Metformin Amplifies Chemotherapy-Induced AMPK Activation and Antitumoral Growth

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Abstract

Purpose: Metformin is a widely used antidiabetic drug whose anticancer effects, mediated by the activation of AMP-activated protein kinase (AMPK) and reduction of mTOR signaling, have become noteworthy. Chemotherapy produces genotoxic stress and induces p53 activity, which can cross-talk with AMPK/mTOR pathway. Herein, we investigate whether the combination of metformin and paclitaxel has an effect in cancer cell lines.

Experimental Design: Human tumors were xenografted into severe combined immunodeficient (SCID) mice and the cancer cell lines were treated with only paclitaxel or only metformin, or a combination of both drugs. Western blotting, flow cytometry, and immunohistochemistry were then used to characterize the effects of the different treatments.

Results: The results presented herein show that the addition of metformin to paclitaxel leads to quantitative potentialization of molecular signaling through AMPK and a subsequent potent inhibition of the mTOR signaling pathway. Treatment with metformin and paclitaxel resulted in an increase in the number of cells arrested in the G2–M phase of the cell cycle, and decreased the tumor growth and increased apoptosis in tumor-bearing mice, when compared with individual drug treatments.

Conclusion: We have provided evidence for a convergence of metformin and paclitaxel induced signaling at the level of AMPK. This mechanism shows how different drugs may cooperate to augment antigrowth signals, and suggests that target activation of AMPK by metformin may be a compelling ally in cancer treatment. Clin Cancer Res; 17(12); 3993–4005. ©2011 AACR.

Metformin is an oral hypoglycemic agent used as first-line therapy for type 2 diabetes, which is now prescribed to almost 120 million people in the world. There are a large number of epidemiologic studies indicating that diabetics have an increased risk of cancer and cancer mortality (1, 2). Increasing evidence also supports a decreased risk of cancer mortality associated with metformin use in patients with type 2 diabetes (3–6). Furthermore, metformin has been shown to inhibit the growth of cancer cells in vitro and in vivo (7–12) and, while there are still no randomized control trials of metformin as a therapy for cancer, there is intriguing evidence that metformin may enhance chemotherapy for established tumors (13, 14).

Metformin has been found to activate AMP-activated protein kinase (AMPK) signaling (15), and this has become an important focus of interest in carcinogenesis, because AMPK has been implicated in the regulation of mTOR activity, which is frequently activated in cancer (16–20). AMPK is the downstream component of the tumor suppressor, LKB1, which acts as a sensor of cellular energy charge, being activated by increasing AMP, coupled with falling ATP (21). The AMP/LKB1-dependent activation of AMPK results from pathologic stresses such as heat shock, hypoxia, glucose deprivation, and metformin administration (15, 21). AMPK is also activated through Ca2+/calmodulin (CaM)-dependent protein kinase kinase (CaMKK), which in contrast to that mediated by AMP/LKB1, is mediated by calcium increases and functions independently of AMP (22, 23). Once activated, AMPK phosphorylates acetyl-CoA carboxylase (ACC) and switches on energy-producing pathways at the expense of energy-depleting processes (24).

Another direct consequence of AMPK activation is the inhibition of the mTOR kinase signaling pathway. mTOR catalytic activity is halted by AMPK activation of the TSC1–TSC2 complex, which inactivates the Rheb GTPase (25, 26). In addition, mTOR activity is positively regulated by growth factors and nutrients (amino acids). PI3K/Akt signaling regulates mTOR through phosphorylation/inactivation of mTOR’s negative regulator, TSC2 (17, 27). mTOR activation results in the phosphorylation of the...
serine/threonine kinase p70S6K and the translational repressor eukaryotic initiation factor (eIF) 4E binding protein (4E-BP1), which have an essential role in regulating cell growth and proliferation by controlling mRNA translation and ribosome biogenesis (28).

To achieve normal cell growth and proliferation, it is critical for cells to have robust antigrowth signaling systems. AMPK has a major role as an antigrowth signal, because it is activated by p53, a sensor of DNA damage stress (29). Recently, the genotoxic stress effect was further evaluated and it has been suggested that the inhibition of mTOR activity occurs through the p53-dependent upregulation of sestrins (SESN1 and SESN2) and consequent mTOR activity occurs through the p53-dependent upregulation of sestrins (SESN1 and SESN2) and consequent activation of AMPK (30). These observations indicate that metformin acts synergistically with chemotherapeutic drugs that increase genotoxic stress through a convergent signaling of metformin-mediated LKB-1/AMPK activation and chemotherapeutic drug activation of SESNs, culminating in an increased AMPK activation and mTOR inhibition. Thus, this study was designed to investigate whether metformin potentiates paclitaxel antitumor effects, a well-known chemotherapeutic drug frequently used in breast and lung cancer patients (31, 32), and to observe whether these drugs share common intracellular signal transduction pathways and to determine whether these signaling systems modulate each other’s actions in different cancer cell lineages and in xenografted tumor cells in mice.

