron transport chain (ETC) complex 1 in the mitochondria without significantly altering expression of other components of the ETC complexes (Supplementary Table S1 and Fig. 3B). Other components of the ETC or tricarboxylic acid (TCA) cycle were unaffected (Supplementary Tables S1, S2 and Fig. 3C). This selective effect of metformin on ETC1 could limit the extraction of electrons from NADH thereby suppressing the mitochondrial ATP-generating capacity.

In contrast, AICAR alone or in combination with 2DG significantly induced expression of multiple genes in the TCA cycle and the ETC complex I, II, III, and IV and F$_0$F$_1$-ATPases that could coordinately increase the efficiency of OXPHOS (Supplementary Table S1 and Fig. 3D and E).
Furthermore, expression of a set of genes involved in gluconeogenesis and vacuolar type ATPases that consume ATP was concomitantly decreased (Fig. 3D and E, Supplementary Table S2). This suggests that activation of AMPK by an AMP-mimetic drug may increase mitochondrial ATP production by augmenting the efficiency of the TCA cycle, ETC, and OXPHOS, enabling cells to maintain bioenergetic homeostasis.
Metformin and AICAR exert opposite effects on mitochondrial energy generation

The disparate effects of AICAR and metformin on expression of components of the mitochondrial respiratory chain suggested that they would have differential effects on mitochondrial energy generation capability such as transmembrane potential (ΔΨm), the proton motive force that drives mitochondrial ATP production (30, 31). Consistent with the mRNA microarray data (Fig. 3D and E), AICAR alone or combined with 2DG increased mitochondrial function as shown by a significant increase in ΔΨm (Fig. 3G). In contrast, metformin, in the presence or absence of 2DG induced a time-dependent decrease in ΔΨm (Fig. 3G) and mitochondrial shrinking with acquisition of an electron dense matrix (Fig. 3F). Similar to the effects of metformin, accumulation of mitochondrial electron dense deposits has been previously reported after a decrease in ETC complex I and III functions (32).

Consistent with the alterations in mitochondrial ΔΨm, we found that 2DG induced a modest time-dependent decrease in cellular ATP levels (Fig. 3H). As predicted, metformin induced a decrease in cellular ATP levels whereas AICAR did not significantly alter cellular ATP levels (Fig. 3H). The combination of 2DG and metformin reduced cellular ATP by 70%, suggesting that decreasing ATP levels below a critical threshold could result in the cell death associated with 2DG and metformin (Fig. 2A and B). Strikingly, AICAR significantly reversed the effects of 2DG on ATP levels at delayed time points (48 hours), compatible with the changes in gene expression and augmented mitochondrial function described earlier.

MP rescues energetic stress-induced cell death by metformin plus 2DG

Because energetic stress activates AMPK and suppresses mTORC1 thereby inducing autophagy (19), we examined cellular ATP levels, AMPK and mTORC1 signals, and autophagy over time of treatment with metformin and 2DG. The combination of metformin and 2DG induced progressive depletion of ATP (Fig. 4A) and increased LC3-II levels indicative of sustained autophagy (Fig. 4B) compatible with the prolonged activation of AMPK and suppression of mTORC1 signals (Fig. 4C). These changes were reflected by a time-dependent decrease in cell size, an accumulation of autophagosomal vesicles and electron dense bodies, and a lack of characteristics typical of apoptosis or necrosis (Fig. 4D and E).

Metformin compromises but does not completely block mitochondrial ATP production, because it selectively inhibits ETC1 while leaving ETC complex II intact (33, 34). Thus, administering high levels of a substrate that can provide electrons to the ETC complex II should reverse, at least in part, the effects of metformin. We exploited MP, a growth factor independent exogenous cell permeable energy substrate that bypasses glycolysis and directly enters the mitochondrial TCA cycle (35–37). In theory, MP should lead to the production of FADH₂ in the TCA cycle with an electron being extracted from complex II and transferred in the ETC, thus producing ATP even when ETC1 is compromised by metformin. Indeed, MP increased intracellular ATP levels in cells treated with 2DG and metformin (Fig. 5A), indicating a partial bypass of the effects of metformin. Compatible with the increase in ATP levels, MP markedly suppressed the increase in phosphorylation of AMPK and ACC induced by 2DG and metformin (Fig. 5B), indicating that AMPK activation by 2DG and metformin is likely a consequence of a compromise in energy production rather than due to a direct effect of either compound on AMPK activity. In agreement with the ability of MP to restore cellular ATP levels and attenuate AMPK activity, MP reduced the formation of GFP-LC3 punctate vesicles as well as the number of autophagosomes (Fig. 5C and D). Consistent with the effects on GFP-LC3, there was a reduced accumulation of LC3-II on Western blotting in cells treated with MP (Fig. 5E). Importantly, MP decreased cell death induced by 2DG plus metformin (Fig. 5F). The ability of MP to attenuate the effects of 2DG plus metformin were confirmed in U2OS cells in terms of autophagy, cellular morphology, cell death, and ATP levels (Supplementary Fig. S2). Thus, the ability of the combination of 2DG and metformin to induce autophagy, AMPK activation, and cell death can be attributed to decreased energy production due to a partial but coordinated compromise of glycolysis and mitochondrial function.

