Inhibition of polo-like kinase 1 (Plk1) enhances the anti-neoplastic activity of metformin in prostate cancer

Chen Shao1, Nihal Ahmad2, Kurt Hodges3, Shihuan Kuang4, Tim Ratliff5, and Xiaoqi Liu1,5*

1 Department of Biochemistry, Purdue University, West Lafayette, IN 47907
2 Department of Dermatology, University of Wisconsin, Madison, WI 53706
3 Department of Pathology and Laboratory Medicine, Indiana University School of Medicine, Indianapolis, IN 46202
4 Department of Animal Sciences, Purdue University, West Lafayette, IN 47907
5 Purdue Center for Cancer Research, Purdue University, West Lafayette, IN 47907

Running title: Plk1 inhibition potentiates metformin

Keywords: Plk1, metformin, prostate cancer, p53, xenograft

*To whom correspondence should be addressed: Department of Biochemistry, Purdue University, 175 S. University Street, West Lafayette, IN 47907 Tel: 765-496-3764; Fax: 765-494-7897; Email: liu8@purdue.edu.

Disclosure of Potential Conflicts of Interest: No potential conflicts of interest are disclosed by the authors.
**Background:** Both inhibition of Plk1 and usage of metformin are reported to achieve strong anti-neoplastic functions in many cancers including prostate cancer (PCa).

**Results:** Plk1 inhibitor BI2536 synergizes with metformin in controlling PCa cell growth.

**Conclusion:** Plk1 inhibition improves the anti-neoplastic function of metformin in PCa through both signaling pathways and metabolic pathways.

**Significance:** The proposed combinational therapy shows great potential for clinical trials.

**ABSTRACT:**

The widely used anti-diabetic drug metformin has been shown to exert strong anti-neoplastic actions in numerous tumor types, including prostate cancer. In this study, we show that BI2536, a specific Plk1 inhibitor, acts synergistically with metformin in inhibiting prostate cancer cell proliferation. Furthermore, we also provide evidence that Plk1 inhibition renders prostate cancer cells carrying wild type p53 to be much more sensitive to low dose metformin treatment. Mechanistically, we found that co-treatment with BI2536 and metformin induces p53-dependent apoptosis and further activates the p53/Redd-1 pathway. Moreover, we also show that BI2536 treatment inhibited metformin-induced glycolysis and glutamine anaplerosis, both of which are survival responses of cells against mitochondrial poisons. Finally, we confirmed the cell-based observations using both cultured cell-derived and patient-derived xenograft studies. Collectively, our findings support another promising therapeutic strategy by combining two well-tolerated drugs against prostate cancer proliferation and the progression of androgen-dependent prostate cancer to castration-resistant stage.

**INTRODUCTION:**

Prostate cancer (PCa), the most common form of malignancy in men, causes the second leading cancer-related death of males in the United States (1). Because PCa cells require androgen for proliferation and development, androgen deprivation (castration) is an effective treatment for patients with late stage PCa. However, although nearly 80% of the patients initially respond to castration therapy well, castration-resistant prostate cancer (CRPC) eventually occurs in most of these patients after several years and then progresses to metastatic diseases (2-4). With very limited methods to treat advanced PCa, novel drugs with new cellular targets are urgently needed.

Metformin is a widely used anti-diabetic drug to almost 120 million people to treat type 2 diabetes. It alleviates hyperglycemia by lowering hepatic glucose production and increasing glucose uptake by peripheral tissues (5). Recently, increasing evidence suggests that metformin also decreases viability of various cancer cells and inhibits xenograft tumor growth in nude mice (6-10). Of note, metformin usage was also shown to reduce the development of CRPC by one clinical trial, in which 3000 CRPC patients were analyzed (11). More importantly, the drug is inexpensive, well tolerated and actively participates in metabolism, which make it quite attractive in cancer therapeutics (12,13). However, there are still many issues need to be addressed before metformin can be widely used in cancer treatments. First, the detailed anti-neoplastic mechanisms behind this drug remain to be elucidated. Previously, the fundamental effect of metformin in cancer treatment was believed to be due to its activation of AMP-activated protein kinase (AMPK) and the subsequent inhibition of both cell cycle progression and mTORC1 (mammalian target of rapamycin complex I), a critical regulator of protein synthesis and cell proliferation (14-17). However, metformin was also shown to achieve the same effect via the p53/Redd-1 pathway.
independent of AMPK in PCa cells (6,18). Furthermore, metformin inhibits mTORC1 in a Rag GTPase-dependent manner (19). More recently, another group reported that the inhibition of mTOR1 by metformin was due to the enhanced binding between PRAS40 and Raptor, both of which are components of the mTORC1, thus independent of AMPK (20). The second concern is that the intake of metformin into the cell requires the expression of organic cation transporter 1 (OCT1) (21,22), and the concentrations of metformin used in the current in vitro or preclinical anti-proliferative studies were much higher than the recommended therapeutic dosage in humans (23). In other words, the positive results in animal studies cannot indicate the success in clinical trials if we are not using a similar dose level of metformin. Thus, how to increase the efficacy of this drug to avoid the high dosage-induced side effects and efficiently deliver it to desired organs should be the major tasks to make metformin a real candidate for cancer therapy.

