METFORMIN AMPLIFIES CHEMOTHERAPY-INDUCED AMPK ACTIVATION AND ANTITUMORAL GROWTH

Running Title: Metformin Sensitizes Cancer Cells to Paclitaxel

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TRANSLATIONAL RELEVANCE

Targeted therapies are being increasingly investigated as new treatment options in oncology. Metformin is a widely-used antidiabetic drug whose anti-cancer effects represent a promising and novel approach for the treatment of cancer. Chemotherapy produces genotoxic stress and induces p53 activity, which can cross-talk with AMPK/mTOR pathway through SESNs. We tested the hypothesis that the combination of metformin and paclitaxel could show a synergistic effect in cancer cell lines. The findings presented here suggest that combined treatment is more effective in arresting cells in the G2/M-phase of the cell cycle, decreasing tumor growth and increasing apoptosis in tumor-bearing mice through a signaling convergence of metformin and paclitaxel at the level of AMPK. Our findings, therefore, demonstrate that different drugs may cooperate to increase anti-growth signals, and suggests that target activation of AMPK may be an alternative therapeutic strategy in cancer treatment.
ABSTRACT

Purpose: Metformin is a widely-used antidiabetic drug whose anti-cancer effects, mediated by the activation of 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 SCID mice and the cancer cell lines were treated with only paclitaxel or 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, demonstrate 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, decreased the tumor growth and increased apoptosis in tumor-bearing mice, when compared to individual drug treatments.

Conclusion: We have provided evidence for a convergence of metformin and paclitaxel induced signaling at the level of AMPK. This mechanism illustrates how different drugs may cooperate to augment anti-growth signals, and suggests that target activation of AMPK by metformin may be a compelling ally in cancer treatment.
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 epidemiological 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, whilst 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, since AMPK has been implicated in the regulation of mammalian target of rapamycin (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 pathological stresses such as heat shock, hypoxia, glucose deprivation and metformin administration [15, 21]. AMPK is also activated through Ca\(^{2+}\)/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, as well as 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 anti-growth signaling systems. AMPK has a major role as an anti-growth signal, since 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 (SESNs) 1 and 2 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, the present 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], as well as 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.
Methods

Antibodies, Chemicals and Buffers

All the reagents were from Sigma-Aldrich unless otherwise specified. Paclitaxel was from Laboratório Químico Farmacêutico Bergamo Ltda. (São Paulo, SP, Brazil). Anti-phospho-mTOR, anti-mTOR, anti-phospho-p70S6K, anti-p70S6K, anti-phospho-4E-BP1, anti-4E-BP1, anti-phospho-AMPKα, anti-AMPKα, 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 (Beverly, MA, USA), anti-p53 and anti-SESN2 antibodies for immunoblotting were from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and anti-SESN1 and anti-SESN3 antibodies for immunoblotting were from Abcam (Cambridge, MA, USA).

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, (Philadelphia, PA, USA). MCF-7 and A549 cells were cultured in DMEM containing 10% fetal bovine serum with the addition of antibiotics or fungicides. Both cell lines were maintained at 37°C in a humid atmosphere and 5% CO2.

Transfection

3x10^5 cells were seeded in a tissue culture plate in complete growth medium and incubated overnight. On the day of transfection, 200pmol 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 (10mM) and paclitaxel (1uM) and incubated for an additional 6 h. siRNA for AMPK was 5’-AAUUACUUCUGGUGCAGCAUAAGCGG-3’ forward and 5’-CCGCUAUGCUGCACCAGAACAUU-3’ reverse, for SESN1 was 5’-GAACCUCUCAGAGCUUGAACUG-3’ forward and 5’-CAGUUCAAGCAGCUGAGAGGUUC-3’ reverse and for SESN2 was 5’-GGAUAGCGAGUAGCCAUGGUCUUCC-3’ forward and 5’-GGAAGACCAGGGCUACCGCUACCC-3’ reverse.

Cell Viability Assay

Cells were seeded at a density of 2 x 10⁴ cells/well in 24-well plates containing 1mL 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 using trypan blue staining or they were treated with 0.3mg/mL of [3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium-Bromide] (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 200µL of 70% ethanol and stored at -20°C until use. Cells were centrifuged and pellets resuspended in 200µL of PBS and 10µg/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 50µg/mL propidium iodide). Cell cycle analysis
was performed by flow cytometry (FACScalibur). Data were analyzed using ModFit LT software.