Metformin plus 2DG treatment inhibits tumor growth and metastasis in xenograft tumor models

We next examined the *in vivo* efficacy of 2DG and metformin by using 2 different xenograft mouse models. First, MDA-MB-231 cells were injected into the mammary fat pad of nude mice and allowed to establish measurable tumors for 14 days. Mice were randomized to different treatment groups and treated intraperitoneally daily for 36 days at which time the experiment was terminated because of excessive tumor volume in control mice. As indicated in Fig. 6A, 2DG or metformin, at doses delivered, did not alter tumor growth. In contrast, the 2DG and metformin combination significantly decreased tumor growth from day 21 to the termination of the study at day 36 (Fig. 6B). Second, using a tumorigenic line selected from p-SK4 cells (s-SK4), we determined whether the effect of 2DG and metformin on *in vivo* tumor growth of MDA-MB-231 cells could be generalized to other lineages. Two weeks of treatment with metformin and 2DG decreased subcutaneous tumor growth of s-SK4 (Fig. 6C). The decrease in tumor volume was confirmed by MRI during the course of treatment (see Fig. 6D, for a representative pair). As with the MDA-MB-231 model, the decrease in tumor growth induced by 2DG and metformin was confirmed by weighing the tumor at the termination of the study (Fig. 6E). Notably, immunohistochemical analysis of the tumor sections revealed substantial decreases in p-S6 and cyclin D1 expression levels.
recapitulating the effects of metformin and 2DG observed in vitro (Fig. 6F).

Discussion

The Warburg effect is an attractive therapeutic target because many cancers are highly glycolytic and is associated with tumor aggressiveness and clinical outcomes. Despite exciting preclinical studies, however, 2DG failed to fulfill its expectations in clinical trials.

Likewise in our studies, 2DG at clinically achievable doses did not substantially activate AMPK, reflecting insufficient suppression of tumor bioenergetics. These effects were inadequate to induce death of most cancer
cell lines assessed or to inhibit tumor growth in vivo. This prompted us to explore whether the activation of AMPK, thus mimicking energy stress, would lead to increases in cell death. We, therefore, used metformin and AICAR, pharmacologic mediators that have been reported to activate AMPK (24–29).

Activation of AMPK has been reported to lead to suppression of mTORC1 signaling (10, 11), cell cycle exit due to stabilization of p27KIP1 (18), and a decrease in energy consumption through diminishing protein and lipid biosynthesis (13–15, 38). Our study suggests an additional role for AMPK activation in tumor bioenergetics. Direct activation of AMPK by the AMP-mimetic AICAR decreased expression of mRNA for enzymes involved in gluconeogenesis, induced expression of mRNA for multiple proteins involved in the ETC and OXPHOS, and increased mitochondrial respiration. This suggests that AMPK induces a switch from dependence on glycolysis for ATP production to energy transduction through mitochondrial respiration. In the current study, we directly showed a role for AMPK in the ability to bypass effects of inhibition of glycolysis; MEFs lacking AMPK were exquisitely sensitive to the effects of glucose deprivation as well as to 2DG (Supplementary Fig. S3A and S3B). Thus, in the context of energy stress, activation of AMPK could limit the effects of glycolysis inhibitors, suggesting a novel therapeutic combination would be required to override the energetic protective effect of AMPK.