Polo-like kinase 1 (Plk1) is an essential serine-threonine kinase involved in many mitotic events, such as mitotic entry, bipolar spindle formation, and sister chromatid segregation (24). Plk1 is over-expressed in many types of human cancers and Plk1 inhibitors have been preclinically evaluated as potential drugs for cancer treatment (25). BI2536, the first Plk1 inhibitor to enter clinical trials, has already been studied in phase I and II, and it is well tolerated in humans regardless of the limited therapeutic effects in some types of tumors (26-28). Of note, it was recently reported that Plk1 is up-regulated in androgen-insensitive PCa cells and its inhibition leads to necroptosis (29). In addition, Plk1 not only promotes androgen receptor signaling (30), but also acts as a negative regulator of tumor suppressor p53 (31,32), which is crucial for mediating metformin treatment of PCa (18).

In this report, we investigated a new therapeutic strategy against PCa using combinatorial treatment of metformin and Plk1 inhibitor BI2536. In addition to both in vitro and in vivo observations, we also provide possible mechanisms for the synergy in signaling pathways and metabolic pathways.

EXPERIMENTAL PROCEDURES

Chemicals—BI2536 was purchased from Symansis NZ Ltd, New Zealand and dissolved in DMSO as working solution. Metformin was purchased from Sigma (D150959) and dissolved in distilled water.

Cell Culture, virus infection and RNAi—LNCaP, C4-2, DU145, PC3, 293A and RWPE-1 cells were purchased from ATCC and cultured in 37°C with 5% CO2. LNCaP and C4-2 cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 100 units/ml penicillin and 100 units/ml streptomycin. DU145, PC3 and HEK293A cells were cultured in Dulbecco’s Modified Eagle’s Medium. RWPE-1 cells were cultured in keratinocyte serum free medium (Gibco). Lentivirus constructs were generated and virus infections were performed as described previously (33). Adenovirus was generated using the pAdEasy XL adenoviral vector system (Stratagene, La Jolla, CA) based on manufacturer’s instructions. Then the virus was amplified using the following procedures: HEK293A cells were infected with adenovirus for 3 days, harvested, re-suspended in 1 ml sterile PBS, and lysed by four rounds of freeze/thaw using a dry ice-methanol bath and a 37°C water bath. The supernatant was collected after centrifugation at 12000g for 10 minutes as virus stocks and stored in -80°C. p53 shRNA construct was transfected into the cells with Lipofectamine 2000 reagent (Invitrogen). Puromycin (Clontech) was used to select single positive clones after transfection using the method described before (33). After 2 months’ selection,
monoclonals were picked up and p53-deleted stable cell lines were generated.

**Western blotting**—After cells were lysed in TBSN buffer (20mM Tris, pH 8.0, 150mM NaCl, 1.5mM EDTA, 5mM EGTA, 0.5% Nondiet P-40, 0.5mM Na$_3$VO$_4$) complemented with protease inhibitors (Sigma), Western blotting were performed with antibodies against cleaved-PARP (AB3565; Millipore), total PARP (9542P;Cell Signaling), pAKT (4060;Cell Signaling), AKT (9272; Cell Signaling), pS6(4858; Cell Signaling), S6(2217; Cell Signaling), Plk1 (sc-17783; Santa Cruz), β-actin (A5441; Sigma), α-tubulin (T6199; Sigma), Erk2 (sc-154; Santa Cruz), Redd-1 (10638-1-AP; Proteintech), p53 (sc-126; Santa Cruz) and PKM2 (3198; Cell Signaling).

**Cell viability assay**—Cells were grown in 96-well plates, and viable cell numbers were determined by assaying conversion of MTT to formazan. The IC50 values were obtained from the average viability curves generated by four independent measurements of each condition. The combination index of BI2536 and metformin was measured with the following equation: c.i. = (Am)$_{50}$/ (As)$_{50}$ + (Bm)$_{50}$/ (Bs)$_{50}$, where (Am)$_{50}$ is the IC50 of metformin in the combination with half of the concentration of BI2536 IC50, (As)$_{50}$ is the concentration of metformin that will produce the identical level of effect alone, (Bm)$_{50}$ is the IC50 of BI2536 in the combination with half of the concentration of metformin IC50 and (Bs)$_{50}$ is the IC50 of BI2536 after single administration. Antagonism is indicated when c.i.>1, c.i.=1 indicates an additive effect and a c.i.<1 indicates synergy.

**Glucose/Lactate assays**—Glucose consumption and lactate production were calculated by measuring the remaining glucose and lactate in the cell culture medium using Eton Biosciences glucose and L-lactate assay kits following the procedures described by the manufacturer.

**Intracellular ATP measurement**—Control and treated cells were counted and then harvested for testing intracellular ATP level following the procedure described by the manufacturer (Roche).

**α-ketoglutarate assay**—α-ketoglutarate level in cultured cells was measured using BioVision α-ketoglutarate colorimetric assay kit following the procedure described by the manufacturer.