**Complex I oxygen consumption**

Measurement of oxygen consumption by MCF-7 and A549 after treatment for 24 h with metformin (10mM), paclitaxel (1uM) or the combination of both drugs was performed by a Oxygraph equipped with a Clark-type electrode (Hansatech Instruments Limited, Norfolk, United Kingdom) in a closed chamber equipped with magnetic stirrer and temperature control at 37°C. Approximately 2,5x10^6 of viable MCF-7 cells/mL, and 4x10^6 of viable A549 cells/mL, permeable with 10 µM of digitonin, were added in 2 ml of reaction medium containing 125 mM sucrose, 65 mM KCl, 10 mM HEPES, 2.0 mM K2HPO4, 1.0 mM MgCl2 (pH 7.2); 50 µM EGTA, and complex I substrates: (2.0 mM malate, 1.0 mM α-ketoglutarate, 1.0 mM pyruvate and 1.0 mM glutamate). Analyses of oxidative phosphorylation and respiratory activity of mitochondria were made by sequential additions of 100 µM ADP, 2 µg/ml CAT, 100 nM carbonylcyanide p-trifluoromethoxyphenylhydrazone (FCCP), 5 mM succinate, 0,5 µM antimycin and 200 µM N,N,N′,N′-tetramethyl-p-phenylenediamine (TMPD)/ascorbate. The data was reproduced and calculated by the device’s specific software.

**Human tumor xenograft models**

4 weeks old male SCID mice were provided by the State University of Campinas - Central Breeding Center. Animals were inoculated in the dorsal region, subcutaneously, with 1 x 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 and using the formula \( V = W \times L \times \left( \frac{W+L}{2} \right) \times 0.52 \). Each group contained 15 animals.

Treatments began when tumors reached 50-100 mm\(^3\). Metformin was given daily by gavage at 500 mg/kg body weight. Paclitaxel was given once a week by intraperitoneal 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 mM Tris, pH 7.4, containing 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, 2 mM 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 used.

**Protein analysis by immunoblotting**

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

**Immunohistochemistry**
To detect Ki-67 and cleaved Caspase 3, microwave postfixation was carried out using a domestic oven which was delivered to slides immersed in 0.01 mol/l citrate buffer, pH 6.0, in two 7-min doses separated by a 2-min break. Sections were then incubated at 4°C overnight with primary monoclonal mouse anti-human Ki-67 clone MIB-1 from Dako (Glostrup, Denmark) (diluted 1:100) and anti-cleaved Caspase3 from Cell Signalling Technology (Beverly, MA, USA). The slides were then incubated with avidin-biotin complex LSAB+ Kit from Dako Cytomation (Carpinteria, CA, USA) for 30 min followed by the addition of diaminobenzidine tetrahydrochloride (DAB) as a substrate-chromogen solution. After hematoxylin counterstaining and dehydration, the slides were mounted in Entellan from Merck (Darmstadt, Germany).

**TUNEL assay**

Terminal deoxynucleotidyl Transferase Biotin–d UTP Nick End Labeling (TUNEL) staining was performed using a commercial apoptosis detection kit (Roche), according to the recommendations of the manufacturer. Analysis and documentation of results were performed using a Leica FW 4500 B microscope.

**Statistical analysis**

Data are presented as means ± SEM of at least three independent experiments. All groups were studied in parallel and differences between groups were analyzed using ANOVA, as appropriate and Bonferroni post hoc tests for multiple un-pairwise 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 (1-50mM) for different periods of time (0-8 hours). Metformin treatment resulted in the activation of AMPK, with increased phosphorylation of AMPKα 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 to untreated cells (Figure 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 for the metformin treatment, 2-DG led to activation of AMPK and inactivation of the mTOR signaling pathway (Supplementary Figure 1A-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 uM until 100 uM with ACC phosphorylation and mTOR and p70S6K inactivation (Supplementary Figure 2). 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 Figure 2).
**Paclitaxel activates AMPK and inhibits mTOR in MCF-7 breast cancer cells and A549 lung cancer cells.**

