Targeting Cancer Cell Metabolism: The Combination of Metformin and 2-Deoxyglucose Induces p53-Dependent Apoptosis in Prostate Cancer Cells

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Abstract

Targeting cancer cell metabolism is a promising strategy to fight cancer. Metformin, a widely used antidiabetic agent, exerts antitumoral and antiproliferative action. In this study, the addition of metformin to 2-deoxyglucose (2DG) inhibited mitochondrial respiration and glycolysis in prostate cancer cells leading to a severe depletion in ATP. The combination of the two drugs was much more harmful for cancer cells than the treatment with metformin or 2DG alone, leading to 96% inhibition of cell viability in LNCaP prostate cancer cells. In contrast, a moderate effect on cell viability was observed in normal prostate epithelial cells. At the cellular level, the combination of metformin and 2DG induced p53-dependent apoptosis via the energy sensor pathway AMP kinase, and the reexpression of a functional p53 in p53-deficient prostate cancer cells restored caspase-3 activity. In addition to apoptosis, the combination of metformin and 2DG arrested prostate cancer cells in G2-M. This G2-M arrest was independent of p53 and correlated with a stronger decrease in cell viability than obtained with either drug. Finally, metformin inhibited 2DG-induced autophagy, decreased beclin 1 expression, and triggered a switch from a survival process to cell death. Our study reinforces the growing interest of metabolic perturbators in cancer therapy and highlights the potential use of the combination of metformin and 2DG as an anticancerous treatment. Cancer Res; 70(6); 2465–75. ©2010 AACR.

Introduction

Current treatments for advanced prostate cancer are limited, with drug resistance and toxicity requiring novel molecular therapeutics drugs directed against new cellular targets. Alterations in cancer cell metabolism are intricately linked to the principal hallmarks of cancer (1). The first tumor-specific alteration consists of an increase in glycolysis, which is maintained even in high oxygen conditions (2). As a consequence, most cancer cells use elevated amount of glucose for anabolism, because it inhibits hexokinase, the first rate-limiting enzyme of glycolysis (3). It leads to intracellular ATP depletion (4) and induction of autophagy, a cell survival process in response to nutrient deprivation (5). Because of the tumor dependence on glycolysis for energy generation provides a biochemical basis to preferentially kill the malignant cells by inhibition of glycolysis.

2-Deoxyglucose (2DG) is an inhibitor of glucose metabolism, because it inhibits hexokinase, the first rate-limiting enzyme of glycolysis (3). It leads to intracellular ATP depletion (4) and induction of autophagy, a cell survival process in response to nutrient deprivation (5). Because of the tumor dependence on glycolysis, 2DG has been considered as a potential anticancer agent and association of chemotheapeutic agents and radiation therapy in hypoxic environment (1). Furthermore, the increased dependency on glycolysis for energy generation provides a biochemical basis to preferentially kill the malignant cells by inhibition of glycolysis.

Metformin is a widely used antidiabetic drug prescribed to almost 120 million people for the treatment of type 2 diabetes. It lowers hyperglycaemia by inhibiting hepatic glucose production (7). Recently, numerous studies have shown that metformin decreases cancer cell viability and tumor growth in xenograft models (8–11). Furthermore, retrospective epidemiologic studies revealed a decrease in the incidence of cancer in patients treated with metformin (12–14). Similarly to 2DG, metformin inhibits the energy-sensitive signaling pathway mTOR and affects cell metabolism. It hampers the respiratory chain complex 1 in hepatocytes (15) and inhibits metabolism to generate ATP than on mitochondrial metabolism. These biological alterations present a major challenge in cancer treatment, as exemplified by the resistance of cancer cells to chemotheapeutic agents and radiation therapy in hypoxic environment (1). Furthermore, the increased dependency on glycolysis for energy generation provides a biochemical basis to preferentially kill the malignant cells by inhibition of glycolysis.
oxygen consumption in colon cancer cells (9), which is consistent with the inhibition of oxidative phosphorylation.