**Colony formation assay**—500-1000 Cells were seeded in 6-well plates and cultured with medium or medium containing different drugs for 20 days with medium refreshment every two days. After culturing, cells were fixed using 10% formalin and stained with 0.5% crystal violet, then colony numbers were counted.

**FACS analysis**—Cells were harvested by trypsination, fixed in 75% ethanol, stained with propidium iodide solution at a final concentration of 50 μg/ml, and then subjected to fluorescence-activated cell sorting (FACS) analysis.

**Mouse xenograft model**—LNCaP cells (5 X 10$^6$ cells per mouse) were mixed with an equal volume of Matrigel (Collaborative Biomedical Products) and inoculated into the right flank of 24 athymic nude mice (Harlan Laboratories). One week later, the mice were randomly separated into 4 groups (6 mice in each group). At day 17 after implantation, castration was performed with all the 24 mice. Metformin was dissolved in distilled water and given to the mice using gavage. BI2536 was dissolved in 0.1 N HCl, diluted with 0.9% NaCl, and injected into the tail vein. Both drugs were injected twice a week for 10 weeks during which body weights and tumor volumes were followed. Tumor volumes were estimated using the formula: $V=L \times W^2/2 (V, \text{mm}^3; L, \text{mm}; W, \text{mm})$. The length and width were measured using digital calipers twice a week.

**Patient-derived xenograft model**—Mice carrying LuCaP35CR tumors were obtained from Dr. Robert Vessella at University of Washington.
(34). Tumors were amplified by cutting the original tumors into about 20~30 mm$^3$ pieces and then implanted into pre-castrated nude mice. After amplifying enough tumors, tumors were harvested and cut into about 20~30 mm$^3$ pieces before implanting into 16 pre-castrated nude mice. When tumors reached 250 to 300 mm$^3$, mice were randomly separated into 4 groups (4 mice in each group) for different treatments.

**Serum PSA measurement**—Blood was collected from mice by retro-orbital bleeding once a week for determining the serum PSA level. PSA level was measured using PSA (Human) ELISA kit (KA0208; Abnova) following the procedure described by the manufacturer.

**Tumor Western blotting**—Tumors were harvested and frozen in -80°C before conducting further analysis. For Western blotting, tumors were melted on ice and homogenized using RIPA buffer (150 mM NaCl, 1 mM PMSF, 1 mM EDTA, 1% Triton X100, 0.1% SDS) complemented with protease inhibitors (Sigma). Then proteins were detected using specific antibodies following the procedure described above.

**Statistical analysis**—Standard 2-tailed Student t tests were performed to determine the significance of differences between experimental conditions and treatments.

**RESULTS**

**Inhibition of Plk1 and metformin decrease viability and colony formation of PCa cells synergistically**—To test whether inhibition of Plk1 synergizes with metformin in inhibiting PCa cell proliferation, we conducted cell viability assays to calculate the combination indices of metformin and BI2536, a well-recognized Plk1 inhibitor, in two PCa cell lines: androgen dependent LNCaP and LNCaP-derived but androgen-independent C4-2. Combination indices of two drugs in both cell lines are about 0.4, suggesting that the two drugs act in a synergistic manner in both cell lines (Tables 1, 2).

To further confirm these initial observations, we performed extensive Western blotting analysis to follow the protein level of cleaved-PARP, a marker for apoptosis. Consistent with the cell viability assays, we found that in both LNCaP and C4-2 cells there is a significantly increased level of cleaved-PARP after the cells were treated with both metformin and BI2536 in comparison to the monotherapy (Fig. 1A-C). However, we did not observe a similar result in PC3, a p53-null PCa cell line (Fig. 1D). More importantly, we also found that these two drugs, either working alone or together, failed to induce detectable apoptosis in non-transformed prostate epithelial cell RWPE-1 (Fig. 1E). Finally, we also found that B2536 and metformin act synergistically to inhibit colony formation of LNCaP (Fig. 1F) and C4-2 cells (Fig. 1G).