In order to investigate the mechanisms underlying the anti-proliferative 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], as well as the amount of SESN 1, 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 (Figure 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 SESN 2 (Figure 2B). This increase in SESN 2 in paclitaxel-treated cells was followed by an increase in the phosphorylation of Thr-172 of AMPK (Figure 2C) and inhibition of mTOR (Figure 2D), p70S6K (Figure 2E) and 4E-BP1 (Figure 2F), when compared to metformin or 2-DG treatments alone. Even though metformin or 2-DG treatment do not increase SESN 2 (Figure 2B), we observe an increase in AMPK phosphorylation (Figure 2C) and inhibition of mTOR (Figure...
2D), p70S6K (Figure 2E) and 4E-BP1 (Figure 2F) when compared to vehicle treated cells. In A549 cells, we also observed an increase in acetyl-Lys379 p-53 and in the amount of SESN 2 only in the paclitaxel-treated cells (Figure 2G and 2H), and this was correlated with an increase in the Thr-172 phosphorylation of AMPK (Figure 2I) and decrease in the activation of mTOR (Figure 2J), p70S6K (Figure 2K) and 4E-BP1 (Figure 2L) when compared to control and metformin and 2-DG treatments alone. Once again, metformin and 2-DG treatments did not increase SESN 2 (Figure 2H) but they were able to increase AMPK phosphorylation (Figure 2I) and inhibit mTOR (Figure 2J), p70S6K (Figure 2K) and 4E-BP1 (Figure 2L) when compared to 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 3 (A and B), both metformin and paclitaxel inhibited cell viability, as related to vehicle treated cells. The metformin treatment was statistically significant at 10mM for the 48-hour treatment and at 1mM for the 72-hour treatment (Figure 3A) in MCF-7 cells and that a 1mM dose of metformin was capable of lowering A549 cells proliferation by approximately 20-30% for both the 48- and 72-hour treatment. The paclitaxel treatment was effective only at the dose of 10nM for both 48- and 72-hour treatment (Figure 3B). Figures 3C and D show that in the combined treatment metformin potentiates paclitaxel action on MCF-7 (Figure 3C) and A549 (Figure 3D) cells, as a dose of 1nM of paclitaxel is statistically different from the metformin and vehicle treated cells for both the 48- and 72-hour treatment. At 10nM of paclitaxel, metformin no longer potentiates paclitaxel treatment on MCF-7 cells. However, in A549 cells, we observed that at the 48-hour treatment
metformin (10mM) and 10nM paclitaxel is more effective on lowering cell growth than paclitaxel alone treatment (Figure 3D). We then analyzed cell viability by trypan blue staining of both cell lines (Figure 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 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 (Figure 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 to either treatment alone. Cells treated with metformin and paclitaxel demonstrated a reduction in G1-phase arrest, compared to 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 to 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 to 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 to 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 no longer undergoing division.

We then examined the protein levels of Caspase 3 and cleaved Caspase 3, of Cyclin D1, of p27 and of phosho-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 (Figure 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 (Figure 3G). Cyclin D1 levels were only reduced in metformin treatment in both cell lines (Figure 3G). p27 expression was increased in metformin treatment while phosphorylation of Rb was reduced in metformin treatment (Figure 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 its 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 (Figure 3H). The combined treatment showed no significant difference from the metformin treatment alone (Figure 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 SESN 2 expression. On the other hand, AMPK shows a further increase in activation after double or triple therapies compared to 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 2 and analyzed SESN 1 and 2 expressions and AMPK, mTOR, p70S6K and 4E-BP1 phosphorylation. Figure 4C and D show that treatment with SESN 1 and 2 siRNA dampers SESN 1 and 2 expressions respectively and reduces AMPK phosphorylation, with an increase in mTOR, p70S6K and 4E-BP1 phosphorylation. Treatment with AMPK siRNA does not reduce SESN 1 or 2 expressions but abolishes AMPK phosphorylation and expression, which increases mTOR, p70S6K and 4E-BP1 phosphorylation.

Thus, these results clearly demonstrate 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 mm\(^3\) 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 mm\(^3\) being very close to the tumor volume at the beginning of the treatment, as compared with the final volumes of the control (377 mm\(^3\)), metformin (203 mm\(^3\)) and paclitaxel (137 mm\(^3\)) 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 to the double therapy (Figure Supplementary 3A and B).

The reduced tumor growth following metformin and paclitaxel treatment is due to the reduced proliferation of tumor cells, as demonstrated by Ki67 staining and quantification (Figure 5C and D) and increased apoptosis, as quantified by TUNEL staining (Figure 5E and F) and cleaved Caspase 3 staining (Figure 5G and H). In the control group, Ki67 positive cells were 25.5% (± 2.8) of the total, in metformin these cells were 13.4% (± 1.1), while in paclitaxel these cells were 10.8% (± 1.2) and metformin and paclitaxel presented 7.2% (± 0.4) Ki67 positive cells (Figure 5 C and D). The results of the TUNEL staining experiments show that the control group presented a 9.5% (± 1.1) apoptosis, while metformin apoptosis was 16.7% (± 4.5), and with paclitaxel apoptosis was 31.8% (± 1.8) and metformin and paclitaxel presented a 35.9% (± 4.8) apoptosis (Figure 5 E and F). Similarly, in the cleaved Caspase 3 staining experiments, the control group presented a 6.5% (± 1.0) positive cells, while metformin positive cells was 22.3% (± 0.8), and paclitaxel presented 27.4% (± 2.1) positive cells and metformin and paclitaxel combined presented 39.9% (± 3.6) cells positive for cleaved caspase 3 (Figure 5G and H). These data indicate a reduced proliferation and increased apoptosis in the combined treatment and are consistent in demonstrating 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 to control or metformin (Figure 6B). The phosphorylation of AMPK at Thr172 was also higher in the metformin and paclitaxel treatment, when compared to paclitaxel alone, metformin alone or control (Figure 6C). Both treatments with paclitaxel and metformin also activated AMPK, as compared to the control. Similarly, phosphorylation of mTOR (Figure 6D), and its direct substrates p70S6K (Figure 6E) and 4E-BP1 (Figure 6F), were reduced following metformin and paclitaxel treatments.
DISCUSSION

In the present 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 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, whilst 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 2DG, which like metformin, leads to intracellular ATP depletion [37, 38], severely inhibited the mTOR signaling pathway.

