

The Role of PI3K and Wnt signaling Pathways and CSC Markers in Melanoma (B16F10) Therapy

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Abstract

Background

Metformin has been the subject of recent studies aimed at the treatment of melanoma cancer. In this study, the anti-cancer effects of metformin, an antidiabetic drug, was investigated *in-vitro* using the B16F10 melanoma cell line.

Methods

Melanoma cells were treated for 24 h with various concentrations of metformin, alone or in combination with dacarbazine. The effects of these two treatment agents on cell viability were evaluated by MTT assay. In addition, stemness and the activation of specific signaling pathways were evaluated by FACS and immunoblotting.

Results

Metformin induced β -catenin phosphorylation and decreased mTOR and PARP expressions. Also, a normal dose of metformin was found to reduce the phosphorylation levels of 4E-BP1, AKT, and S6p. In this study, we evaluated the potential of metformin as a therapeutic agent against CSCs in the adjuvant setting.

Conclusion

Our data indicate that some transcriptional regulators and proteins in the above-mentioned pathways were associated with cancer progression and inhibited by adjuvant chemotherapy with metformin. Metformin significantly inhibited cell growth and proliferation pathways, including Wnt and PI3K/AKT/mTOR. These findings show the potential of metformin in cancer treatment.

Introduction

Melanoma is one of the most belligerent and treatment-resistant cancer. The number of melanoma-related deaths remains high despite the advances made in drug discovery and delivery. Although malignant melanoma counts for 5% of all skin cancers, it causes 80% of skin cancer deaths [1]. Melanoma is one of the most invasive forms of skin cancer with resistance to cytotoxicity agents and high metastatic potential. For these reason, it has been a focus for the development of novel and efficient therapies [2].

Several studies suggest the presence and involvement of CSCs in the initiation, propagation, invasion, and metastasis of this malignancy [3]. Therefore, identifying melanoma stem

cells upon stemness markers and regulatory pathways might develop a new therapeutic strategy to treat the progression of this lethal form of cancer. Gene expression indexing of cancer stem cell markers has the potential to improve current diagnostic and to reveal insight into the intrinsic CSC physiology [4]. Hence, according to the change of CSCs markers within various stages of tumor life, targeting alternative CSC biomarkers for the treatment of tumors is necessary [5, 6].

Increasing evidence has shown that some CD biomarkers like CD34, CD44, CD133 [4] are related to promoting invasion and migration in a variety of solid tumors by activation of the intracellular signal pathway [7, 8]. CD34 is an important adhesion molecule and hematopoietic stem cells often used as a marker of tumor vasculature [9, 10]. Also, CD44 has a critical role in regulating the properties of CSCs, like tumor initiation, self-renewal, metastasis, and chemo or radio resistance [7]. New findings suggested that the interplay between some intracellular pathways like the Wnt signaling pathway and PI3K/AKT/mTOR pathway is associated with the induction of cancer stem cell properties linked to CD34 and CD44 expression [11].

Dacarbazine (DTIC) is a chemotherapy medicine used in the treatment of several cancers like melanoma. Dacarbazine belongs to a group of drugs called alkylating agent [12]. Metformin (1, 1-dimethylbiguanide hydrochloride) is used for diabetes mellitus type 2 first line therapy. The primary effect of metformin is based on interfering with respiratory complex I, reducing ATP production and activation of adenosine monophosphate-activated kinase (AMPK) that can activate intracellular pathways [13]. These intracellular pathways cause cell changes to stemness and irregular proliferation. Therefore metformin therapy significantly reduces the risk of melanoma cancer by preventing the propagation pathways and stemness [14, 15]. The reduction in proliferation observed in vitro in B16 murine melanoma cells with either PI3K or WNT signaling [2, 9].

The main focus of this work was to understand possible mechanisms underlying the inhibitory effect of metformin on CSCs and intracellular pathway. The population of CSCs after treatment with metformin and dacarbazine was significantly reduced. Combination therapy can reduce CSCs fraction, and probably there is a significant relationship between stemness and intracellular reproduction pathways. Also, some oncogenic signaling pathways involved in this disease were evaluated. Our data showed that this antidiabetic agent, metformin, exerts antitumor effects via inhibition of Wnt and mTOR signaling.