We decided to combine 2DG and metformin, two drugs that target the two sources of cell energy and may represent a major advantage over traditional chemotherapies. However, the effect of this combination on cancer cell metabolism and growth is presently unknown.

Here, we show that metformin and 2DG act synergistically to induce a massive ATP depletion in prostate cancer cells. It led to a stronger inhibitory effect on cell viability than the drugs alone. When used individually, metformin arrested cell cycle in G0-G1 and 2DG induced autophagy; their combination blocked cell cycle in G2-M and induced p53-dependent apoptosis via the AMP kinase (AMPK) pathway. Finally, we showed that metformin shifted the 2DG response from autophagy to apoptosis.

Materials and Methods

Cell lines and culture conditions. LNCaP and P69 cells were cultured in RPMI 1640 and PC-3 and DU145 in DMEM containing 25 mmol/L glucose supplemented with 10% fetal bovine serum (FBS) and 100 units/mL penicillin, 100 mg/mL streptomycin, and 2 mmol/L glutamine at 37°C and 5% CO2.

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The P69 cell line was derived by immortalization of human prostate cancer cells. Metformin decreased complex 1 activity by 70% in LNCaP cells, whereas it had a modest effect in P69 cells. Rotenone, a specific inhibitor of complex 1, inhibited the activity in both cell lines (Fig. 1A). When mitochondrial oxidation is impaired, cells compensate and increase aerobic glycolysis to improve their bioenergetics (19). In agreement, metformin accelerated glucose depletion from medium more severely in LNCaP than P69 cells (Fig. 1B) and consequently lactate concentration (one of the end products of aerobic glycolysis) augmented by 73% in the culture medium of LNCaP compared with 28% in P69 (Fig. 1C). These results show that, as a consequence of complex 1 inhibition, metformin significantly increases glycolysis in prostate cancer cells and to a lesser extent in normal cells. To block this effect, cells were treated with 2DG, an inhibitor of glycolysis. 2DG (1 mmol/L) decreased lactate production and prevented metformin-induced lactate production in LNCaP and P69 (Fig. 1C). Altogether, these results suggest that metformin and 2DG inhibit the two main sources of cellular ATP. This was indeed the case because metformin and 2DG alone decreased intracellular ATP concentration by 60% in LNCaP and had a slight effect in P69 (Fig. 1C). Importantly, the combination of the two drugs robustly diminished ATP concentration by >90% in LNCaP.

Results

Metformin hampers prostate cancer cell metabolism and aggravates ATP depletion induced by 2DG. To determine whether metformin affects cancer or normal prostate cell metabolism, we analyzed its effects on mitochondrial complex 1 activity in LNCaP and the normal P69 cell line (immortalized human primary prostate epithelial cells). Metformin decreased complex 1 activity by 70% in LNCaP cells, whereas it had a modest effect in P69 cells. Rotenone, a specific inhibitor of complex 1, inhibited the activity in both cell lines (Fig. 1A). When mitochondrial oxidation is impaired, cells compensate and increase aerobic glycolysis to improve their bioenergetics (19). In agreement, metformin accelerated glucose depletion from medium more severely in LNCaP than P69 cells (Fig. 1B) and consequently lactate concentration (one of the end products of aerobic glycolysis) augmented by 73% in the culture medium of LNCaP compared with 28% in P69 (Fig. 1C). These results show that, as a consequence of complex 1 inhibition, metformin significantly increases glycolysis in prostate cancer cells and to a lesser extent in normal cells. To block this effect, cells were treated with 2DG, an inhibitor of glycolysis. 2DG (1 mmol/L) decreased lactate production and prevented metformin-induced lactate production in LNCaP and P69 (Fig. 1C). Altogether, these results suggest that metformin and 2DG inhibit the two main sources of cellular ATP. This was indeed the case because metformin and 2DG alone decreased intracellular ATP concentration by 60% in LNCaP and had a slight effect in P69 (Fig. 1C). Importantly, the combination of the two drugs robustly diminished ATP concentration by >90% in LNCaP.
cells and ~50% in other prostate cancer cell lines (DU-145 and PC3) but only by 20% in P69 (Fig. 1D; Supplementary Fig. S1). These results show that metformin and 2DG initiate a strong metabolic stress in prostate cancer cells compared with normal cells.

