# RESEARCH ARTICLE

# Metformin Inhibits Growth of Hepatocellular Carcinoma Cells by Inducing Apoptosis Via Mitochondrion-mediated Pathway

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## Abstract

Recently, population-based studies of type 2 diabetes patients have provided evidence that metformin treatment is associated with a reduced cancer incidence and mortality, but its mode of action remains unclear. Here we report effects of metformin on hepatocellular carcinoma (HCC) Hep-G2 cells and details of molecular mechanisms of metformin activity. Our research indicates that metformin displays anticancer activity against HCC through inhibition of the mTOR translational pathway in an AMPK-independent manner, leading to G1 arrest in the cell-cycle and subsequent cell apoptosis through the mitochondrion-dependent pathway. Furthermore, we showed that metformin strongly attenuated colony formation and dramatically inhibited Hep-G2 tumor growth in vivo. In conclusion, our studies suggested that metformin might have potential as a cytotoxic drug in the prevention and treatment of HCC.

**Keywords:** Metformin - HCC - mTOR - apoptosis - signaling pathway

## Introduction

HCC is the fifth most common cancer with poor prognosis, and is the fourth leading cause of cancer death worldwide (Avila et al., 2006). The incidence of HCC is rising all over the world due to the increasing opportunities of hepatitis B infection (Daniels et al., 2009). Surgery remains the first choice for HCC treatment. However, only a small proportion of patients are suited to surgical resection. Chemotherapy is one of the palliative approaches for unresectable tumors, but the efficacy of current HCC chemotherapy is only modest and HCC remains an unmet medical need.

Metformin, a biguanide, is the most widely used drug for a long time in the treatment of typeII diabetes (Kahn et al., 2005). It reduces the level of glucose by decreasing liver glucose production, increasing fatty acid oxidation and glucose utilization. A surprising finding in recent studies was that metformin may reduce cancer risk or improve cancer prognosis. For example, In a case–control study, a dose–response relationship between higher metformin use, duration of use, number of prescriptions, and amount dispensed and lower rates of cancer was identified. Research showed that metformin usage decreased the risk of cancer (Evans et al., 2005; Lee et al., 2011; Zhu et al., 2011). Compared with patients treated with metformin, patients with type II diabetes exposed to sulfonylureas had a significantly increased risk of cancer mortality (Bowker et al., 2006; Currie et al., 2009). A study among new metformin users with type

II diabetes over a 10-year period indicated a 37% lower incidence of cancer (Duncan et al., 2009; Libby et al., 2009). The protective effect of metformin has been found in pancreatic and prostate cancer as well (Li et al., 2009; Wright et al., 2009). Furthermore, There have been series reports of anticancer activity of metformin in experimental models. For example, low dose of metformin, inhibits cellular transformation and selectively kills cancer stem cells in breast cancer. Besides, the combination of metformin and doxorubicin, kills both cancer stem cells and non–stem cancer cells in vitro and in vivo (Hirsch et al., 2009). In endometrial cancer cell lines, metformin inhibits cell proliferation through AMPK activation and subsequent inhibition of the mTOR pathway (Cantrell et al., 2010). The role of metformin in glucose and fatty acid metabolism is well known. However, there has been little work evaluating the potential utility of metformin in vivo models of cancer and how metformin induces apoptosis remains unknown. Here, we describe experiments carried out to investigate the hypothesis that metformin exhibits direct antiproliferative actions on HCC cells both in vitro and in vivo.

## Materials and Methods

### Chemicals and reagents

Chemicals, cell-culture materials were obtained from Invitrogen (Burlington, ON, Canada). Anti-phospho-AMPKα (Thr172), anti-AMPKa, anti-phospho-p70S6K (Thr389), anti-p70S6K, anti-phospho-mTOR

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(Ser2448), anti-mTOR, anti-ß-actin, anti-bcl-2, anti-bax, anti-caspase-3 were purchased from Cell Signaling Technology(Beverly,MA,USA). Horseradish eroxidase-conjugated anti-rabbit IgG, anti-mouse IgG, and enhanced chemiluminescene (ECL) reagents were from Pharmacia-Amersham (Santa Cruz,USA). Metformin (1, 1-Dimethylbiguanide hydrchloride) and compound C (the AMPK inhibitor) were obtained from Sigma (St.Louis, MO, USA).