Materials and Methods

Antibodies, chemicals, and buffers

All the reagents were from Sigma-Aldrich unless otherwise specified. Paclitaxel was obtained from Laboratório Químico Farmacêutico Bergamo Ltda. Anti–phospho-mTOR, anti–mTOR, anti–phospho-p70S6K, anti–p70S6K, anti–phospho-4E-BP1, anti–4E-BP1, anti–phospho-AMPKa, anti–AMPKa, anti–β-actin, anti–acetyl-lys379-p53, anti–phospho-p53, anti–phospho-ACC, anti– caspase 3, anti–cleaved caspase 3, anti–p27, and anti–phospho-Rb antibodies for immunoblotting were from Cell Signaling Technology; anti–p53 and anti–SESN2 antibodies for immunoblotting were from Santa Cruz Biotechnology; and anti–SESN1 and anti–SESN3 antibodies for immunoblotting were from Abcam.

Cell culture

The human breast cancer cell line MCF-7 (LKB1-positive) and human lung cancer cell line A549 (LKB1-negative) were obtained from ATCC. MCF-7 and A549 cells were cultured in Dulbecco’s Modified Eagle’s Medium containing 10% FBS with the addition of antibiotics and fungicides. Both cell lines were maintained at 37°C in a humid atmosphere and 5% CO2.

Transfection

A total of 3 × 10⁵ cells were seeded in a tissue culture plate in complete growth medium and incubated overnight. On the day of transfection, 200 pmol of siRNA was diluted into OPTI-MEM (Life Technologies) and mixed with 10 μL of Lipofectamine 2000 (Life Technologies) according to supplier’s protocol. The transfection medium was then replaced by complete medium and after 24 hours cells were treated with metformin (10 mM/L) and paclitaxel (1 μmol/L) and incubated for an additional 6 hours. siRNA for AMPK was 5′-AAUUAUCUUCGUGCCACGCAUAGCCG-3′ forward and 5′-CCGCUAUUCCUGGACCCAGAAUAAUU-3′ reverse; for SESN1, 5′-GAACCCUUCIGUCA-GAGUGCUUGAACUUG-3′ forward and 5′-CAGUUCACGACUUCUGAGAAGGGUUC-3′ reverse; and for SESN2, 5′-GGGAACGAGCAUGACUGGCUUCC-3′ forward and 5′-GGAGACCAGGGCGUACUAAUCC-3′ reverse.

Cell viability assay

Cells were seeded at a density of 2 × 10⁴ cells/well in 24-well plates containing 1 mL of complete medium in triplicate. Cells were allowed to attach overnight before treating with the indicated dose of metformin and paclitaxel for 24 hours. Subsequently, viable cells were counted by using trypan blue staining or they were treated with 0.3 mg/mL of MTT for 4 hours and MTT-formazan conversion was analyzed by spectrophotometry at 570 nm after culture medium was removed and ethanol was added.

Cell-cycle analysis

Cells were trypsinized, washed in PBS, centrifuged, and pellets were fixed in 200 μL of 70% ethanol and stored at −20°C until use. Cells were centrifuged and pellets resuspended in 200 μL of PBS, and 10 μg/mL of RNase A was incubated for 1 hour at 37°C. Subsequently, cells were resuspended in propidium iodide solution (0.1% sodium citrate, 0.1% Triton X-100, and 50 μg/mL propidium iodide). Cell-cycle analysis was carried out by flow cytometry (FACS-calibur). Data were analyzed by ModFit LT software.
Complex I oxygen consumption

Measurement of oxygen consumption by MCF-7 and A549 after treatment for 24 hours with metformin (10 mmol/L), paclitaxel (1 μg/mL), or the combination of both drugs was carried out by an Oxygraph equipped with a Clark-type electrode (Hansatech Instruments Limited) in a closed chamber equipped with magnetic stirrer and temperature control at 37°C. Approximately 2.5 × 10^6 of viable MCF-7 cells/mL, and 4 × 10^6 of viable A549 cells/mL, permeable with 10 μmol/L of digitonin, were added in 2 mL of reaction medium containing 125 mmol/L sucrose, 65 mmol/L KCl, 10 mmol/L HEPES, 2.0 mmol/L K2HPO4, 1.0 mmol/L MgCl2 (pH 7.2), 50 μmol/L EDTA, 100 mmol/L EGTA, and complex I substrates (2.0 mmol/L malate, 1.0 mmol/L α-ketoglutarate, 1.0 mmol/L pyruvate, and 1.0 mmol/L glutamate). Analyses of oxidative phosphorylation and respiratory activity of mitochondria were made by sequential additions of 100 μmol/L ADP, 2 μg/mL chloramphenicol acetyltransferase, 100 mmol/L carbonylcyanide p-trifluoromethoxyphenylhydrazone, 5 mmol/L succinate, nicotin acetyltransferase, 100 nmol/L carbonyl cyanide m-chlorophenylhydrazone, 30 μmol/L antimycin, and 200 μmol/L N,N,N′,N′′-tetramethyl-p-phenylenediamine/ascorbate. The data were reproduced and calculated by the device’s specific software.