**The combination of metformin and 2DG exerts a deleterious effect on cancer cell viability.** To determine if this effect on metabolism affects cell viability, we analyzed cell viability by trypan blue staining using two concentrations of metformin (1 and 5 mmol/L) and 1 mmol/L 2DG in LNCaP and P69 cells. The combination of the two drugs induced a 95% inhibition of cell viability in LNCaP, whereas metformin (5 mmol/L) and 2DG alone decreased cell viability by 70% and 37%, respectively (Fig. 2A). In P69 cells, metformin had almost no effect whereas 2DG inhibited cell viability by 28%, and the combination of the two drugs led to a small additive inhibitory effect compared with 2DG alone. Similar results were obtained using XTT cell viability (data not shown). These results show that the combination of metformin and 2DG is very toxic for cancer cells but had few effects in normal cells.

To establish a direct relationship between the decrease in cell viability and the inhibition of cell proliferation, cell viability was followed over 3 days after the addition of metformin and/or 2DG in LNCaP and P69 cells. We did not observe any effect of the drugs alone, whereas the combination slightly decreased cell viability in P69 cells. By contrast, metformin and 2DG alone similarly reduced cell viability compared with control, and their combination decreased cell viability below cell seeding density in LNCaP cells (Fig. 2B), suggesting a massive cell death. These results show that the combination of metformin and 2DG displays strong antiproliferative effects. Furthermore, it establishes a correlation between a strong metabolic stress and an important antiproliferative effect.

**The combination of metformin and 2DG induces apoptosis via AMPK.** To determine if the deleterious effect of the combination of the drugs is due to apoptosis, we analyzed caspase-3 activity in LNCaP cells. Metformin or 2DG did not activate caspase-3 (Fig. 3A). In contrast, the combination of metformin and 2DG increased caspase-3 activity and Annexin V-positive cells and initiated caspase-3 cleavage (Fig. 3A, C; Supplementary Fig. S2A, B). As expected AICAR (control) induced a strong increase of caspase-3 activity. Z-VAD-fmk (a pan-caspase inhibitor) abolished caspase-3 activity in cells treated with the combination of metformin and 2DG, as well as AICAR (Fig. 3A). We then analyzed the expression of major proapoptotic and antiapoptotic genes by quantitative reverse transcription–PCR using a dedicated “apoptosis plate” (20). We found that proapoptotic genes were upregulated after cotreatment with metformin and 2DG, whereas antiapoptotic genes were downregulated. In accordance, BCL2 and GADD45 expression was abolished or upregulated, respectively (Supplementary Fig. S2C). To determine the implication of apoptosis, we treated cells with the pan-caspase inhibitor Z-VAD-fmk, which had no effect on cell viability per se and did not alter the effect of metformin or 2DG. Importantly, the caspase inhibitor prevented the additive effect of the combination of metformin and 2DG and restored cell viability to the level of metformin or 2DG treatment (Fig. 3B). To examine if AMPK, the major energy...
sensor kinase, is implicated in metformin/2DG-induced apoptosis, we analyzed the phosphorylation of AMPK. The combination of metformin and 2DG induced a stronger activation of AMPK and a total inhibition of phosphorylated S6 ribosomal (a marker of mTOR activity; Fig. 3C; Supplementary Fig. S3) compared with the drugs alone, suggesting a correlation between the level of ATP depletion and activation of AMPK. To determine if AMPK is involved in apoptosis induced by the combination of the drugs, we knocked down the α1 and α2 catalytic unit of AMPK (8). siRNAs partially inhibited total AMPK-α and AMPK phosphorylation; however, this inhibition was sufficient to prevent caspase-3 cleavage and activity (Fig. 3C, D). To confirm the implication of AMPK, we used compound C, an AMPK inhibitor (21), which prevented the inhibition of P56 ribosomal and inhibited caspase-3 cleavage (Supplementary Fig. S3). These results suggest that apoptosis induced by the energetic stress caused by the combination of metformin and 2DG is mediated by AMPK.