Materials And Methods

2 – 1. Cell culture

The metastatic melanoma cell line B16F10 was obtained from the Pastor Institute (Tehran-Iran). Cells were frozen and stored in liquid nitrogen and were slowly thawed for the conduction of experiments. To achieve 75% confluence on the culture plate, the cells were maintained at 37°C in a humidified atmosphere with 5% CO₂ in DMEM (10% fetal bovine serum) and 1% penicillin/streptomycin and rested for 24 h. The cells were prepared for experiments by detaching using trypsin with EDTA.

2–2. cell viability

For the viability experiment, the cells were incubated in 96 well flat-bottom plates (1×10^4 cell/well). Cells were rested for 24 h and then treated with appropriate concentration of metformin hydrochloride (0.5 mM, 2mM, 8mM) and/ or dacarbazine (134 $\mu\text{g/ml}$)(Positive Control Group), a chemotherapy drug, and a group of dacarbazine with increased doses of metformin hydrochloride (0.5 mM, 2mM, 8mM). There is a Sham Group (Negative Control Group) without any treatment. After treatment with dacarbazine, metformin, or combination therapy, the mitochondrial dehydrogenase activity, as the index of cell viability, was determined by MTT (5mg/ml) that incubated for 2 h after was added to each well. The formazan crystal formed was dissolved with 100 μl of DMSO. The MTT absorbance, proportionate to the number of viable cells, was measured in ELISA (Enzyme-linked immunosorbent assay) reader at 570 nm by a microplate spectrophotometer (BD Biosciences, San Jose, CA). Briefly, this was a colorimetric assay. In this assay, metabolically active cells ferment the yellow tetrazolium salt, to the form of the purple Formosan dye.

2–3. Flow cytometry

All of the groups with different treatments for 24 h were assessed by flow cytometry for expressing CD34 and CD44 marker protein. Cell suspensions of the B16F10 cell line were incubated with 1 μL of CD44 Rat anti-Human, Mouse PE, Clone (ebioscience Ins-sandiego, USA), and anti-mouse CD34 FITC conjugated (ebioscience Ins-sandiego, USA), individually. Anti Rat IgG2b-PE isotype control for CD44, Rat IgG2a K isotype control FITC for CD34, and unstained cells were used as controls. The phenotype was assessed using a FACS caliber flow cytometer (BD, Heidelberg, Germany).

2–4. Western Blot

For extracting total protein, cells were resuspended in lysis buffer containing 150mmol/L NaCl, 1%SDS, and 50 mmol/L Tris-Cl pH 8, 2 $\mu\text{g/ml}$ aprotinin 2 $\mu\text{g/ml}$ leupeptin, 40mg/mL of phenylmethylsulfonyl fluoride (PMSF), 2mmol/L dithiothreitol (DTT). Cells were centrifuged at 12000 rpm for 15 min and supernatant were then quickly frozen at -80°C until use.

The protein concentration was distinguished by the Bradford assay (Biorad, Hercules, and CA). Thirty micrograms of cell extracted protein from B16F10 were loaded onto a 10% SDS- polyacrylamide gel subjected to electrophoresis and electro-transferred onto a nitrocellulose membrane. The immunoblot was incubated 1 h at room temperature with 5% nonfat milk, and then the membrane was then probed with the primary antibody overnight at 4°C . The next day, Blots were washed twice with Tris buffered saline/Tween 20(TBST) and the secondary appropriate antibody at room temperature was added for 1 h. Blots were again washed with TBST before development. The visualization blot of the signal was obtained using ECL (Bio-Rad Clarity Western ECL). Band densitometry was measured by Image J software, Protein Simple and semi quantitative data were normalized for GAPDH and Tubulin.

2–5. Statistical analysis

Differences between study groups for MTT were analyzed by One-way analysis of variance (ANOVA) with a post-hoc method and for FACS and western blotting, nonparametric Kruskal-Wallis, Mann-Whitney. Results represent the average of three independent experiments ($n = 3$; mean \pm SD) for MTT and FACS and triplicate experiments for western blotting. The value of $P < 0.05$ was considered significant.

Results

Combined treatment with metformin and dacarbazine decreased the viability of melanoma cells.