### Cell lines and culture conditions

The human HCC cell line MHCC97-H cells were obtained from liver cancer institute of Fudan university in Shanghai of China. All the other cell lines were purchased from Shanghai institute of Cell Biology, Chinese Academy of Sciences. Cells were cultured in DMEM, supplemented with 10% fetal bovine serum (FBS) and 100 units/ml gentamycin at 37 °C and 5% $CO_2$. Cells were passaged by 0.25% Trypsin-EDTA when they reached 80% confluence.

### Cell proliferation assay

The effect of metformin on HCC cell lines was evaluated by MTT assay. Briefly, 7000 cells were seeded into 96-well tissue culture plates and treated with different doses of metformin for 24 h, 48 h, 72 h respectively. MTT reagent was then added to each well, and the cells were further incubated for 4 h, absorbance was measured in an automated microplate reader at 450 nm.

### Cell cycle analysis

Hep-G2 cells were serum starved for 24 h and then treated with metformin with different doses for 48 h. The cells were then washed with PBS (pH7.4) and fixed with 70% ice-cold ethanol at 4 °C overnight. After fixation, the cells were stained with PI at 1 mg/ml for 30 min at room temperature. The stained cells were analyzed by flow cytometry.

### Detection of apoptosis

Briefly, Hep-G2 cells were treated with metformin in different doses in complete medium for 48 h. The cells were harvested and rinsed twice with PBS (pH7.4) at 4 °C. A total of $1 \times 10^5$ cells were stained with Annexin V and PI for 30 min in dark. The stained apoptotic cells were counted by flow cytometry.

### Mitochondrial and cytosolic fractionation

Isolation of mitochondria and cytosol was performed using the Cell Mitochondria Isolation Kit (Beyotime Institute of Biotechnology, China) according to the standard procedure. Samples of cytosol and mitochondria were dissolved in lyses buffer and proteins were subjected to Western blotting respectively.

### Western blot analysis

Hep-G2 cells were grown in 100 mm tissue culture dishes with metformin at different concentrations. Cells were lysed in a radioimmunoprecipitation assay buffer (RIPA) (9.1 mM dibasic sodium phosphate, 1.7 mM monobasic sodium phosphate, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 0.2 mM sodium vanadate, 0.2 mM phenylmethylsulfonyl fluoride, and aprotinin at 0.2 U/ml). Clarified protein lysates (50 g) were resolved electrophoretically on denaturing SDS-polyacrylamide gels (8–10%), and transferred to nitrocellulose membranes. The membranes were then blocked with 1% bovine serum albumin at room temperature for 1 h and then incubated with the indicated specific primary antibodies for 3 h, followed by incubation with the respective alkaline phosphatase-conjugated secondary antibodies for 1 h. Finally, Antigen–antibody complexes were detected using the ELC system.

### Soft-agar growth

Hep-G2 cells ($1 \times 10^3$ cells/dish) were plated in 35-mm-diameter dishes with a top layer of 0.3% agar and a bottom layer of 0.6% agar in medium. 0.3 ml of medium was supplemented every three days and the dishes were examined under microscope. After 20 days, cell clusters were photographed.

### Hep-G2 tumor xenograft

Hep-G2 cells ($5 \times 10^6$) were injected into the right flank of 30 BALB/c nude mice (supplied by Experimental Ainimal Department of Xiamen University, Fujian, China). Seven days later, 25 mice with size of ~100mm3 were randomly distributed into five groups, i.e. control group (PBS), low-dose metformin (40 mg/kg/day) group, high-dose metformin(200 mg/kg/day) group, cisplatin (5 mg/kg/day) group and metformin (40 mg/kg/day) + cisplatin (5 mg/kg/day) group. Tumor volume (mean values and 95% confidence intervals) was measured at various times after the initial injection. At the end of the experiment, mice were sacrificed and tumor weight was measured. All the mouse experiments were performed in accordance with Institutional Animal Care and Use Committee procedures and guidelines.