**p53 regulates metformin/2DG induction of apoptosis.** We asked whether p53, which plays a central role in apoptosis (22), is required for metformin/2DG-induced apoptosis in LNCaP cells. 2DG alone did not affect p53 protein level, whereas metformin slightly increased its expression. Interestingly, their combination strongly induced p53 expression (Fig. 4A). To determine the role of p53 in metformin/2DG-induced apoptosis, we measured caspase-3 activity in prostate cancer cells deficient (PC3) or mutated (DU145) for p53. The combination of metformin and 2DG did not affect caspase-3 activity in both cell lines, whereas staurosporine strongly induced it (Fig. 4B). Therefore, functional p53 seems to be important for metformin/2DG-induced apoptosis. To confirm this role, we expressed wild-type p53 in DU145 and PC3 using an adenoviral vector (Fig. 4C). The reexpression of p53 induced caspase 3 activity in PC3 and DU145 cells upon metformin/2DG treatment, whereas no caspase activity was detected in cells transduced with the control adenoviral vector (Fig. 4C). Altogether, these results suggest that p53 is required for apoptosis induced by the combination of metformin and 2DG.

**Metformin/2DG treatment induces cell cycle arrest in G2-M independently of p53.** Because the decrease in cell viability induced by the combination of metformin and 2DG is not entirely due to apoptosis (Fig. 3B), we asked whether cell cycle was affected. LNCaP cells were treated for 24 h in the presence of metformin or/and 2DG. Metformin or 2DG led to a cell cycle arrest in G0-G1 (Fig. 5A). Interestingly, the combination of metformin and 2DG led to the accumulation of LNCaP in G2-M with 32% of cells arrested at this stage (Fig. 5A). To determine if cells were arrested in mitosis (M), we analyzed microtubules organization by confocal microscopy with α-tubulin antibodies but did not find accumulation of mitotic cells (data not shown). We then examined the protein level of cyclins B1 and A in proliferating cells.
Figure 3. Metformin and 2DG combination induces apoptosis in LNCaP cells. A, LNCaP cells were treated with metformin (5 mmol/L), 2DG (1 mmol/L), metformin + 2DG, or AICAR (5 mmol/L) for 48 h. Relative caspase-3 activity in the presence or absence of 100 μmol/L of ZVAD-fmk is represented. B, LNCaP cells were treated with metformin (5 mmol/L), 2DG (1 mmol/L), or their combination in the presence or absence of ZVAD-fmk, and a viability assay (XTT) was performed 3 d after the addition of the agents. The addition of ZVAD-fmk significantly increased cell viability with $P < 0.05$ (*). C, immunobloting of the indicated proteins. D, caspase-3 activity in LNCaP cells treated with lipofectamine, transfected with control siRNA or α1 and α2 AMPK siRNA.
After 24 hours, cyclin B1 was slightly decreased by metformin, and was notably reduced in cells treated with the combination of metformin/2DG (Fig. 5B). Cyclin A was decreased with metformin or 2DG and strongly affected in metformin/2DG-treated cells after 24 hours (Fig. 5B). These results show that, in addition to apoptosis, the combination of metformin and 2DG blocked cell cycle in G2.

To test whether p53 is required for the G2-M arrest, cell cycle was analyzed in DU145 and PC3. 2DG did not affect cell cycle, whereas metformin induced a slight increase in the percentage of cells in G0-G1 in DU145 and cells accumulated in G2-M in PC3 (Fig. 5C; data not shown). Importantly, the combination of the drugs, similar to LNCaP cells, led to a G2-M arrest in p53-deficient cells (Fig. 5C) and a decrease in cyclin B1 expression (data not shown). In conclusion, these results suggest that p53 is not required for cell cycle arrest in G2-M.