This study is in the following of previous research that performed in Molecular Medicine of Institute Superiore di Sanità, Rome[16]. B16F10 mouse melanoma cells were exposed to different doses of dacarbazine, and their cell proliferation and viability were examined after 24 h (Fig. 1A). The results which is in accordance with our previous report showed the 24 h IC₅₀ value of dacarbazine was calculated to be 134 μ g/ml[16].

Combined treatment with metformin and dacarbazine decreased melanoma cancer stem cell (CSC) markers.

Combined treatment with metformin and dacarbazine decreased melanoma CSC markers. The expression of multiple CSC surface markers correlates with metastatic potential[17]. In this study, the expression of stem cell markers in B16 melanoma cells was investigated. It has been shown that CD34 and CD44 play a key role in the regulation of several properties of CSCs, such as self-renewal, metastasis, tumor initiation, chemo- and radio-resistance, and colonization [18, 19]. For this reason, in the present study, the expression levels of two cell-surface markers, i.e. CD34 and CD44, were examined before and after treatment with metformin by flow cytometry. CD34 is a glycoprotein expressed on hematopoietic stem cells and capillary endothelial cells. CD34 has been found to be over expressed in aggressive tumor cells and this has been verified by molecular methods [12]. In this study, as suggested in some previous studies, we assumed that this molecule could be related to endothelial and stemness phenotype in tumor cells and facilitate cell migration [6, 18].

The results showed that metformin, alone or in combination with dacarbazine, reduced the expression of CSC markers, as determined by FACS. Treatment with metformin for 24 hours did not decrease the expression of CD44 except at a high dose of 8 mM, which led to chemoresistance. Our findings also showed that dacarbazine, alone or in combination with metformin (2 mM), significantly reduced the expression of CD44, but no significant difference was observed at other doses of metformin. CD44 expression in some other metastatic cancer cell lines is about 90% [20]. In our experiment, high levels of CD44 were detected in all samples, suggesting that a high expression of CD44 in malignant melanoma cells may be associated with increased metastatic risk (Fig. 2).

Furthermore, our findings showed that all doses of metformin in combination with dacarbazine reduced the CD34⁺ CSC population. Also, metformin (8 mM) significantly reduced the expression of CD34 marker.

Loading [MathJax]/jax/output/CommonHTML/jax.js ($p < 0.05$) decrease in CD34 expression in cells treated with

dacarbazine chemotherapy (37.86%) and combination therapy, especially at anormal dose (DTIC + metformin 2 mM) (52.26%).However, there was no significant change in the CD34 expression in cells treated with metformin alone (0.5 and 2 mM) (Fig. 3).Therefore, it can be concluded that combination therapy with metformin and dacarbazine, compared to chemotherapy,can lead to better results.

Dacarbazineaffected intracellular pathways and protein expression in melanoma cells.

CSCs can induce neoplastic transformation and cell proliferation. The role of several key molecular pathways involved in melanoma onset, progression, and proliferationhasnot been clearly determined.Thus, to further investigate therole of these molecular pathways, we assessed the effects of treatment with metformin, alone and in combination with chemotherapy, on the expression of mitoticproteins. The results showedthat combinedtreatment with metformin and dacarbazine reduced the expression of p-AKT compared with that of AKT (Fig. 4-B). Also, significant decrease was observed in S6rp/phosphorylated-S6 expression in all groups. Combined treatment with metformin and dacarbazine inhibited thephosphorylation of S6rp, which, in turn, led to a significant decrease in the expression of S6rp, especially at normal doses of metformin with dacarbazine (Fig. 4-C). As Fig. 4-A shows, while there was a decreasein4E-binding protein 1 (4E-BP1)/phosphorylated-4E-BP1 expression at low and normal doses of metformin,there was a significant increase in the expression of this protein at high doses of metformin (8mM).A comparison of the results for groups 5 and 8 showed that therewasno clear explanation for the above findings.

In addition, the results showed that treatment with a high dose of metformin in combination with dacarbazinecan phosphorylate β -catenin,priming it for ubiquitination by the E3 ubiquitin ligase and degradation through proteasome (Fig. 5-B). This ubiquitination and degradation of phosphorylated β -catenin inhibited Wnt signaling pathway (Fig. 5-B). Metformintreatment ata high dose and in combination therapy significantly increased the amount of phosphorylated β -catenin compared to non-phosphorylated β -catenin; however, there was no significantchangein cyclin D1 levels. Moreover, while treatment withdacarbazine alone increased the cyclin D1 level, metformin treatment decreased the level of this protein (Fig. 5-C).