**Plk1 status affects cellular response to metformin**—Because inhibition of Plk1 potentiated metformin-mediated inhibition of proliferation in LNCaP and C4-2 cells, two PCa cell lines with elevated levels of Plk1, we then asked whether overexpression of Plk1 in RWPE-1, which has an almost non-detectable level of Plk1, affects its response to metformin. Accordingly, we overexpressed Plk1 in RWPE-1 cells by infecting with adenovirus. One immediate outcome of Plk1 overexpression in RWPE-1 cells is the activation of the PI3K/AKT/mTOR cell survival pathway, as indicated by the increased phosphorylation levels of AKT and S6, two major downstream effectors of PI3K (Fig. 2A). Plk1 elevation-mediated activation of the PI3K/AKT/mTOR pathway is addressed in detail in a separate study (35). Interestingly, we observed that Plk1 overexpression renders RWPE-1 cells, which normally do not respond to even very high concentrations of metformin, to be much more sensitive to this drug (Fig. 2B and 2C). And this is not due to the cell cycle effect because Plk1 overexpression does not change the cell cycle of...
RWPE-1 cells (Fig. 2D). In contrast, we found an opposite trend in prostate cancer cell line LNCaP, i.e., Plk1-depleted LNCaP cells (Fig. 2E) were much more sensitive to metformin than control cells with a normal Plk1 level, indicated by both cleaved-PARP Western blotting (Fig. 2F) and cell viability assays (Fig. 2G). Importantly, the increased cell death is not due to the Plk1 inhibition itself as cells infected with Plk1 depletion virus did not have a significant higher apoptosis level compared with the cells infected with control virus (Fig. 2H). In addition, the protein level of Redd-1, which was induced after high concentrations of metformin treatment (18), was much lower in Plk1-overexpressed cells than in Plk1-depleted cells (Fig. 2E). Furthermore, Plk1 depletion also potentiates metformin-associated cell death in C4-2 cells (Fig. 2I). Consistent with the observation in Fig. 2E, we found that co-treatment with BI2536 further enhanced the level of Redd-1 induced by metformin in LNCaP cells, but not in PC3 and DU145 (Fig. 2J), both of which do not have wild type p53. Taken together, these data suggest that the roles of Plk1 in cellular responses to metformin are different between prostate cancer cells and non-transformed prostate epithelial cells. And p53 status may play an important role in the cellular response to the combination treatment of Plk1 inhibition and metformin.

Metformin and BI2536 induce a p53-dependent apoptosis—Because p53 is likely involved in mediating the synergic effect of BI2536 and metformin and because a p53-dependent synergy between metformin and 2-deoxyglucose was reported previously (36), we asked whether the p53 level was changed upon BI2536 and metformin treatment. Although we did not detect any obvious change of p53 level in LNCaP cells using the lower drug concentrations (Fig. 3A), a significant increase of p53 level of combinational treatment than single drug treatments was observed when we increased the concentrations of both metformin and BI2536 to about their IC50 values (Fig. 3B). Furthermore, the combinational treatment led to a significant increase of the p53 level in C4-2 cells even at the lower concentrations (Fig. 3C). Of note, we repeatedly noticed that C4-2 cells were much more sensitive to BI2536 than LNCaP cells during the experimentation, suggesting that Plk1 is much more critical for CRPC cell survival. Next, to further validate whether p53 is important for the combinational effect, we generated several p53-depleted LNCaP cell lines (Fig. 3D) and tested their sensitivities to different drug treatments. Comparing with WT LNCaP cells, p53-depleted LNCaP cells showed similar sensitivities to either metformin or BI2536 alone, but much less sensitive to combinational treatment, as indicated by cell viability after drug treatments (Fig. 3E). Consistent with this observation, we found that there was a significant decrease of the level of cleaved-PARP in p53-depleted LNCaP cells than in control cells under the same combination treatment (Fig. 3F). Furthermore, we conducted colony formation assay to confirm the results (Fig. 3G and 3H).

BI2536 hinders glycolysis and glutamine anaplerosis induced by metformin—As metformin actively participates in many important metabolic pathways, we investigated the changes of cellular metabolism after adding Plk1 inhibitor together with metformin. Similar to previous reports on the effect of metformin on glycolysis, we found that metformin treatment significantly up-regulated glucose consumption and lactate production in LNCaP cells (Fig. 4A), whereas addition of BI2536 down-regulated metformin-induced glycolysis, indicated by the decrease of glucose consumption and lactate production (Fig. 4B). Mechanistically, metformin treatment increased the level of pyruvate kinase M2 (PKM2), a rate-limiting factor of glycolysis, in LNCaP cells, but addition of
BI2536 antagonized such an effect (Fig. 4C). Recently, glutamine anaplerosis was found to be another important cell survival pathway after metformin treatment (37). Thus, we asked whether Plk1 inhibition has any effects on glutamine metabolism as well. Upon a high concentration of metformin (2.5mM) treatment, the majority of cellular α-ketoglutarate is generated from glutamine, but not from the glucose metabolism in PCa cell lines (37). We investigated whether BI2536 inhibits glutamine anaplerosis by measuring cellular α-ketoglutarate level under this condition. As expected, treatment with 2.5mM metformin up-regulated glutamine anaplerosis in LNCaP cells, but adding BI2536 together significantly antagonized this effect (Fig. 4D). Taken together, these data suggest that inhibition of Plk1 negatively affects the two crucial cellular anti-stress metabolic responses induced by metformin treatment, consequently resulting in rapid cell death due to energy crisis.

**BI2536 and metformin acts synergistically in LNCaP xenograft model**—To assess the in vivo effect of metformin and BI2536 treatment, alone or in combination, we conducted LNCaP xenograft model study. In order to mimic the progression of tumors into androgen-independent stage, we performed castration to the mice after detectable tumors were formed. As indicated in Fig. 5A, 5B and 5D, neither metformin nor BI2536 treatment alone had an obvious effect in preventing the tumor growth whereas a strong inhibition of tumor growth was detected in the combinational group, supporting a synergistic effect of metformin and BI2536 in vivo. Moreover, the trend was similar to tumor volume measurements when the serum prostate specific antigen (PSA) levels of these mice were followed (Fig. 5C).