To determine if this blockade is associated with an effect on cell viability, we treated DU145 and PC3 with metformin or/and 2DG. Metformin inhibited cell viability by 20% and 25% in PC3 and DU145, respectively (Fig. 5D). 2DG alone had almost no effect in DU145 and inhibited cell viability by 32% in PC3. Importantly, the combination of the drugs had a significant and stronger effect on cell viability than
either drug with a decrease in cell viability of 75% and 51% in PC3 and DU145, respectively (Fig. 5D). Although the effect on cell viability was less important in PC3 and DU145 than in LNCaP (75% and 51% versus 95%), our results show that p53 was not required for the additive effect of the combination. These data suggest that the effect of the combination of metformin/2DG may also be due to a G2-M cell cycle arrest which occurs independently of the status of p53.

![Figure 5](cancerres.aacrjournals.org)
Metformin inhibits 2DG-induced autophagy. PC3 and LNCaP cells induce autophagy as a survival response to 2DG (5). We assessed 2DG induced autophagy using different approaches. First, we showed by electron microscopy that 2DG (20 or 1 mmol/L) induced the formation of autophagosomes (Fig. 6A). Second, 2DG induced the accumulation of LC3 II, the autophagic form of LC3 (Fig. 6B; Supplementary Fig. S4). Finally, following transfection of LNCaP with RFP-LC3 construct, the formation of RFP puncta in cells treated with 2DG was observed (Fig. 6D).

We then investigated the effect of metformin on the autophagic response. Unlike 2DG, metformin did not induce the accumulation of LC3 II (Fig. 6B). However, interestingly, the addition of metformin to 2DG led to a total extinction of LC3-I and LC3-II (Fig. 6B), suggesting that metformin inhibits autophagy. To confirm this effect, we analyzed the expression of beclin 1, a major regulator of autophagy (23). Cells were treated with 2DG and metformin (0.5, 1, and 5 mmol/L). Metformin decreased LC3 protein levels in a dose-dependent manner and diminished beclin 1 expression in cells treated with 2DG (Fig. 6C). Noteworthy, we observed the same inhibition in PC3 cells (data not shown), suggesting that this effect is not dependent of p53. We then visualized the formation of RFP puncta, and in accordance with our previous results, metformin decreased 2DG induced autophagy as measured by the quantification of cells with RFP-LC3 positive dots (Fig. 6D). These results show that metformin inhibits 2DG-induced autophagy and suggest that inhibition of autophagy could trigger apoptosis in LNCaP cells.

Discussion

Metformin alone exhibits a strong antiproliferative action in numerous cancer cell lines (8, 9, 11, 24), and 2DG by itself sensitizes cancer cells to the action of radiation or chemotherapeutics agents (25–29). Here we show that the combination of the two drugs has a much stronger deleterious effect than either drug. Combining metformin and 2DG led to a drastic reduction of intracellular ATP through the inhibition of the mitochondrial complex 1 and glycolysis. Similarly, 3-bromopyruvate, which inhibits glycolysis and mitochondrial respiration (30), inhibits hepatocellular carcinoma cell growth (31) and displays antitumoral action in liver (32). However, its action on other cancers has not been proved yet, and as an alkylating agent, it causes numerous side effects. Metformin and 2DG have the clinical advantage of inducing no adverse side effect per se, although metformin could very rarely cause lactic acidosis (33).

Our study suggests that the induction of apoptosis is correlated with a drop in ATP. Indeed, metformin or 2DG induces a moderate decrease of ATP, a slight activation of AMPK, and a modest decrease of mTOR activity compared with the strong effect of their combination, which induces apoptosis. In P69 cells, where the combination of the drugs decreases moderately the intracellular ATP concentration, cell viability was moderately affected. Therefore, in agreement with others, we propose that the ATP level regulates the onset of apoptosis (34, 35). Furthermore, we make a connection between the metabolism-sensitive pathway AMPK and apoptosis. The decrease in ATP concentration induces apoptosis, and the inhibition of the "metabolic sensor" kinase AMPK blocks caspase-3–dependent cell death. Such implication of AMPK in apoptosis has already been described for AICAR, which mimics energy depletion and induces apoptosis in B-cell chronic lymphocytic leukemia and pancreatic β cells (36, 37). Induction of apoptosis is a major challenge, because it may improve the effects of classic anticancer agents. Noteworthy, ongoing clinical trial are currently testing the association of metformin and chemotherapy in breast cancer (14), whereas others have concluded for a beneficial effect of 2DG in combination with radiation in glioma (28). Because we show that the combination of metformin and 2DG induces apoptosis and exerts an additive antiproliferative compared with either drug, we expect a more efficient effect on cancer.