Although combination therapy with metformin and dacarbazine reduced the expression of mammalian target of rapamycin (mTOR) in all groups, except for group 8, the change was not significant. Metforminat a dose of2 mMhada stronger effect on reducing mTOR expression. Therresultsfurther showed that combined treatment with metformin and dacarbazine, especiallyat a high dose, increased mTOR expression while treatment with dacarbazine alone decreased the expression of mTOR(Fig. 4-D).Poly (ADP-ribose) polymerase (PARP) is a nuclear DNA binding protein that is activated by DNA strand breaks. Once PARP is cleaved by caspase during apoptosis, its DNA repair function is impaired. Cell apoptosis increased in combination therapy due tothe increase in the expression of cleaved PARP compared to that of non-cleaved PARP (Fig. 5-A).

Discussion

Previous experiment showed that metformin, at doses of 2mM and higher, inhibited the proliferation of B16F10 melanoma cells. A significant increase in cytotoxicity was observed in cells treated with a fixed dose of dacarbazine (IC50) and increasing doses of metformin. All doses of metformin, especially at 2 mM, in combination with dacarbazine (134µg/ml) inhibited the growth of B16F10 melanoma cells after 24 h[16]. Although metformin alone has increased cell viability, Combination therapy has reduced it so that changes compared to dacarbazine alone are not significant (Fig. 1B) and in following melanoma progression and pathways described. Our finding in accordance with previous confirmed combination therapy is more effective than dacarbazine therapy.

Combined treatment with metformin, as an antidiabetic drug, and dacarbazine, a well-known chemotherapeutic drug, has shed new light on the molecular pathways involved in cancer progression and the expression levels of CSC markers, such as CD34 and CD44. It has been shown that CSCs, unlike bulk tumor cells, are capable of inducing tumor growth and forming colonies in culture [16, 21]. The expression levels of multiple CSC surface markers have been found to associate with invasion potential [17]. In recent years, some studies have focused on the high expression of these CSC marker genes in cancer cell lines and solid tumors [22, 23]. These results indicate that when B16F10 cancer cells were treated with different concentrations of metformin for 24 h, the expression of CSC markers was reduced and a cellular response was triggered, which, in turn, inhibited cell proliferation and metastasis. Therefore, it can be concluded that metformin, alone or in combination with dacarbazine, can reduce melanoma cancer cells. Also, it can be stated that the changes in the expression of CSC markers are a good indicator of the changes in intracellular pathways.

The reduction in CD44, despite being significant, is not so great ($p < 0.05$). Based on these data, it can be stated that combination therapy with metformin and dacarbazine cannot reduce the expression of this marker after 24 h and that the changes in its expression level must be evaluated over a longer time period. It is suggested to consider a longer incubation time in future researches. Some studies have shown that the CD44 ligand-receptor complexes on dacarbazine-treated melanoma cells are less stable than on untreated cells. However, the CD44 receptor could still be activated, which, in turn, could lead to the activation of intracellular signaling and, consequently, a cellular response [12].

Flow cytometric analysis of B16F10 cells revealed that combination therapy with metformin significantly decreased the expression of CD34 which is in accordance with our previous reports [5, 24]. In this study, as expected, cells treated with dacarbazine and metformin at a physiological dose showed a reduction in the expression levels of CD34 CSC marker. Thus, the combination therapy used in this study can be considered as an effective treatment for melanoma cancer. These findings are consistent with those of other studies that have shown the effects of dacarbazine on reducing the expression of CD34 marker. Our data revealed a new role for CD34 in the growth of cancerous tumors and provides new insights into a treatment that could inhibit tumor progression. In our previous study, the cells treated with metformin and dacarbazine generated fewer colonies than those treated with only metformin. Colonization reflects the proliferation of CSCs, and it has been shown that combination therapy with metformin and

Loading [MathJax]/jax/output/CommonHTML/jax.js d stemness[16].