Human tumor xenograft models

Four-week-old male severe combined immunodeficient (SCID) mice were provided by the State University of Campinas—Central Breeding Center. Animals were inoculated in the dorsal region, subcutaneously, with 1 × 10^6 A549 cells. The mice had ad libitum access to food and water. Once tumors became palpable, tumor volume (V) was calculated daily by measuring the length (L) and width (W) of the tumor with calipers by using the formula: V = [W × L × (W + L)/2] × 0.52. Each group contained 15 animals.

Treatments began when tumors reached 50 to 100 mm^3. Metformin was given daily by gavage at 500 mg/kg body weight. Paclitaxel was given once a week by intraperitoneal (i.p.) injection of 10 mg/kg body weight. All experiments were approved by the Ethics Committee of the State University of Campinas.

Tissue extracts

Mice were anesthetized with sodium amobarbital (15 mg/kg body weight, i.p.). Tumors were removed, minced coarsely and homogenized in extraction buffer [1% Triton-X 100, 100 mmol/L Tris (pH 7.4), containing 100 mmol/L sodium pyrophosphate, 100 mmol/L sodium fluoride, 10 mmol/L EDTA, 10 mmol/L sodium vanadate, 2 mmol/L PMSF and 0.1 mg of aprotinin/mL]. The extracts were centrifuged at 11,000 rpm and 4°C and the supernatants of these tissues were used.

Protein analysis by immunoblotting

Whole tissue extracts and cell pellets were homogenized in extraction buffer, treated with Laemmli sample buffer containing 100 mmol/L DTT and heated in a boiling water bath. For total extracts, similar-sized aliquots (50 μg protein) were subjected to SDS-PAGE. Proteins were resolved on 8% to 15% SDS gels and blotted onto nitrocellulose membranes (Bio-Rad). Band intensities were quantified by optical densitometry of developed autoradiographs by using Scion Image software (Scion Corporation).

Immunohistochemistry

To detect Ki-67 and cleaved caspase 3, microwave post-fixation was carried out by a domestic oven which was delivered to slides immersed in 0.01 mol/L citrate buffer, pH 6.0, in two 7-minute doses separated by a 2-minute break. Sections were then incubated at 4°C overnight with primary monoclonal mouse anti-human Ki-67 clone MB-1 from Dako (diluted 1:100) and anti-cleaved caspase 3 from Cell Signaling Technology. The slides were then incubated with avidin-biotin complex LSAB+ Kit from Dako Cytoplasmation for 30 minutes followed by the addition of diamobenzidine tetrahydrochloride as a substrate-chromogen solution. After hematoxylin counterstaining and dehydration, the slides were mounted in Entellan from Merck.

Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay

Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining was done by a commercial apoptosis detection kit (Roche), according to the recommendations of the manufacturer. Analysis and documentation of results were carried out by a Leica FW 4500 B microscope.

Statistical analysis

Data are presented as mean ± SEM of at least 3 independent experiments. All groups were studied in parallel and differences between groups were analyzed by ANOVA, as appropriate, and Bonferroni post hoc tests for multiple unpairwise comparisons of means. The level of significance adopted was P < 0.05.

Results

Metformin activates AMPK and inhibits mTOR in MCF-7 breast cancer cells and A549 lung cancer cells

To examine the effect of metformin on cancer cell growth, MCF-7 breast cancer and A549 lung cancer cell lines were treated with various concentrations of metformin (1–50 mmol/L) for different periods of time (0–8 hours). Metformin treatment resulted in the activation of AMPK, with increased phosphorylation of AMPKα1 at Thr-172 in a time- and dose-dependent manner. Activation of AMPK is associated with decreased activation of mTOR and p70S6K, a critical translational pathway for protein synthesis (10). Metformin treatment resulted in attenuated activation of mTOR, as shown by the decreased phosphorylation of mTOR, p70S6K, and 4E-BP1, in a time- and dose-dependent manner in treated cancer cells, compared with untreated cells (Fig. 1A–D).

We also observed the effect of 2-deoxy-D-glucose (2-DG), another AMPK activator, in both cell lines at various concentrations, and for different periods of time. As observed
Figure 1. Metformin and paclitaxel activate AMPK and inhibit mTOR in MCF-7 and A549 cells. MCF-7 cells were treated (A) with 10 mmol/L metformin for the indicated time and (B) for 6 hours with the indicated doses. The lysates were immunoblotted (IB) with the indicated antibodies. A549 cells were treated (C) with 10 mmol/L metformin for the indicated time and (D) for 6 hours with the indicated doses. The lysates were immunoblotted with the indicated antibodies. MCF-7 cells were treated (E) with 1 μmol/L paclitaxel for the indicated time and (F) for 6 hours with the indicated doses. The lysates were immunoblotted with the indicated antibodies. A549 cells were treated (G) with 1 μmol/L paclitaxel for the indicated time and (H) for 6 hours with the indicated doses. The lysates were immunoblotted with the indicated antibodies. Data are representative of at least 3 experiments.
for the metformin treatment, 2-DG led to activation of AMPK and inactivation of the mTOR signaling pathway (Supplementary Fig. S1A–D).