### Statistical analysis

All experiments, except the nude mice study, were repeated at least three times and the values were expressed as the mean ± SD. Statistical significance was determined by Student's t-test. P-value< 0.05 was considered statistically significant.

## Results

### Metformin inhibits the proliferation of Hep-G2 cells in a dose- and time-dependent manner

Metformin induced significant proliferation inhibition on Hep-G2 cells lines in a dose and time dependent manner (Figure 1A). Moreover, the proliferation of several other HCC cell lines were also inhibited after they were treated with metformin for 48 h (Figure 1B).

### Metformin induces apoptosis and G0/G1 phase arrest in Hep-G2 cells

The effect of Hep-G2 on cell cycle profile was analyzed by PI staining and flow cytometry analysis. Treatment of metformin for 48h showed a dose dependent increase in G0/G1 phase cells. For example, Treatment with different doses of metformin (10 mM, 20 mM) for 48h resulted in
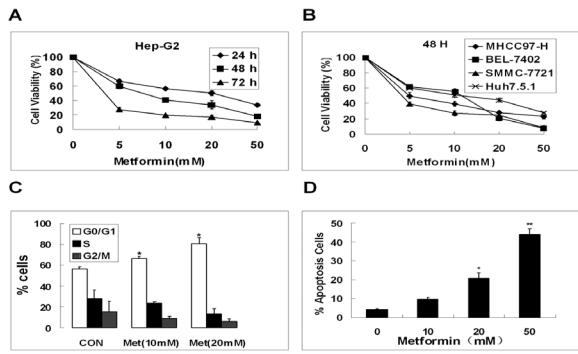
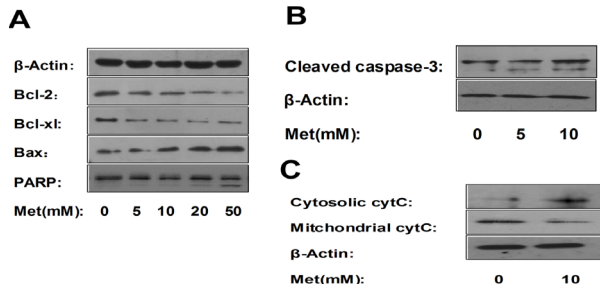**Figure 1. Metformin Inhibits the Proliferation and Induces Apoptosis of Hep-G2 Cells**



**Figure 2. Metformin Induces Apoptosis of Hep-G2 Cells Mainly Through Mitochondrion-Mediated Pathways**



**Figure 3. Metformin Inhibits the Proliferation of Hep-G2 Cells Independenly of AMPK**



**Figure 4. Metformin Inhibits Colony Formation and Decreases Hep-G2 Xenograft**

an increment of G0/G1 population in Hep-G2 cells, which were 66.4% and 80.3% respectively, compared to 56.6% of untreated cells (Figure 1C). To determine whether the cell death was due to apoptosis induced by metformin, The percentage of apoptotic cells was determined by cell flow cytometric analysis following PI staining. Compared with untreated cells, cells treated with metformin for 48h resulted in apoptosis in a dose dependent manner (Figure 1D).

*Metformin induces the apoptosis mainly through mitochondrion-mediated pathways*

The treatment of metformin decreased the expression of Bcl-2, Bcl-xl and increased the expression of Bax in a dose dependent manner in Hep-G2 cells by Western blot analysis. (Figure 2 A). In the downstream of apoptosis signaling pathways, there is a significant cleavage-activation of caspase-3, PARP. Besides, metformin induced the release of cytochrome c from mitochondria to cytosol (Figure 2C). The results confirmed our prediction that metformin induces apoptosis mainly through mitochondrion-mediated internal pathway.