Typically, melanoma metastasis is associated with the activation of signaling pathways that are essential for embryogenesis [25]. In recent years, some molecular pathways have been reported to be involved in many cancers. Identifying the pathways that are responsible for embryogenesis and melanoma progression and metastasis is a key step toward a more effective treatment for melanoma cancer. Some data suggest that intracellular pathways are strongly associated with malignant melanocytic lesions, which are caused by a combination of genetic and epigenetic changes involved in neoplastic transformation [11, 26]. Growing evidence indicates that metabolic reprogramming is a downstream consequence of tumor progression and that it is induced by oncogene activation. It also has been reported that metabolic reprogramming can change metabolism and enhance oncogenic signaling to promote tumor malignancy [27].

The results of this study showed that metformin inhibited the proliferation of melanoma cells by suppressing the phosphorylation of AKT pathway proteins ($p < 0.05$), facilitated the phosphorylation of β -catenin, and increased the expression of cleaved PARP ($p < 0.05$).

According to Fig. 6 the activated p-AKT has the potential to phosphorylate other proteins, which can lead to increased cancer cell proliferation. Glycogen synthase kinase 3β (GSK3 β) is another kinase that is inhibited when phosphorylated by p-AKT [28]. As a result of GSK3 β inhibition, free β -catenin can accumulate and translocate to the nucleus and up regulate the expression of oncogenic genes, such as cyclin D1 [29]. In addition, activated p-AKT can increase the expression of c-MYC by activating mTOR. The activation of mTOR, a kinase implicated in the regulation of apoptosis, can lead to the phosphorylation and activation of S6rp and initiation factor 4E-BP1, thus promoting protein translation and cell growth. Through these regulatory mechanisms, p-AKT exerts an anti-apoptotic effect and promotes cancer progression. mTOR activation functions as a growth factor in melanoma cells. Our results, together with the above data, show the inactivation of mTOR and a decrease in its activity after therapy, suggesting that the mTOR pathway can be used as a target for the treatment of melanoma. Also according to the study performed by Baradaran et al, it has been reported that when metformin is transported into cells, it inhibits mitochondrial complex I (NADH) [30]. Thus, it can be said that metformin decreases ATP synthesis and activates the AMPK pathway. Metformin has been shown to directly inhibit insulin-induced malignant cell growth in a PI3K/AKT/mTOR- and Wnt-dependent manner via the AMPK pathway. Metformin has also been found to cause a significant increase in AMPK activity by the phosphorylation of Thr and inhibition of DNA synthesis, cell mitosis, and proliferation [31]. Furthermore, metformin has been shown to activate AMPK, which, in turn, can block protein synthesis and mTOR signaling in many cancer cells [32]. Our studies have demonstrated that loss of mTOR and S6rp is associated with marked dependence on AKT. The evaluation of the effects of treatment with metformin and dacarbazine on melanoma cells shows that these agents have strong inhibitory effects on AKT, mTOR, 4E-BP1, and S6rp pathways. Treatment with metformin, alone or in combination with dacarbazine, reduced AKT, mTOR, and rpS6 phosphorylation and negatively regulated the activities of these pathways. Our studies show that combination therapy with metformin and dacarbazine, compared to treatment with dacarbazine alone, can lead to a more significant decrease in the expression of phosphorylated AKT, S6rp,

and 4E-BP1, indicating that combined treatment with metformin is likely to enhance the therapeutic effects of dacarbazine.

Wnt signaling has a key role in cell survival and differentiation. Another study reported that cytoplasmic β -catenin destruction complex with APC, Axin, and GSK can cause the ubiquitination and degradation of β -catenin. Metformin activates the AMPK pathway, and this pathway can inhibit β -catenin nuclear translocation by binding to β -catenin in the cytosol [31, 33]. In the present study similar to others findings, the elevated levels of phosphorylated β -catenin following combination therapy and the activation of small molecules inhibited the proliferation of cancer cells. However, the changes in cyclin D1 levels were inconsistent, and metformin treatment, especially at normal doses, increased the level of this protein. The phosphorylation of β -catenin may block the translocation of β -catenin into the nucleus. An increased ratio of phosphorylated to non-phosphorylated β -catenin indicates the effectiveness of combination therapy in inhibiting the Wnt signaling pathway ($p < 0.05$).