A549 lung cancer cell line is negative for LKB1 and recent reports have shown that LKB1 deficiency in hepatocytes impairs metformin action (33). However, in this study we show that AMPK activation in A549 cells after metformin treatment is independent of LKB1 and is dose-responsive, starting from 1 to 100 μmol/L with ACC phosphorylation and mTOR and p70S6K inactivation (Supplementary Fig. S2). The same pattern of AMPK and ACC activation and mTOR and p70S6K inactivation is seen on MCF-7 cell line, which is LKB1 normal (Supplementary Fig. S2).

**Paclitaxel activates AMPK and inhibits mTOR in MCF-7 breast cancer cells and A549 lung cancer cells**

To investigate the mechanisms underlying the antiproliferative effects of paclitaxel, we characterized the effects of paclitaxel on AMPK and the mTOR pathway. As recently reported, genotoxic stress increases the amount of SESNs, and this effect leads to AMPK activation (30). Our results show that paclitaxel treatment increased the acetylation of p53 at Lys-379, the phosphorylation of p53 at Ser 15, which are known markers of genotoxic stress (34, 35), and the amount of SESN1, SESN2, and SESN3 in a time- and dose-dependent manner in both cell lines. Paclitaxel treatment also resulted in increased phosphorylation of AMPKα at Thr-172, in a time- and dose-dependent manner. The increased activation of AMPK led to inactivation of mTOR as evidenced by diminished phosphorylation of mTOR, p70S6K, and 4E-BP1 also in a time- and dose-dependent manner (Fig. 1E–H).

**Effect of combined treatment of AMPK activators and paclitaxel on cancer cell lines**

We next sought to determine the effects of the combined treatment of AMPK activators with paclitaxel. In MCF-7 cells, as shown in Figure 2A, paclitaxel treatment led to a higher increase in acetyl-Lys 379 p53 than metformin or 2-DG treatment, as well as an increase in the amount of SESN2 (Fig. 2B). This increase in SESN2 in paclitaxel-treated cells was followed by an increase in the phosphorylation of Thr-172 of AMPK (Fig. 2C) and inhibition of mTOR (Fig. 2D), p70S6K (Fig. 2E), and 4E-BP1 (Fig. 2F), when compared with metformin or 2-DG treatments alone. Even though metformin or 2-DG treatment do not increase SESN2 (Fig. 2B), we observe an increase in AMPK phosphorylation (Fig. 2C) and inhibition of mTOR (Fig. 2D), p70S6K (Fig. 2E), and 4E-BP1 (Fig. 2F), when compared with vehicle-treated cells. In A549 cells, we also observed an increase in acetyl-Lys379 p-53 and in the amount of SESN2 only in the paclitaxel-treated cells (Fig. 2G and H), when compared with control and metformin and 2-DG alone, and this was correlated with an increase in the Thr-172 phosphorylation of AMPK and decrease in the activation of mTOR, p70S6K, and 4E-BP1 when compared with control. Once again, metformin and 2-DG treatments did not increase SESN2 (Fig. 2H) but they were able to increase AMPK phosphorylation (Fig. 2I) and inhibit mTOR (Fig. 2J), p70S6K (Fig. 2K), and 4E-BP1 (Fig. 2L), when compared with vehicle-treated cells.

**Metformin and paclitaxel inhibit cell viability**

To examine the effects of metformin and paclitaxel on cancer cell growth, we treated MCF-7 and A549 cell lines with metformin or paclitaxel alone or in combination and cell viability was determined. As shown in Figure 3A and B, both metformin and paclitaxel inhibited cell viability, as related to vehicle-treated cells. The metformin treatment was statistically significant at 10 mmol/L for the 48- and 72-hour treatment (Fig. 3A) in MCF-7 cells and that a 1 mmol/L dose of metformin was capable of reducing A549 cells proliferation by approximately 20% to 30% for both the 48- and 72-hour treatments. The paclitaxel treatment was effective only at the dose of 10 mmol/L for both 48- and 72-hour treatments (Fig. 3B). Figure 3C and D shows that in the combined treatment metformin potentiates paclitaxel action on MCF-7 (Fig. 3C) and A549 (Fig. 3D) cells, as a dose of 1 nmol/L of paclitaxel is statistically different from the metformin and vehicle-treated cells for both the 48- and 72-hour treatments. At 10 mmol/L of paclitaxel, metformin does not further potentiate paclitaxel treatment on MCF-7 cells. However, in A549 cells, we observed that at the 48-hour treatment metformin (10 mmol/L) and 10 mmol/L paclitaxel is more effective on reducing cell growth than paclitaxel-alone treatment (Fig. 3D). We then analyzed cell viability by trypan blue staining of both cell lines (Fig. 3E), which showed that both metformin and paclitaxel inhibited cell viability, as related to vehicle-treated cells, and the combined treatment was more effective than either treatment alone.