*Decreased phosphorylation of mTOR and P70S6K following metformin treatment in an AMPK-independent manner*

Western Blotting was conducted to assess the effect of metformin on AMPK, mTOR, Akt and P70S6K phosphorylation. Hep-G2 cells were treated with different doses of metformin for 48h, increased phosphorylation of AMPK together with decreased phosphorylation of mTOR and P70S6K was observed. Moreover, P-Akt expression were also significantly inhibited after cells were treated with metformin (Figure 3A). In order to determine whether the antiproliferative effect of metformin is mediated by
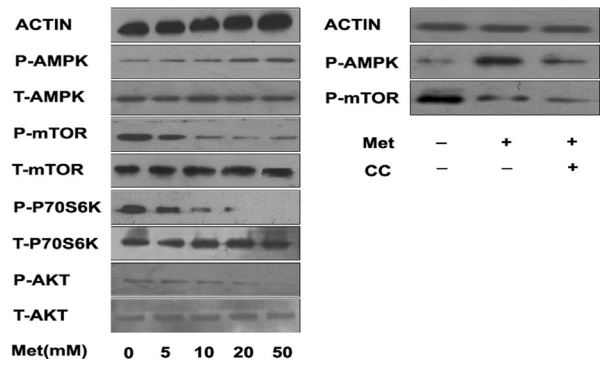
the AMPK pathway, compound C, an inhibitor of AMPK, was used to block this pathway. We found that compound C decreased the metformin-induced phosphorylation of AMPK. However, it could not prevent the inhibition of mTOR expression by metformin (Figure 3B). These results demonstrate that metformin inhibited the proliferation of Hep-G2 cells through a downregulation of mTOR pathway in an AMPK-independent manner.

*Metformin suppresses soft agar growth and in vivo tumor proliferation*

To determine whether metformin inhibits anchorage-independent growth, we performed a soft-agar colony formation assay with or without metformin renewed daily. We found that metformin decreased the number of the colonies in a dose dependent manner in Hep-G2 cells (Figure 4A). These results indicate that metformin inhibits anchorage-independent growth of HCC cells.

In order to determine whether metformin could affect tumor growth, the effect of metformin on tumor growth inhibition was studied in vivo via intraperitoneal injection. Our study found that tumor growth was obviously inhibited in high-dose metformin group and combination group (Figure 4B). Tumor volume of the high-dose metformin group and combination group was $611.2\pm50.8$ mm$^3$ and $281.2\pm20.0$ mm$^3$ respectively. Compared with the control group $1032.7\pm41.6$ mm$^3$, the tumor growth inhibition rate was 40.8% in high-dose metformin group ($P < 0.01$) and 72.8 % in combination group ($P < 0.01$) (Figure 4B). Body weight reduction is an indicator of drug toxicity. In the high-dose metformin group and combination group, body weight was $23.00\pm2.51$ g and $23.98\pm2.36$ g respectively, which is comparable to the control group.

## Discussion

Metformin has a long history of human use, with less toxicity and relatively cheap.It is now widely used as a first-line treatment for typeIIdiabetes (Bailey et al., 1996). Of interest, recent data suggest that metformin could protect patients with type II diabetes from cancer and inhibit cancer cells proliferation in vitro (Gotlieb et al., 2008; Hirsch et al., 2009; Cantrell et al., 2010; Goodwin et al., 2011; Soranna et al., 2012). According to our research, We have confirmed that metformin is a potent inhibitor of cell proliferation in Hep-G2 cell line. For example, Hep-G2 cells treated with 20 mM metformin for 48h led to cell proliferation inhibition with 62% decrease of cell viability. We then asked whether metformin affects cell cycle.To confirm this hypothesis, proliferating Hep-G2 cells were treated with metformin for 48h at different concentrations. As shown in Fig.1C, an increasing number of cells accumulate in G0/G1. In parallel, a reduction percentage of cells in the S and G2/M phases was observed. In order to determine whether metformin induces apoptosis in Hep-G2 cells, we performed an annexin V-FITC labeling assay and found that metformin at concentration less than 5 mM did not affect the amount of annexin V positive cells (data not shown). However, metformin at the concentration larger than 5mM increased the percentage of cell positive for annexin V. These results show that metformin can induce apoptosis but only at high concentrations.