**BI2536 and metformin acts synergistically in a patient-derived xenograft model**—To better mimic the tumor growth condition and follow the situation in the relatively late CRPC stage, we conducted a patient-derived xenograft study using LuCaP35CR model, which was originally generated from a 66-year-old patient (34). Of note, this model is not indicated to harbor any p53 mutation in a whole-exome sequencing study of the LuCaP series (38). After about 40 days drug treatment, we found that metformin treatment alone nearly had no effect and that BI2536 alone showed a very limited effect in inhibiting the tumor growth. In contrast, we observed a significantly lower growth rate of the tumors treated with both metformin and BI2536 in comparison with monotherapies (Fig. 6A, 6B and 6D). Importantly, this result was also consistent with the serum PSA levels and Western blotting analysis of the harvested tumors (Fig. 6C and 6E). Thus, these experiments support the notion that BI2536 and metformin also have a strong synergy in a patient-derived xenograft model.

**DISCUSSION**

Metformin, with its high safety and remarkable functions in inhibiting growth of multiple tumor cell lines, favors physicians to consider its potential use in the treatment of cancer. However, in addition to the undefined molecular action, there are many other concerns about the dose and cellular uptake of the drug when considering the clinical applications. Thus, how to achieve the anti-neoplastic function with recommended therapeutic dosages for diabetic patients or even lower dosages for non-diabetic patients is one challenging task for physicians. Herein, we presented a novel therapeutic strategy which can dramatically increase the efficiency of metformin in inhibiting the growth of PCa by combining inhibition of Plk1.

Beyond the well identified cell-cycle related functions, Plk1 also plays important roles in many pivotal signaling pathways of cancer cells. In
addition to the previous finding that Plk1 acts as a negative regulator of p53, we also showed that Plk1 contributes to activation of the PI3K/AKT/mTOR pathway (Fig. 2A), one of the most frequently activated cell surviving pathways in numerous cancer cells (39-41). Thus, we proposed to challenge the hypothesis that Plk1 inhibition could potentially be one novel avenue to increase metformin’s efficiency against PCa.

First of all, we found that Plk1 inhibitor BI2536 and metformin showed a strong synergy in LNCaP cells and its androgen-independent derivative C4-2 cells (42), as we noticed that BI2536 strikingly increased metformin-associated lethality in these two cell lines. For LNCaP cells, we can even use about 5 µM to induce apoptosis and prevent colony formation when combing with a low concentration of BI2536 (Fig. 1B, 1F). The other interesting observation is that C4-2 cells were much more sensitive to Plk1 inhibition (IC50 of 8nM) than LNCaP cells (IC50 of 90nM) even they share the same genetic background (Table 2, Fig. 1C, 1G), suggesting that Plk1 plays more and more important roles as PCa cells progress to castration-resistant stage. In contrast, normal prostate cells were quite resistant to these two drugs (Fig. 1E and 2C), thus offering selectivity for this proposed therapeutic strategy against cancer cells. Consistent with these observations, Plk1 depletion sensitized LNCaP cells to low concentrations of metformin (Fig. 2F and 2G). Furthermore, we observed that overexpression of Plk1 in RWPE-1 cells somehow made these non-transformed cells more sensitive to metformin (Fig. 2B and 2C). Consistent with our previous findings that Plk1 negatively regulates p53, we found that p53 was indeed down-regulated when we overexpressed Plk1 (Fig. 2B, bottom panel). However, a significant induction of p53 was observed in the Plk1-overexpressing RWPE-1 cells upon metformin treatment (Fig. 2B, top panel). One possible explanation is that Plk1 overexpression changes the cellular signaling network, such as the PI3K/AKT/mTOR pathway, in normal prostate epithelial cells, somehow makes them behave like cancer cells, which are more sensitive to metformin, and that p53 induction correlates with the onset of apoptosis.

Although mitochondrial inhibition acts as the premise for metformin’s downstream functions is well-accepted, the notion that the drug exerts its anti-tumor functions through the AMPK pathway is plausible as alternative pathways are supported by increasing evidences. Particularly in PCa, activation of the p53/Redd-1 axis was found to be crucial in inhibition of both cell cycle progression and mTOR pathway (18). Even though the exact role of p53 in metformin treatment is still under debate as opposite results were observed in colon cancer (43), we don’t feel that these two observations actually counteracted with each other. Because colon cancer cells without p53 cannot efficiently make metabolic alternations in response to the mitochondrial stress induced by metformin, they are more sensitive to metformin than colon cancer cells with WT p53 (43). However, for the p53/Redd-1 axis in PCa cells carrying WT P53, positive regulation of p53 will lead to an enhanced repression of cell proliferation. Indeed, after LNCaP and C4-2 cells were co-treated with BI2536 and metformin, the p53 and Redd-1 levels are both up-regulated (Fig. 2J, Fig. 3B and 3C). Since Redd-1 was found to be a potent tumor suppressor downstream of p53 (44-46), we propose that the up-regulated p53/Redd-1 pathway could be one of the explanations for the observed synergy in PCa. Consistently, we found that p53 knockdown makes LNCaP cells significantly less sensitive to the combination treatment of BI2536 and metformin although we haven’t figured out the exact molecular mechanism (Fig. 3E-3G). Interestingly, we also found that BI2536 treatment significantly inhibited metformin-induced glycolysis and glutamine anaplerosis (Fig. 4B-4D), two important cell survival responses upon
mitochondrial stress (37,47). Similar to our findings, an inhibitor of glycolysis strongly synergizes with metformin in PCa cells (36). But the difference is that in comparison with metformin treatment alone, we did not observe a sharp decrease of ATP level when combining metformin and BI2536 together (data not shown). In short, we propose that inhibition of Plk1 promotes the cytotoxicity of metformin to PCa cells through both signaling pathways and metabolic pathways.