**Effect of metformin, 2-DG, and paclitaxel on cell cycle**

To evaluate the mechanism of growth inhibition by metformin, 2-DG, and paclitaxel, the cell-cycle profile was analyzed by flow cytometry after treatment with metformin, 2-DG, or paclitaxel alone, or the combination of the drugs. Vehicle treatment presented the majority of cells in the G1 phase of the cell cycle (MCF-7, 68.8%; A549, 71.2%), a small part in the G2–M phase (MCF-7, 12.4%; A549 8.6%) and the rest of the cells were found to be in the S-phase (MCF-7 18.8%, A549, 20.2%). 2-DG treatment resulted in a slight increase in cells in G1 phase arrest (MCF-7, 72.1%; A549, 77.4%), with a decrease in S-phase (MCF-7, 15.3%; A549, 15.4%) and no significant alteration in G2–M phase (MCF-7, 12.6%; A549, 7.2%). Metformin treatment resulted in an increase in the number of cells in the G1 phase (MCF-7, 80.6%; A549, 81.2%) with almost similar number of cells in the G2–M phase of MCF-7 cells (MCF-7, 9.5%; A549, 8.7%) and a reduction in the number of cells in the S-phase in both cell lines (MCF-7, 9.9%; A549, 10.2%). Metformin combined with 2-DG (MET + 2-DG) treatment resulted in a decrease in the number of cell in G1 phase (MCF-7, 53.5%; A549, 52.1%), and increase in cells in the G2–M phase arrest (MCF-7, 33%; A549, 35.3%) and a
Figure 2. Effect of combined treatment with 2-DG, metformin, and paclitaxel on MCF-7 and A549 cells. Cells were treated with 10 mmol/L metformin, 10 mmol/L 2-DG, and 1 μmol/L paclitaxel for 6 hours, and cells were prepared as described in Materials and Methods. The lysates of MCF-7 cells were immunoblotted (IB) with (A) acetyl-Lys-379 p53 and p53, (B) SESN2 and β-actin, (C) pAMPKα and AMPKα, (D) pmTOR and mTOR, (E) pp70S6K and p70S6K, and (F) p4E-BP1 and 4E-BP1. The lysates of A549 cells were immunoblotted (IB) with (G) acetyl-Lys-379 p53 and p53, (H) SESN2 and β-actin, (I) pAMPKα and AMPKα, (J) pmTOR and mTOR, (K) pp70S6K and p70S6K, and (L) p4E-BP1 and 4E-BP1. Data (mean ± SEM; n = 3 experiments in triplicate) are presented as relative to control. *, P ≤ 0.05 vs. control; #, P ≤ 0.05 vs. paclitaxel; †, P ≤ 0.05 vs. metformin.
Metformin Sensitizes Cancer Cells to Paclitaxel

Figure 3. Metformin (MET) and paclitaxel (PTX) inhibit cell viability. A, cell viability of MCF-7 and A549 cells treated with increasing doses of metformin for 48 or 72 hours measured by MTT assay. B, cell viability of MCF-7 and A549 cells treated with increasing doses of paclitaxel for 48 or 72 hours measured by MTT assay. Data (mean ± SEM; n = 3 experiments in triplicate) are presented as relative to vehicle. *, P < 0.05 vs. vehicle. C, cell viability of MCF-7 cells treated with increasing doses of paclitaxel or associated with metformin (10 mmol/L) for 48 or 72 hours measured by MTT assay. D, cell viability of A549 cells treated with increasing doses of paclitaxel or associated with metformin (10 mmol/L) for 48 or 72 hours measured by MTT assay. Data (mean ± SEM; n = 3 experiments in triplicate) are presented as relative to vehicle. *, P < 0.05 vs. vehicle; #, P < 0.05 vs. metformin. E, cell viability of MCF-7 and A549 cells treated with metformin (10 mmol/L), paclitaxel (1 umol/L), or a combination, as measured by trypan blue staining. Cells were treated for 48 hours with respective drugs. Data (mean ± SEM; n = 3 experiments in triplicate) are presented as relative to vehicle. *, P < 0.05 vs. vehicle; #, P < 0.05 vs. metformin; †, P < 0.05 vs. paclitaxel. F, cell-cycle analysis of MCF-7 and A549 cells treated with metformin (10 mmol/L), paclitaxel (1 umol/L), or a combination, as measured by flow cytometry (FACScalibur). Cells were treated for 24 hours with respective drugs. G, MCF-7 and A549 cells were treated with the indicated drugs for 24 hours and cell lysates were immunoblotted (IB) with the indicated antibodies. H, complex I oxygen consumption rates of MCF-7 and A549 cells treated for 24 hours with metformin (10 mmol/L), paclitaxel (1 umol/L), or a combination, as measured by a Oxygraph equipped with Clark-type electrode. Data (mean ± SEM; n = 3 experiments) are presented as relative to vehicle. *, P < 0.05 vs. vehicle; †, P < 0.05 vs. paclitaxel.
A decrease in cells in S-phase (MCF-7, 13.4%; A549, 12.6%) as related to vehicle-treated cells. Paclitaxel treatment, as expected, caused an increase in the number of cells in the G2-M phase (MCF-7, 21.5%; A549, 18.8%) with a reduction in the number of cells in the G1 phase (MCF-7, 64%; A549, 64.7%) and in the S-Phase (MCF-7, 14.5%; A549, 16.5%) in both cell lines (Fig. 3F).