Some previous studies have shown that chemotherapy drugs are critical mediators of alternative metabolic pathways because their use leads to some coordinate changes in phosphorylated AKT, phosphorylated β -catenin, phosphorylated S6rp, and phosphorylated 4E-BP1 [33]. This is consistent with the results of the present study. Furthermore, it has been noted that the inactivation of Wnt/ β -catenin and PI3K pathways and the activation of AMPK following combined treatment with metformin and dacarbazine can lead to a decrease in the expression of stemness genes and cell proliferation. Stemness markers are often upregulated in melanoma tumors and promote tumor progression and metastasis. These cell-surface markers are associated with CSCs and are thought to drive the growth of many cancers which is in accordance with our previous results [34]. Tumor growth and metastasis are determined by the complex interplay between several factors, including CD34 and CD44 markers and intracellular pathways. Our results revealed a novel role for CD34 in both cell proliferation and stemness. The results of this study also indicated that the Wnt and PI3K pathways were involved in the upregulation of stemness markers such as CD34 at the transcriptional level, conferring cancer stem cell-like properties to melanoma cells. In this respect, combination therapy with metformin and dacarbazine represents a potential therapeutic option for the treatment of melanoma cancer because this combination therapy can suppress the above-mentioned pathways.

PARP is a nuclear enzyme involved in DNA repair. PARP hyper activation induced NAD^+ depletion, which led to mitochondrial dysfunction and cell death [35]. According to the results of this study, metformin increased the expression of PARP after 24 h, which can reflect an attempt by the cells to prevent apoptosis during treatment ($p < 0.05$). In some other studies, the expression of PARP increased after 48 or 72 h following treatment with metformin. The characteristic ability of PARP to be activated by DNA strand breaks makes poly(ADP-ribose)lation an immediate and drastic cellular response to DNA damage as induced by ionizing radiation, alkylating agents, and oxidants [36]. In this study, although metformin increased the expression of PARP expression, given that it inhibited colonization and proliferation pathways and increased the expression of cleaved PARP, metformin treatment can be considered an

Conclusion

The results of this study demonstrated that combined treatment with metformin and dacarbazine inhibited proliferation pathways, including PI3K, Wnt, and mTOR. In addition to preventing the conversion of stem cells into CSCs, metformin altered metabolic pathways. Metformin, at a high dose (8 mM) alone and at all doses (0.5, 2, and 8 mM) in combination with dacarbazine increased β -catenin phosphorylation, inhibited Wnt signaling pathway, and suppressed cancer cell proliferation. Combined treatment with metformin, at all doses (0.5, 2, and 8 mM), and dacarbazine decreased the phosphorylation of AKT, which led to the inhibition of PI3k/AKT/mTOR signaling pathway and cancer cell proliferation.

Furthermore, the results showed that the CD34 marker, unlike CD44, could be a good indicator of changes in stem cell properties since there was a correlation between the decrease in the CD34 expression and the suppression of intracellular signaling pathways. Metformin suppressed cancer cell proliferation and enhanced cancer cell death by inhibiting cell growth signaling pathways, particularly mTOR and Wnt signaling pathways. Based on the results of this study, it can be concluded that metformin is an effective adjunct to cancer chemotherapy.

Abbreviations

CSCs Cancer stem cells MTT assay The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide CD biomarker Cluster of differentiation WntWingless-related integration site PI3K Phosphatidylinositol-3-kinase AKT=PKB Ak strain transforming =Protein kinase B mTOR Mammalian target of rapamycin AMPK Adenosine monophosphate-activated kinase DTICDacarbazine S6rp S6 ribosomal protein 4E-BP1 4E-binding protein 1 PARP Poly (ADP-ribose) polymerase GSK3 β Glycogen synthase kinase 3 β APC Adenomatous polyposis coli

Declarations

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Authors' contributions

All Authors read and approved the manuscript.

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Availability of data and materials

All data generated or analyzed during this study are available for consultation and can be requested from authors.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

The authors declare that they have no conflict of interest.