Previously, the lowest dose of metformin used in mouse xenograft studies was 40 mg/kg per day (23) and there was a detectable reduction of tumor growth in LNCaP xenograft model using 1 mg/day metformin treatment (6). To determine whether we could significantly reduce the dose of metformin when combining BI2536 treatment together, we injected 5 mg/kg metformin for just two times per week, which equals to about 0.03 mg/day. Of note, the dose of BI2536 was also significantly lower than the previously reported dosage in PCa xenograft study (48). Upon castration, all the tumors of LNCaP xenograft study were growing much slower and neither of these two single treatments had obvious effect in tumor repression compared with the control group. In contrast, the tumors treated with both drugs nearly stopped growing anymore upon castration (Fig. 5B), strongly suggesting that these two drugs act together in preventing the progression from androgen sensitive to androgen-independent stage. LuCaP35CR is the androgen-independent form of LuCaP35, a new patient-derived xenograft tumor model for studying the progression to androgen independence (34). For our purpose, LuCaP35CR could be an excellent model to mimic the relatively late stage of PCa. Because these tumors grow much faster than tumors generated from LNCaP cells, we increased the doses of metformin and BI2536 to 30 mg/kg and 15 mg/kg (2 injections per week), respectively. Similar to the LNCaP xenograft study, we did not detect any obvious change in single drug treatment as both of the doses were still significantly lower than what has been generally used in mouse studies. In striking contrast, the mice treated with both drugs not only had smaller tumors and lower serum PSA levels (Fig. 6B and 6C), but also showed reduced angiogenesis potentials as the tumors became transparent (Fig. 6A).

In summary, our in vitro and in vivo data support a strong synergy of inhibition of Plk1 and metformin in inhibiting PCa cell proliferation. In addition, we are the first to provide evidence that metformin can still achieve its anti-neoplastic function at doses significantly lower than those used in others’ studies when comibing with BI2536. Thus, the combinational strategy can be considered for clinical trials to increase the efficiency of metformin in PCa.
References


**FOOTNOTES**

This work was supported by NIH grant R01CA157429 (X.L.), NIH grant R01AR059130 (N.A.), NSF grant MCB-1049693 (X.L.) and ACS grant RSG-13-073 (X.L.). C. Shao was financially supported by China Scholarship Council (CSC). Xenograft data were acquired by a Purdue Center for Cancer Research facility supported by P30 CA023168.

**ABBREVIATIONS**

PLK1, polo-like kinase 1; PCa, prostate cancer; CRPC, castration-resistant prostate cancer; AMPK, AMP-activated protein kinase; mTOR1, mammalian target of rapamycin complex I; PARP, Poly (ADP-ribose) polymerase; PKM2, pyruvate kinase M2; PI3K, phosphatidylinositol 3-kinase
Figure legends

FIGURE 1. **BI2536 and metformin inhibit PCa cell proliferation in a synergistic manner.** A-E, inhibition of Plk1 potentiates the lethality of metformin in p53 WT PCa cells, but not in PC-3 and non-transformed prostate cells. LNCaP (A, B), C4-2 (C), PC3 (D) or RWPE-1 (E) cells were treated with metformin, BI2536 (BI) or both at the indicated concentrations for the indicated time, and harvested for immunoblotting (IB) with antibodies against cleaved form of poly(ADP-ribose) polymerase (c-PARP), a marker for apoptosis. In E, 50uM sodium arsenite (As) was used as a positive control for cell death. F, BI2536 and metformin inhibit colony formation synergistically in LNCaP cells. LNCaP cells \(1 \times 10^3\) were seeded on soft agar in 6-well plates, treated with 5uM metformin, 10nM BI2536 or both for 3 weeks, and stained with 0.005% crystal violet. The bottom panel is quantification results. *p<0.05, **p<0.01. G, BI2536 and metformin inhibit colony formation synergistically in C4-2 cells. C4-2 cells \(1 \times 10^3\) were seeded on soft agar in 6-well plates, treated with 0.5mM metformin, 1nM BI2536 or both for 3 weeks, and stained with 0.005% crystal violet. The bottom panel is quantification results. *p<0.05, **p<0.01.