The combined treatment of 2-DG and paclitaxel, of metformin and paclitaxel and the triple therapy resulted in a synergistic effect of G2-M cell-cycle arrest. Cells treated with 2-DG and paclitaxel showed a reduction in G1 phase (MCF-7, 57.1%; A549, 60.4%), there was no significant alteration in the number of cells in S-phase (MCF-7, 15.1%; A549, 16.9%); however, there was an increase in G2–M phase (MCF-7, 27.8%; A549, 22.7%) compared with either treatment alone. Cells treated with metformin and paclitaxel showed a reduction in G1 phase arrest, compared with either treatment alone (MCF-7, 54.6%; A549, 45.5%). This treatment resulted in no significant alteration in the number of cells in the S-phase compared with either treatment alone (MCF-7, 13.5%; A549, 12.8%). Additionally, when we analyzed the G2–M phase, we observed a significant increase in the number of cells in this phase in the metformin combined with paclitaxel treatment, as compared with either treatment alone (MCF-7, 31.9%; A549, 41.8%). Finally, the triple therapy resulted in a decrease in the number of cells in G1 phase as compared with MET + 2-DG treatment or paclitaxel treatment alone (MCF-7, 40.9%; A549, 39.7%), a slight decrease in the number of cells in S-phase (MCF-7, 10.9%; A549, 12.1%) and a significant increase in the number of cells in G2-M phase (MCF-7, 48.1%; A549, 48.2%). Thus, Figure 3F shows an increase in cell-cycle arrest in the G2–M phase, during the combined treatment of MET + 2-DG, 2-DG and paclitaxel, metformin and paclitaxel, and the triple therapy and a decrease in the G1 phase, indicating that cells submitted to these combined treatments were not further undergoing division.

We then examined the protein levels of caspase 3 and cleaved caspase 3, of cyclin D1, of p27, and of phospho-Rb in the cells. After 24 hours, caspase 3 was slightly decreased in the metformin and paclitaxel treatments and notably reduced in both cell lines treated with the combination of metformin and paclitaxel (Fig. 3G). Cleaved caspase 3 was slightly increased with metformin or paclitaxel treatments, and strongly increased in metformin and paclitaxel combined treatment after 24 hours in both cell lines (Fig. 3G). Cyclin D1 levels were only reduced in metformin treatment in both cell lines (Fig. 3G). p27 expression was increased in metformin treatment whereas phosphorylation of Rb was reduced in metformin treatment (Fig. 3G).

Cancer cell metabolism is unaffected by paclitaxel and hampered by metformin

To determine whether metformin, paclitaxel, or the combined treatment affect cancer cell metabolism we analyzed their effects on mitochondrial complex I oxygen consumption in MCF-7 and A549 cell lines. Metformin decreased complex I oxygen consumption by 58% in MCF-7 and by 92% in A549, whereas paclitaxel had a modest effect in both cell lines (Fig. 3H). The combined treatment showed no significant difference from the metformin treatment alone (Fig. 3H).

AMPK is implicated in the synergistic effect of metformin and paclitaxel

Figure 4A and B shows that p53 is activated with paclitaxel treatment and that further stimulation with metformin + paclitaxel, 2-DG + paclitaxel (double therapy), or a combination of metformin + 2-DG + paclitaxel (triple therapy) does not increase its activation. The same pattern is seen in SESN2 expression. On the contrary, AMPK shows a further increase in activation after double or triple therapies compared with paclitaxel only and vehicle treatments. Additionally, mTOR and its direct substrates p70S6K and 4EBP-1 are inhibited with paclitaxel treatment and further inhibition is observed with the double or triple treatments.

To further evidence the role of AMPK and SESNs in the combination treatment, we treated MCF-7 and A549 cells with siRNA to AMPK and to SESN1 and SESN2 and analyzed SESN1 and SESN2 expressions and AMPK, mTOR, p70S6K, and 4E-BP1 phosphorylation. Figure 4C and D show that treatment with SESN1 and SESN2 siRNA dampers SESN1 and SESN2 expressions, respectively, and reduces AMPK phosphorylation, with an increase in mTOR, p70S6K, and 4E-BP1 phosphorylation. Treatment with AMPK siRNA does not reduce SESN1 or 2 expressions but abolishes AMPK phosphorylation and expression, which increases mTOR, p70S6K, and 4E-BP1 phosphorylation.