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References

1. Maio M. (2012) Melanoma as a model tumour for immuno-oncology, *Ann Oncol.* **23 Suppl 8**, viii10-4.
2. Janjetovic K, Harhaji-Trajkovic L, Misirkic-Marjanovic M, Vucicevic L, Stevanovic D, Zogovic N, Sumarac-Dumanovic M, Micic D, Trajkovic V. In vitro and in vivo anti-melanoma action of metformin. *European journal of pharmacology.* 2011;668:373–82.
3. Bapat S. (2008) *Cancer Stem Cells: Identification and Targets.*
4. Hajimoradi Javarsiani M, Sajedianfard J, Haghjooy Javanmard S. (2020) The effects of metformin on the hippo pathway in the proliferation of melanoma cancer cells: a preclinical study, *Arch Physiol Biochem*, 1–6.
5. Breza TS, Magro CM. CD34 expression in primary cutaneous malignant melanoma: apropos of a case and review of the aberrant melanoma phenotype. *J Cutan Pathol.* 2005;32:685–9.
6. Pisacane AM, Picciotto F, Risio M. CD31 and CD34 expression as immunohistochemical markers of endothelial transdifferentiation in human cutaneous melanoma. *Cell Oncol.* 2007;29:59–66.
7. Eyler CE, Foo WC, LaFiura KM, McLendon RE, Hjelmeland AB, Rich JN. Brain cancer stem cells display preferential sensitivity to Akt inhibition. *Stem Cells.* 2008;26:3027–36.
8. Zhang SS, Han ZP, Jing YY, Tao SF, Li TJ, Wang H, Wang Y, Li R, Yang Y, Zhao X, Xu XD, Yu ED, Rui YC, Liu HJ, Zhang L, Wei LX. CD133(+)CXCR4(+) colon cancer cells exhibit metastatic potential and

Loading [MathJax]/jax/output/CommonHTML/jax.js Med. 2012;10:85.

9. Chen L, Lu Y, Wu JM, Xu B, Zhang LJ, Gao M, Zheng SZ, Wang AY, Zhang CB, Zhang WW, Lei N. Ligustrazine inhibits B16F10 melanoma metastasis and suppresses angiogenesis induced by Vascular Endothelial Growth Factor. *Biochemical biophysical research communications*. 2009;386:374–9.
10. Tardío JC. CD34-reactive tumors of the skin. An updated review of an ever-growing list of lesions. *J Cutan Pathol*. 2008;35:1079–92.
11. Manning BD, Toker A. AKT/PKB Signaling: Navigating the Network. *Cell*. 2017;169:381–405.
12. Huang X, He J, Zhang HT, Sun K, Yang J, Wang H, Zhang H, Guo Z, Zha ZG, Zhou C. Effect of dacarbazine on CD44 in live melanoma cells as measured by atomic force microscopy-based nanoscopy. *Int J Nanomedicine*. 2017;12:8867–86.
13. Owen MR, Doran E, Halestrap AP. Evidence that metformin exerts its anti-diabetic effects through inhibition of complex 1 of the mitochondrial respiratory chain. *BIOCHEM J*. 2000;348 Pt 3:607–14.
14. Zhang Y, Storr SJ, Johnson K, Green AR, Rakha EA, Ellis IO, Morgan DA, Martin SG. Involvement of metformin and AMPK in the radioresponse and prognosis of luminal versus basal-like breast cancer treated with radiotherapy. *Oncotarget*. 2014;5:12936–49.
15. Zi FM, He JS, Li Y, Wu C, Yang L, Yang Y, Wang LJ, He DH, Zhao Y, Wu WJ, Zheng GF, Han XY, Huang H, Yi Q, Cai Z. Metformin displays anti-myeloma activity and synergistic effect with dexamethasone in in vitro and in vivo xenograft models. *Cancer Lett*. 2015;356:443–53.
16. Hajimoradi Javarsiani M, Haghjooy Javanmard S, Sajedianfard J. In Vitro Inhibition of Melanoma (B16f10) Viability and Colonization through Combining Metformin and Dacarbazine. *Middle East J Cancer*. 2020;11:159–67.
17. Kim YS, Kaidina AM, Chiang JH, Yarygin KN, Lupatov AY. Cancer stem cell molecular markers verified in vivo. *Biochemistry (Moscow) Supplement Series B: Biomedical Chemistry*. 2017;11:43–54.
18. Maltby S, Freeman S, Gold MJ, Baker JH, Minchinton AI, Gold MR, Roskelley CD, McNagny KM. Opposing roles for CD34 in B16 melanoma tumor growth alter early stage vasculature and late stage immune cell infiltration. *PloS one*. 2011;6:e18160.
19. Sidney LE, Branch MJ, Dunphy SE, Dua HS, Hopkinson A. Concise review: evidence for CD34 as a common marker for diverse progenitors. *Stem cells*. 2014;32:1380–9.
20. Fu C, Zhou N, Zhao Y, Duan J, Xu H, Wang Y. Dendritic cells loaded with CD44(+) CT-26 colon cell lysate evoke potent antitumor immune responses. *Oncol Lett*. 2019;18:5897–904.
21. González-Moles MA, Scully C, Ruiz-Ávila I, Plaza-Campillo JJ. The cancer stem cell hypothesis applied to oral carcinoma. *Oral Oncology*. 2013;49:738–46.
22. Lin J, Ding D. The prognostic role of the cancer stem cell marker CD44 in ovarian cancer: a meta-analysis. *Cancer Cell Int*. 2017;17:8.
23. Zimmerer RM, Matthiesen P, Kreher F, Kampmann A, Spalthoff S, Jehn P, Bittermann G, Gellrich NC, Tavassol F. Putative CD133 + melanoma cancer stem cells induce initial angiogenesis in vivo. *Microvasc Res*. 2016;104:46–54.