It was initially demonstrated 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 of A549 tumor when xenografted in SCID mice, of approximately 50%. These effects were paralleled by a decrease in the central regulator of cell growth and survival, mTOR signaling pathway, as measured by p70S6K and 4EBP-1 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 to 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 EGF, PDGF, KIT and IGF1R, which coordinate tumor growth [42, 43]. The deregulated mTOR pathway is very frequent in human cancer. These alterations include mutational activation of the p110α subunit of PI3K, loss of PTEN function, overexpression of PI3K, Akt, eIF4E 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. Since 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 et al. [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 other hand, 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 two activators of AMPK, metformin and 2-DG, we observed a compelling accumulation of cells in G2/M [36]. Since 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 anti-growth signals, suggesting that target activation of AMPK by metformin may be a compelling ally in cancer treatment.

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REFERENCES


Figure Legends

Figure 1

**Metformin and paclitaxel activate AMPK and inhibit mTOR in MCF-7 and A549 cells.** (A) MCF-7 cells were treated with 10 mM metformin for the indicated time and (B) for 6 hours with the indicated doses. The lysates were immunoblotted with the indicated antibodies. (C) A549 cells were treated with 10 mM metformin for the indicated time and (D) for 6 hours with the indicated doses. The lysates were immunoblotted with the indicated antibodies. (E) MCF-7 cells were treated with 1 uM paclitaxel for the indicated time and (F) for 6 hours with the indicated doses. The lysates were immunoblotted with the indicated antibodies. (G) A549 cells were treated with 1 uM 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.

Figure 2

**Effect of combined treatment with 2-DG, metformin and paclitaxel on MCF-7 and A549 cells.** Cells were treated with 10 mM metformin, 10 mM 2-DG and 1 uM paclitaxel for 6 hours, and cells were prepared as described in 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, (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, (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).

Figure 3

Metformin and paclitaxel 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 mM) 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 mM) 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 mM), paclitaxel (1 μM) 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 mM), paclitaxel (1 μM) 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 mM), paclitaxel (1 μM) 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).

**Figure 4**

**AMPK involvement in the synergistic effect of metformin and paclitaxel.** (A) MCF-7 cells were treated with paclitaxel alone (1 uM) or with metformin/paclitaxel (10 mM/1 uM) or with 2-DG/paclitaxel (10 mM/1 uM) or the triple therapy for 6 hours. The cell lysates were then immunoobloted (IB) with the indicated antibodies. (B) A549 cells were treated with paclitaxel alone (1 uM) or with metformin/paclitaxel (10 mM/1 uM) or with 2-DG/paclitaxel (10 mM/1 uM) or the triple therapy for 6 hours. The cell lysates were then immunoobloted (IB) with the indicated antibodies. (C) MCF-7 cells were transfected with siRNA for AMPKα, for SESN1 or for SESN2 and than treated with metformin/paclitaxel (10 mM/1 uM) for 6 hours. The cell lysates were then immunoobloted (IB) with the indicated antibodies. (D) A549 cells were transfected with siRNA for AMPKα, for SESN1 or for SESN2 and than treated with metformin/paclitaxel (10 mM/1 uM) for 6 hours. The cell lysates were then immunoobloted (IB) with the indicated antibodies.

**Figure 5**

**Metformin and paclitaxel synergize in vivo to reduce A549 tumor growth.** $1.0 \times 10^6$ A549 cells were injected subcutaneously into the flank of SCID mice. Once the tumor reached 50-100 mm$^3$, treatments were initiated, as indicated in Methods. Data are presented as means ± 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 % 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 %TUNEL-positive nuclei of cells per field, 4 fields per tumor section, mean ± SEM. (G) Representative microphotograph of cleaved Caspase3 staining on tumor sections. (H) Graph 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).

Figure 6

Metformin and paclitaxel activate AMPK and inhibit mTOR in vivo. Mice bearing A549 xenografts were treated with only metformin or paclitaxel, or a combination, as described in Methods. The A549 tumor lysates 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, (F) p4E-BP1 and 4E-BP1. Data (mean ± SEM; n = 8) are presented as relative to control (* p ≤ 0.05 vs control; # p ≤ 0.05 vs paclitaxel; ‡ p ≤ 0.05 vs metformin).
Figure 6
Clinical Cancer Research

METFORMIN AMPLIFIES CHEMOTHERAPY-INDUCED AMPK ACTIVATION AND ANTITUMORAL GROWTH

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