Thus, these results clearly show an essential role for SESN and AMPK in the activation of AMPK and inhibition of mTOR, respectively, after the combined treatment of metformin and paclitaxel.

The effect of metformin and paclitaxel on A549 tumor growth in SCID mice

Xenografted SCID mice were treated with control vehicle, metformin, paclitaxel, or metformin and paclitaxel. Treatments began when the tumors presented an average size of 50 mm³ and tumor growth rate was measured daily after the beginning of the treatment. Figure 5A shows that metformin and paclitaxel is clearly more effective in reducing tumor growth, as compared with either paclitaxel alone, metformin alone, or the control. For the entire experiment, the animals treated with combination of metformin and paclitaxel presented almost no tumor growth, with the final tumor volume of 71 mm³ being very close to the tumor volume at the beginning of the treatment, as compared with the final volumes of the control (377 mm³), metformin (203 mm³), and paclitaxel (137 mm³) as shown in Figure 5B. We observed that 2-DG and paclitaxel combination yielded similar results to metformin and paclitaxel combination. However, we did not observe an additive effect with the triple therapy when compared with the double therapy (Supplementary Fig. S3A and B).
The reduced tumor growth following metformin and paclitaxel treatment is due to the reduced proliferation of tumor cells, as shown by Ki-67 staining and quantification (Fig. 5C and D) and increased apoptosis, as quantified by TUNEL staining (Fig. 5E and F) and cleaved caspase 3 staining (Fig. 5G and H). In the control group, Ki-67–positive cells were 13.4% (±1.1) whereas in paclitaxel these cells were 10.8% (±1.2) and metformin and paclitaxel presented 7.2% (±0.4) Ki-67–positive cells (Fig. 5C and D). The results of the TUNEL staining experiments show that the control group presented a 9.5% (±1.1) apoptosis whereas metformin apoptosis was 16.7% (±4.5), paclitaxel apoptosis was 31.8% (±1.8), and metformin and paclitaxel apoptosis was 35.9% (±4.8) (Fig. 5E and F). Similarly, in the cleaved caspase 3 staining experiments, the control group presented a 6.5% (±1.0) positive cells whereas metformin presented 22.3% (±0.8) positive cells, paclitaxel presented 27.4% (±2.1) positive cells, and metformin and paclitaxel combined presented 39.9% (±3.6) cells positive for cleaved caspase 3 (Fig. 5G and H). These data indicate a reduced proliferation and increased apoptosis in the combined treatment and are consistent in showing that there is a significant advantage in the use of combination treatment with metformin and paclitaxel, as compared with treatment with either agent alone.

**Effect of metformin, paclitaxel, and metformin and paclitaxel treatment on AMPK and the mTOR pathway in A549 xenografts**

As treatment with metformin and paclitaxel inhibited tumor growth, we sought to determine the AMPK/mTOR pathway activation status in the tumor tissue of animals treated with metformin, paclitaxel, and the combination of metformin and paclitaxel. Figure 6A shows that both treatments with paclitaxel resulted in a higher acetylation of Lysine 379 of p-53 and increased quantity of SESN2, as compared with control or metformin (Fig. 6B). The phosphorylation of AMPK at Thr172 was also higher in the combined treatment and are consistent in showing that there is a significant advantage in the use of combination treatment with metformin and paclitaxel, as compared with treatment with either agent alone.

**Discussion**

In this study, we show that the combination of metformin and paclitaxel has a major antitumor effect in vivo and induces massive cell-cycle arrest in vitro. These effects are correlated with a potent activation of AMPK. Our results
Figure 5. Metformin (MET) and paclitaxel (PTX) synergize in vivo to reduce A549 tumor growth. A549 cells (1.0 \times 10^6) were injected subcutaneously into the flank of SCID mice. Once the tumor reached 50 to 100 mm³, treatments were initiated, as indicated in Materials and Methods. Data are presented as mean ± SEM. A, tumor growth was measured daily after beginning treatment. B, tumor volume after 3 weeks of treatment. C, representative microphotograph of Ki-67 staining on tumor sections (arrows indicate positive Ki-67 staining). D, graph of percentage of Ki-67–positive cells per field; 4 fields per tumor section; mean ± SEM. E, representative microphotograph of TUNEL staining on tumor sections. F, graph of percentage of TUNEL-positive nuclei of cells per field; 4 fields per tumor section; mean ± SEM. G, representative microphotograph of cleaved caspase 3 staining on tumor sections. H, graph of percentage of cleaved caspase 3–positive nuclei of cells per field; 4 fields per tumor section; mean ± SEM. *, P < 0.05 vs. control; †, P < 0.05 vs. paclitaxel; ‡, P < 0.05 vs. metformin.
show that metformin, which induces a moderate decrease in ATP levels (36), is able to produce molecular activation of AMPK and inactivation of mTOR signaling in breast and lung cancer cells, whereas paclitaxel, through activation of p53 and SESNs, yields similar effects to metformin. Combined treatment with metformin and paclitaxel leads to a quantitative increase in AMPK activation and a drastic reduction of molecular signaling through the mTOR pathway. Likewise, the combination of paclitaxel with 2-DG, which like metformin, leads to intracellular ATP depletion (37, 38), severely inhibited the mTOR signaling pathway.