To further explore the mechanism underlying the apoptosis induced by metformin, we study the signaling pathway involved in cell apoptosis. By our data, we conclude that metformin induces the apoptosis of Hep-G2 cells in vitro mainly through mitochondrion-mediated internal pathway, which is initiated by a variety of apoptosis-inducing signals that break the balance of the major apoptosis regulator, the proteins of the Bcl-2 family, such as Bcl-2, Bcl-xl and Bax. Besides, the pro-apoptotic protein Bax accumulates on mitochondria after being activated and triggers an increase in the permeability of the outer mitochondrial membrane. Consequently, the mitochondria release cytochromec. This, in turn, activates downstream death programs, such as caspase 3 and PARP. The result is similar to that of Luo-weifang et al. Who have reported that metformin significantly induced apoptosis in pancreatic cell lines and they discovered that metformin-induced apoptosis is associated with PARP cleavage activation of caspase-3, -8 and -9 (Wang et al., 2008). Besides, metformin was found to induce apoptosis in vitro in colon cancer cells but only in those p53-deficient cells (Buzzai et al., 2007). Metformin reduces high-fat-induced cardiac cell death, through inhibition of ceramide synthesis. However, it induces proton and lactate accumulation, leading to cell damage that is independent of caspase-3 at high concentrations (An et al., 2006). These studies indicate that metformin mediates its effects through different mechanisms.

To our knowledge, treatment with metformin results in activation of AMPK, the phosphorylated AMPK suppresses the mammalian target of Rapamycin (mTOR) and p70S6K signaling pathway, which plays an important role for protein translational machinery and cell proliferation (Bolster et al., 2002; Wullschleger et al., 2006). Several studies have demonstrated the therapeutic potential of mTOR inhibition, inducing anti-angiogenic effects in pre-clinical models (Yu et al., 1999; Hidalgo et al., 2000; Holland et al., 2004). In our study, metformin induced the phosphorylation of AMPK on Thr172 in Hep-G2 cells and inhibited the phosphorylation of mTOR and p70S6K. These downregulations in turn are known to decrease mRNA translation and protein synthesis (Bolster et al., 2002; Wullschleger et al., 2006), which would explain the antineoplastic effect observed. In order to determine whether the antiproliferative effect of metformin is mediated by the AMPK pathway, compound C was used to block this pathway. We found that compound C could decrease the metformin-induced phosphorylation of AMPK. However, it did not prevent the inhibition of mTOR phosphorylation. These series of experiments demonstrate that metformin inhibits the proliferation of Hep-G2 cells independenly of AMPK. Similar to our study, Ben Sahra et al. (2008) have found that metformin inhibited the prostate cancer cells growth in an AMPK-independent manner. Kalender et al. showed that metformin inhibited mTORC1 signaling independently of AMPK (Kalender et al., 2010). Our study contradicts the results obtained in MCF-7 cells by Zakikhani et al. (2006), who showed that metformin inhibited cell growth via AMPK in MCF-7 breast cancer cells. Dowling et al. found that metformin induced AMPK-dependent dephosphorylation of S6K in MCF-7 but not in LKB1-deficient or TSC2-dificient cells (Dowling et al., 2007). These differences may be due to a cell specific effect.

Our in vivo study showed that both high-dose metformin group and combination group significantly decrease Hep-G2 tumor growth without any significant side effects, such as weight loss, hair loss, dysphoria or lethargy. Our results are of particular importance since it is the first time that metformin is shown to inhibit HCC growth in a xenograft model .Similar to our research, Ben Sahra et al. have found that mteformin inhibited the prostate tumor growth in vivo (Sahra et al., 2008). Some researchers founded that metformin prevented pancreatic cancer in reduced hamsters fed a high-fat diet and exposed to a pancreatic carcinoma (Schneider et al., 2001). Huang X et al. reported metformin delayed the onset of tumors in mice deficient in the PTEN tumor suppressor (Huang et al., 2008).

Thus, in summary, we have demonstrated that the anti-diabetic drug, metformin, selectively induces apoptosis through mitochondrion-mediated internal pathway, and inhibits the mTOR signaling pathway, which may lead to the suppression of protein synthesis machinery in HCC. Although metformin has been shown to inhibit proliferation in vitro in breast, prostate, colon and ovarian cancer cell lines, its effect on HCC was demonstrated for the first time. Our results support the development of pre-clinical experiments to further evaluate the potential role of combining metformin with chemotherapy as a new treatment for HCC. Further studies of metformin may provide more insights into its efficacy.

## Acknowledgements

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