Although the combination of metformin and 2DG did not induce apoptosis in p53-deficient cells, the combination of the two drugs has a significant and additive effect on cell viability in these cells. This additive effect may be due to the strong accumulation of cells in G2-M. Indeed, cell cycle arrest (and more specifically G2-M arrest) is associated with a strong inhibition of cell viability. Thus, Adriamycin, a chemotherapeutic agent, induces a more stringent cell cycle arrest in G2 than in G1 in synchronized cells (38). Noteworthy, the G2-M arrest is the main molecular mechanism of action of Taxol derivatives, the most efficient and most frequently used chemotherapeutic agents in prostate cancer therapy (39). G2-M is also characteristic of the induction of senescence (40), which is controlled by p53, but metformin and 2DG did not induce senescence in LNCaP cells (data not shown).

We show in accordance with Di Paola and colleagues (5) that 2DG alone induced autophagy in PC3 and LNCaP cells. When cells are cultured in the absence of nutrients, a survival process is induced to recycle essential metabolites such as lipids and amino acids for fuelling their bioenergetic machinery (41). Here, we show that the addition of metformin to 2DG induces a shift from a survival process to cell death in LNCaP and PC3 cells. In accordance with our observation, inhibition of autophagy by ATG5 shRNA or chemical inhibitors results in tumor cell death by apoptosis in vitro and in animal models (42, 43). Metformin induces a decrease in beclin 1 expression, a protein required for autophagy (44). Beclin 1, a tumor suppressor gene, overexpression reduces the malignant phenotype and the ability for anchorage-independent growth in MCF-7 breast cancer cells (23). This role is in contradiction with the effect of metformin, which reduces beclin 1 expression and triggers apoptosis in combination with 2DG. Thus, we suggest that beclin 1 has a survival function by blocking the onset of the apoptotic cascade and metformin is the trigger of apoptosis in cells engaged in the survival response induced by 2DG. Similar conclusion has been made in breast cancer cells in response to DNA damage after downregulation of beclin 1 expression with shRNA (45). Metformin as an activator of AMPK and an inhibitor of mTOR should potentially induce autophagy. However, only
Figure 6. Metformin inhibits 2DG induced autophagy. A, electron microscopy of LNCaP cells treated with 20 or 1 mmol/L 2DG for 24 h. Arrows point to autophagosomes; Nu, nucleus. Insets, enlargement of autophagosomes. B, immunoblotting of LC3 in LNCaP cells treated for 48 h with metformin (5 mmol/L) and/or 2DG (10 mmol/L) and 50 nmol/L bafilomycin A (Baf). C, immunoblotting of LC3 and beclin 1 in LNCaP cells treated with 2DG and different concentrations of metformin or 50 nmol/L bafilomycin A. D, cells transfected with RFP-LC3 were analyzed for puncta formation (autophagosomes) 48 h after the treatment. Arrows point to autophagosomes. Transfected cells with RFP-LC3–positive pucta are quantified.
one study shows that metformin induces autophagy in cancer cells (9), suggesting that, depending on cell type, activation of AMPK is not automatically associated with the induction of autophagy. Other activators of AMPK, such as AICAR, do not induce systematically autophagy and are even reported to inhibit autophagy (46).

Our observations highlight the importance of confirming the antitumoral effect of the combination of metformin and 2DG in vivo. Indeed, the remarkable efficiency of their combination to affect cell viability by inducing apoptosis and/or blocking cell cycle may have important implications in the treatment of prostate cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


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