It was initially shown that metformin was capable of reducing proliferation in different types of cancer including, prostate, colon, and breast cancer cell lines. Subsequently, in vivo experiments with metformin resulted in tumor growth inhibition of up to 55% (12, 39). In accordance with these data, we herein show that metformin treatment resulted in a reduction of A549 and MCF-7 cell viability and a decreased tumor volume (approximately 50%) of A549 tumor when xenografted in SCID mice. These effects were paralleled by a decrease in the central regulator of cell growth and survival, mTOR signaling pathway, as measured by p70S6K and 4E-BP1 phosphorylation.

The mechanisms by which cells protect their genetic material during genotoxic stress include the alert of checkpoint proteins and arrest of cell growth and proliferation (40, 41). The major cellular stress-sensing molecule is p53, which halts cell growth and proliferation by increasing SESNs, thus leading to activation of AMPK and inhibition of mTOR (29, 30). Here we show that paclitaxel induces p53 activation in the cancer cells and activates AMPK. AMPK activation resulted in decreased mTOR pathway activity; this effect may be related to the reduction of cell metabolism that is observed during prolonged mitosis, induced by paclitaxel.
Intracellular interactions between different signaling systems may function as mechanisms for enhancing or counter-regulating signaling pathways. In the case of metformin, the cross-talk with paclitaxel-induced signaling pathways resulted in direct interactions between these drug-induced signaling systems at the level of AMPK. Simultaneous treatment with both drugs led to increased phosphorylation of AMPK and a drastic reduction of mTOR signaling pathway. Furthermore, there was no increase of the effects of the combination of metformin and paclitaxel compared with only metformin on tumor cell metabolism. These results suggest that the positive cross-talk between metformin and paclitaxel-induced signaling was due to additive effects on AMPK activation. Further inhibition of mTOR pathway with the triple therapy does not change the antineoplastic effect of metformin and paclitaxel combination.

The mTOR pathway is a crucial pathway, downstream of several growth factor receptors including epidermal growth factor, platelet-derived growth factor, KIT, and insulin like growth factor I receptor, which coordinate tumor growth factors. The deregulated mTOR pathway is very frequent in human cancer. These alterations include mutational activation of the p110α subunit of phosphoinositide 3-kinase (PI3K), loss of PTEN function, overexpression of PI3K, Akt, elf4E, and p70S6K, as well as inactivation of tuberous sclerosis 1 or 2 (42, 43). It was also established that the mTOR pathway can be inactivated by AMPK (44), which acts through a PI3K-independent mechanism. The susceptibility of cancer cells to PI3K inhibitors is highly determined by the presence of mutations in components of the PI3K/Akt/mTOR pathway (45). In contrast, our results showed that a drastic reduction of the mTOR pathway, elicited by the combination of metformin and paclitaxel, yields decreased cell viability in both MCF-7, which has a mutational activation of the PI3K catalytic subunit, and A549 cells, which does not harbor genetic alterations in the PI3K/Akt/mTOR pathway. As the mTOR pathway is essential to cell metabolism and growth, and delayed mitosis induced by paclitaxel is associated with a reduction in gene transcription, our data suggest that the cancer cells may be "pathway addicted," independently of harboring a mutation in the PI3K/Akt/mTOR pathway during paclitaxel-induced cell-cycle arrest. It is interesting to note that the susceptibility of cancer cells to metformin and paclitaxel combination occurred in a LKB1 independent manner. These data are in accordance with Sanli and colleagues (46) that recently showed that metformin can activate AMPK, probably through action of a metabolite derived from complex I inhibition. Thus, our data suggest that metformin antineoplastic effects are effective even when LKB1 is suppressed.

Toxicity elicited by paclitaxel has been linked to irreversible tubulin polymerization, a cell-cycle block at the metaphase–anaphase transition, and cell death (47, 48). On the contrary, in addition to the metabolic activity of AMPK, there is growing evidence that AMPK has a crucial role in the establishment of cell division, and it has been suggested that AMPK may be essential in the coordination between the sensing of energy resources and genome division (49, 50). Our results show that the combination of metformin and paclitaxel has an additive effect on cell viability and, in accordance with a previous study that combined 2 activators of AMPK, metformin and 2-DG, we observed a compelling accumulation of cells in G₂-M (36). As gene transcription is silenced during mitosis and paclitaxel plus metformin induced a more prolonged division, our results suggest that this event leads to a greater decrease in cell viability.

In conclusion, we observed a convergence of paclitaxel-and metformin- induced signaling at the level of AMPK. This mechanism illustrates how different drugs may cooperate to augment antigrowth signals, suggesting that target activation of AMPK by metformin may be a compelling ally in cancer treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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