FIGURE 2. **Plk1 status affects the cellular response to metformin.** A, RWPE-1 cells were infected with adenovirus expressing GFP or GFP-Plk1 for 2 days and harvested for IB with indicated antibodies. B, RWPE-1 cells expressing GFP or GFP-Plk1 were treated with 0.5mM metformin for 24h (top panel) or directly harvested for IB (bottom panel). C, RWPE-1 cells were infected with lentivirus (empty vector or Plk1 overexpression) for 1 day, harvested, and reseeded in 96-well plates under different concentrations of metformin for 3 days, followed by cell viability measurement. D, RWPE-1 cells were infected with lentivirus for 3 days and harvested for FACS analysis. E, LNCaP cells were infected with lentivirus (empty vector, Plk1 overexpression or Plk1 depletion) for 3 days, treated with 5mM metformin for 4h, and
harvested. F, LNCaP cells were infected with lentivirus for 2 days and treated with 0.5mM metformin for 24h. G, LNCaP cells were infected with lentivirus for 1 day, harvested, and reseeded in 96-well plates under different concentrations of metformin for 3 days, followed by cell viability assay. H, 5 x10^5 LNCaP cells were seeded in 6-well plates and infected with lentivirus for 3 days and then cell viability was measured by counting the number of viable cells. I, C4-2 cells were infected with lentivirus for 2 days and treated with 0.5mM metformin for 24h. J, LNCaP, DU145 and PC3 cells were treated with indicated drugs for 4h.

FIGURE 3. Inhibition of Plk1 and metformin induce p53-dependent apoptosis. A & B, LNCaP cells were treated with lower (A) or higher (B) concentrations of drugs for 24h, and harvested for IB. Doxorubicin (Dox) was used as a positive control to induce elevation of p53 level in B. C, C4-2 cells were treated with indicated drugs. D, IB of WT and p53-depleted LNCaP cell clone. E, 5X10^5 LNCaP cells (WT or p53-depleted) were seeded in 6-well plates, treated with 0.5mM metformin, 10nM BI or both for 24h, and harvested for viability assay. *p<0.05. F, LNCaP cells (WT or p53-depleted) were treated with drugs indicated and subjected to IB. G, LNCaP cells (WT and p53-depleted) (1x10^3) were seeded on soft agar in 6-well plates, treated with 0.5mM metformin, 10nM BI2536 or both for 3 weeks, and stained with 0.005% crystal violet. H, Quantification of the colony numbers. The results were normalized to control group of each cell line, *p<0.05.

FIGURE 4. Metformin and BI2536 alter glucose and glutamine metabolism. A, LNCaP cells were treated with metformin for 24h. After cell numbers were counted, the media were collected to measure glucose consumption and lactate production upon normalizing the cell numbers. **p<0.01. B, LNCaP cells were treated with 0.5mM metformin, 10nM BI2536 or both for 24h. After cell numbers were counted, the media were collected to measure glucose consumption and lactate production. *p<0.05, ***p<0.001. C,
LNCaP cells were treated with 0.5mM metformin ± 10nm BI2536 for 24h. D, LNCaP cells were treated with drugs indicated for 24h and harvested to measure α-ketoglutarate levels.

FIGURE 5. **BI2536 and metformin inhibit growth of LNCaP-derived tumors synergistically.** LNCaP cells (5x10⁶) were subcutaneously inoculated into the flanks of Nu/Nu nude mice to form tumors. Mice were castrated at day 17, intravenously injected twice per week with BI2536 (5 mg/kg), given metformin (5 mg/kg) using gavage or both, and followed for 75 days. A, images of the mice at the end of the study. B, tumor growth curves of the study. C, inhibition of PSA level by BI2536 and metformin. Blood was collected beginning from day 30 (once a week) and serum PSA level was measured using the PSA ELISA kit for 6 weeks. D, tumor weight measurement upon sacrifice.

FIGURE 6. **BI2536 and metformin inhibit LuCaP35CR xenografts synergistically.** LuCaP35CR tumors were inoculated into nude mice, which had been castrated 2 weeks ahead. After tumors reached the size of 250 to 300mm³, mice were intravenously injected twice per week with BI2536 (30 mg/kg), given metformin (15 mg/kg) using gavage or both, and followed for additional 43 days. A, images of the mice at the end of the study. B, tumor growth curves of the study. C, serum PSA level of the mice. D, tumor weight measurement upon sacrifice. E, IB of the harvested tumor samples.
**Tables**

Table 1: The IC50 values of BI2536 and metformin in LNCaP cell

<table>
<thead>
<tr>
<th>Drugs</th>
<th>IC50</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>BI2536</td>
<td>91.3nM</td>
<td></td>
</tr>
<tr>
<td>metformin</td>
<td>5mM</td>
<td></td>
</tr>
<tr>
<td>BI2536 (in combination with 2.5mM metformin)</td>
<td>35nM</td>
<td>Combination Index=0.384</td>
</tr>
<tr>
<td>metformin (in combination with 50nM BI2536)</td>
<td>5.5uM</td>
<td></td>
</tr>
</tbody>
</table>
Table 2: The IC50 values of BI2536 and metformin in C4-2