In the absence of glycolysis, mitochondrial energy production is the only potential cellular source of ATP. As described earlier, inhibition of glycolysis and subsequent activation of AMPK results in a coordinated increase in mRNA levels of genes involved in mitochondrial energy production, potentially triggering a switch to dependence on OXPHOS. Metformin, an orally available antidiabetic drug, has been proposed to inhibit complex 1 of the
Figure 4. Prolonged incubation with 2DG and metformin depletes cellular ATP, activates AMPK, suppresses mTORC1 downstream signaling, and sustains autophagy. p-SK4 cells were incubated with 2DG and metformin for the indicated times. A, cellular ATP levels were determined. Data are the mean ± SD (n = 3). B, Western blotting for LC3 expression. β-Actin was used as loading control. C, kinetics of signaling molecules in AMPK and mTORC1 downstream. Western blotting of samples from (4B). D, TEM analysis of morphologic alteration of cells treated with 2DG in combination with metformin. Severe cytoplasmic contraction and resultant membrane blebbing is evident (48 and 60 hours). Note most of the cytoplasm is replaced with autophagosomes (60 hours). Neither typical apoptotic nor necrotic cells are evident (N, nucleus; arrowheads, autophagosome; scale bar, 10 μm for top; 500 nm for bottom). E, autophagic vesicles were counted for 3 randomly selected cells from each electron microscopy section. Data presented mean ± SD.
Figure 5. Exogenous energy substrate MP increases cellular ATP, decreases AMPK activation, and reduces autophagy and cell death. p-SK4 cells were treated with 2DG (4 mmol/L) and metformin (5 mmol/L) with and without MP (10 mmol/L). A, intracellular ATP levels were measured at 72 hours. *, P < 0.01. B, Western blotting (lysates from A) to assess AMPK activation. β-Actin was used as loading control. C, autophagic analysis by fluorescence microscopy of GFP-LC3 punctate patterns (top) and TEM (bottom; N, nucleus; arrowheads, autophagosome; scale bar, 10 μm for top; 500 nm for bottom). D, quantitation of autophagosomes on TEM. Autophagic vesicles were counted for 3 randomly selected cells from each electron microscopy section. Data presented are mean ± SD. *, P < 0.01. E, cell lysates (samples from SA) were subject to Western blotting to assess LC3 expression and lipidation (LC3-II), an autophagy marker. F, cell death assay by trypan blue exclusion. *, P < 0.05. All assays were conducted at 72 hours except C, which was 60 hours. Data are mean ± SD (n = 3).
respiratory chain in the mitochondria (33, 34). Indeed, our
data show that metformin coordinately decreases mRNA
levels for ETC1 components and mitochondrial mem-
brane potential compatible with compromising mitochon-
drial energy transduction. While metformin has been
known to activate AMPK in intact cells (26, 39), this could
be a consequence of its inhibitory effects on mitochondrial
ATP production as shown here. Furthermore, the ability
of metformin and other biguanide analogues such as
phenformin to induce lactic acidosis is compatible with
inhibition of mitochondrial function and consequent pro-
duction of lactate due to elevated levels of pyruvate.

In light of this, we assessed whether inhibition of
glycolysis with 2DG combined with a compromise of
mitochondrial function with the clinically applicable met-
formin would result in cell death. Strikingly, in 13 of 15
cancer cell lines assessed regardless of mutational status,
the combination of 2DG and metformin was more effica-
cious at inhibiting cell growth and inducing cell death
than either drug alone (data not shown).

Recently, Ben Sahra and colleagues reported that the
combination of metformin and 2DG induced p53-dependent
apoptosis in prostate cancer cells (40). In their study,
apoptosis was AMPK-mediated and p53 was required. In
contrast, our results show that AMPK itself prevents cell
death during glucose deprivation or 2DG treatment (See
Supplementary Fig. S3A and S3B). Furthermore, AICAR
combined with 2DG failed to induce cell death in a
number of cancer cell lines, suggesting a critical role of
AMPK as a guardian of cellular bioenergetics. Although,
the distinct results between the 2 studies may be due to the
different tissue of origin or characteristics of cell lines
used, our data suggest that prolonged activation of AMPK
by 2DG and metformin might reflect sustained bioener-
getics stress due to failure of mitochondrial compensation
rather than direct activation of AMPK.
Consistent with the ability of the combination of 2DG and metformin to induce cell death in vitro, the combination significantly suppressed tumor growth in 2 xenograft models in vivo. These results suggest that the tumor cell bioenergetics can be targeted, and the combination of 2DG and metformin warrants further clinical evaluation. Recently, metformin has gained much attention due to its antitumor activity in some cancer types in preclinical assays (40, 41), with AMPK activation being suggested as a major mechanism of action. As our data indicate, however, AMPK activation can promote cancer cell survival in energetically stressed conditions. A recent study showing that metformin can exert its inhibitory action directly on mTORC1 (42), further reinforces the notion that the effects of metformin can be mediated independently of AMPK.

In summary, realizing the clinical benefit of blockade of the Warburg effect may require concomitant inhibition of multiple components of cellular energy pathways. A preemptive blockade of the Warburg effect and compensatory mechanisms may prove to be dominant over the survival and growth-promoting effects of growth factors or activated oncogenes. Because 2DG and metformin are already widely used in PET scan or seizure disorders and in type II diabetes, respectively, the expedited assessment of clinical effect of deprivation of cancer bioenergetics could be immediately tested in cancer patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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