<table>
<thead>
<tr>
<th>Drugs</th>
<th>IC50</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>BI2536</td>
<td>8nM</td>
<td></td>
</tr>
<tr>
<td>metformin</td>
<td>5mM</td>
<td></td>
</tr>
<tr>
<td>BI2536 (in combination with 2.5mM metformin)</td>
<td>2.2nM</td>
<td>Combination Index=0.374</td>
</tr>
<tr>
<td>metformin (in combination with 4nM BI2536)</td>
<td>495 uM</td>
<td></td>
</tr>
</tbody>
</table>
Figure 2

A

RWPE-1

adenovirus

CRTL GFP GFP-Plk1

GFP-Plk1

GFP

AKT

p-AKT-S473

S6

p-S6-S235/6

Erk2

B

RWPE-1 + Metformin

adenovirus

CRTL GFP GFP-Plk1

C

RWPE-1

lv-ctrl lv-Plk1

Plk1

Actin

p53

Actin

D

RWPE-1

Ctrl Plk1

2n 4n 2n 4n

E

LNCaP

lv-ctrl lv-Plk1

Plk1

Redd-1

Actin

F

LNCaP + Metformin

ctrl lv-ctrl lv-Plk1

C-PARP

Actin

G

LNCaP

Viability (%)

0 0.1 1 10 100 1000 10000

Metformin (µM)

H

LNCaP (p53 WT) PC3 (p53-null) DU145 (p53 mutant)

C4-2

lv-ctrl lv-Plk1

Plk1

Actin

p53

C-PARP

Actin

J

LNCaP (p53 WT) DU145 (p53 mutant) PC3 (p53-null)

CTRL 5mM Met 5mM Met + 50nM BI

Redd-1

Tubulin
Figure 4

A

B

C

D

**p=0.014**

Rel. glucose consumption

Metformin (mM)

0 1 5

**p=0.014**

Rel. lactate production

Metformin (mM)

0 1 5

ctrl Met BI Combo

ctrl Met BI Combo

ctrl Met (2.5mM) (30nM)

ctrl Met BI Combo

ctrl Met (2.5mM) (30nM)

*α-ketoglutarate*

Actin

P

PKM2

Metformin (mM)

2.5 2.0 1.5 1.0 0.5 0.0

Rel. glucose consumption

0 1 5

*α-ketoglutarate*

Actin

P

PKM2

Metformin (mM)

2.5 2.0 1.5 1.0 0.5 0.0

Rel. lactate production

0 1 5

ctrl Met BI Combo

ctrl Met (2.5mM) (30nM)

ctrl Met BI Combo

ctrl Met (2.5mM) (30nM)

*α-ketoglutarate*

Actin

P

PKM2

Metformin (mM)

2.5 2.0 1.5 1.0 0.5 0.0

Rel. lactate production

0 1 5

ctrl Met BI Combo

ctrl Met (2.5mM) (30nM)

ctrl Met BI Combo

ctrl Met (2.5mM) (30nM)

*α-ketoglutarate*

Actin

P

PKM2

Metformin (mM)

2.5 2.0 1.5 1.0 0.5 0.0

Rel. lactate production

0 1 5

ctrl Met BI Combo

ctrl Met (2.5mM) (30nM)

ctrl Met BI Combo

ctrl Met (2.5mM) (30nM)

*α-ketoglutarate*

Actin

P

PKM2

Metformin (mM)

2.5 2.0 1.5 1.0 0.5 0.0

Rel. lactate production

0 1 5

ctrl Met BI Combo

ctrl Met (2.5mM) (30nM)

ctrl Met BI Combo

ctrl Met (2.5mM) (30nM)
Figure 5

A

Control  metformin

BI2536  metformin+BI2536

B

Tumor size (mm$^3$)

0 10 20 30 40 50 60 70 80

0 400 800

Castration  Time (d)

B

Serum PSA (ng/ml)

1 2 3 4 5 6

0 100 200 300 400

Weeks

C

Serum PSA (ng/ml)

0 50 100 150 200 250 300 350

CTRL  Metformin  BI2536  Metformin + BI2536

Weeks

D

Tumor weight (mg)

ctrl  Met  BI  Combo

1000 800 600 400 200 0


Figure 6

A) Control, metformin, BI2536, metformin+BI2536

B) Tumor size (mm³) vs. Time (d)

C) Serum PSA (ng/ml) vs. Weeks

D) Tumor weight (mg)

E) Western Blot analysis for c-PARP, Cyclin D1, and Actin
Signal Transduction: Inhibition of Polo-like Kinase 1 (Plk1) Enhances the Anti-neoplastic Activity of Metformin in Prostate Cancer

Chen Shao, Nihal Ahmad, Kurt Hodges, Shihuan Kuang, Tim Ratliff and Xiaoqi Liu
J. Biol. Chem. published online December 10, 2014

Access the most updated version of this article at doi: 10.1074/jbc.M114.5966817

Find articles, minireviews, Reflections and Classics on similar topics on the JBC Affinity Sites.

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/early/2014/12/10/jbc.M114.5966817.full.html#ref-list-1