24. Dietrich A, Tanczos E, Vanscheidt W, Schopf E, Simon JC. High CD44 surface expression on primary tumours of malignant melanoma correlates with increased metastatic risk and reduced survival. *Eur J Cancer*. 1997;33:926–30.
25. Kulikova KV, Posvyatenko AV, Gnuchev NV, Georgiev GP, Kibardin AV, Larin SS. Nuclear β -catenin localization is not sufficient for canonical Wnt signaling activation in human melanoma cell lines. *Mol Biol*. 2011;45:816.
26. Kong Y, Si L, Li Y, Wu X, Xu X, Dai J, Tang H, Ma M, Chi Z, Sheng X, Cui C, Guo J. Analysis of mTOR Gene Aberrations in Melanoma Patients and Evaluation of Their Sensitivity to PI3K-AKT-mTOR Pathway Inhibitors. *Clin Cancer Res*. 2016;22:1018–27.
27. Azzolin L, Panciera T, Soligo S, Enzo E, Bicciato S, Dupont S, Bresolin S, Frasson C, Basso G, Guzzardo V, Fassina A, Cordenonsi M, Piccolo S. YAP/TAZ incorporation in the beta-catenin destruction complex orchestrates the Wnt response. *Cell*. 2014;158:157–70.
28. Hoeflich KP, Merchant M, Orr C, Chan J, Den Otter D, Berry L, Kasman I, Koeppen H, Rice K, Yang NY, Engst S, Johnston S, Friedman LS, Belvin M. Intermittent administration of MEK inhibitor GDC-0973 plus PI3K inhibitor GDC-0941 triggers robust apoptosis and tumor growth inhibition. *Cancer Res*. 2012;72:210–9.
29. Vasudevan KM, Garraway LA. AKT signaling in physiology and disease. *Curr Top Microbiol Immunol*. 2010;347:105–33.
30. Baradaran R, Berrisford JM, Minhas GS, Sazanov LA. Crystal structure of the entire respiratory complex I. *Nature*. 2013;494:443–8.
31. Park SY, Kim D, Kee SH. Metformin-activated AMPK regulates β -catenin to reduce cell proliferation in colon carcinoma RKO cells. *Oncol Lett*. 2019;17:2695–702.
32. Lei Y, Yi Y, Liu Y, Liu X, Keller ET, Qian C-N, Zhang J, Lu Y. Metformin targets multiple signaling pathways in cancer. *Chin J Cancer*. 2017;36:17–7.
33. Park SY, Lee Y-K, Lee WS, Park OJ, Kim Y-M. The involvement of AMPK/GSK3-beta signals in the control of metastasis and proliferation in hepato-carcinoma cells treated with anthocyanins extracted from Korea wild berry Meoru. *BMC Complement Altern Med*. 2014;14:109–9.
34. Zhao B, Tumaneng K, Guan KL. The Hippo pathway in organ size control, tissue regeneration and stem cell self-renewal. *Nat Cell Biol*. 2011;13:877–83.
35. Tentori L, Lacal PM, Graziani G. Challenging resistance mechanisms to therapies for metastatic melanoma. *Trends Pharmacol Sci*. 2013;34:656–66.
36. Haji HA, Sheibak H, Khosravi M, Asadi J. (2016) The effect of metformin on the expression of caspase 3, 8, 9 and PARP-1 in human breast cancer cell line T47D, *Int J Health studies*. 2.

Figures

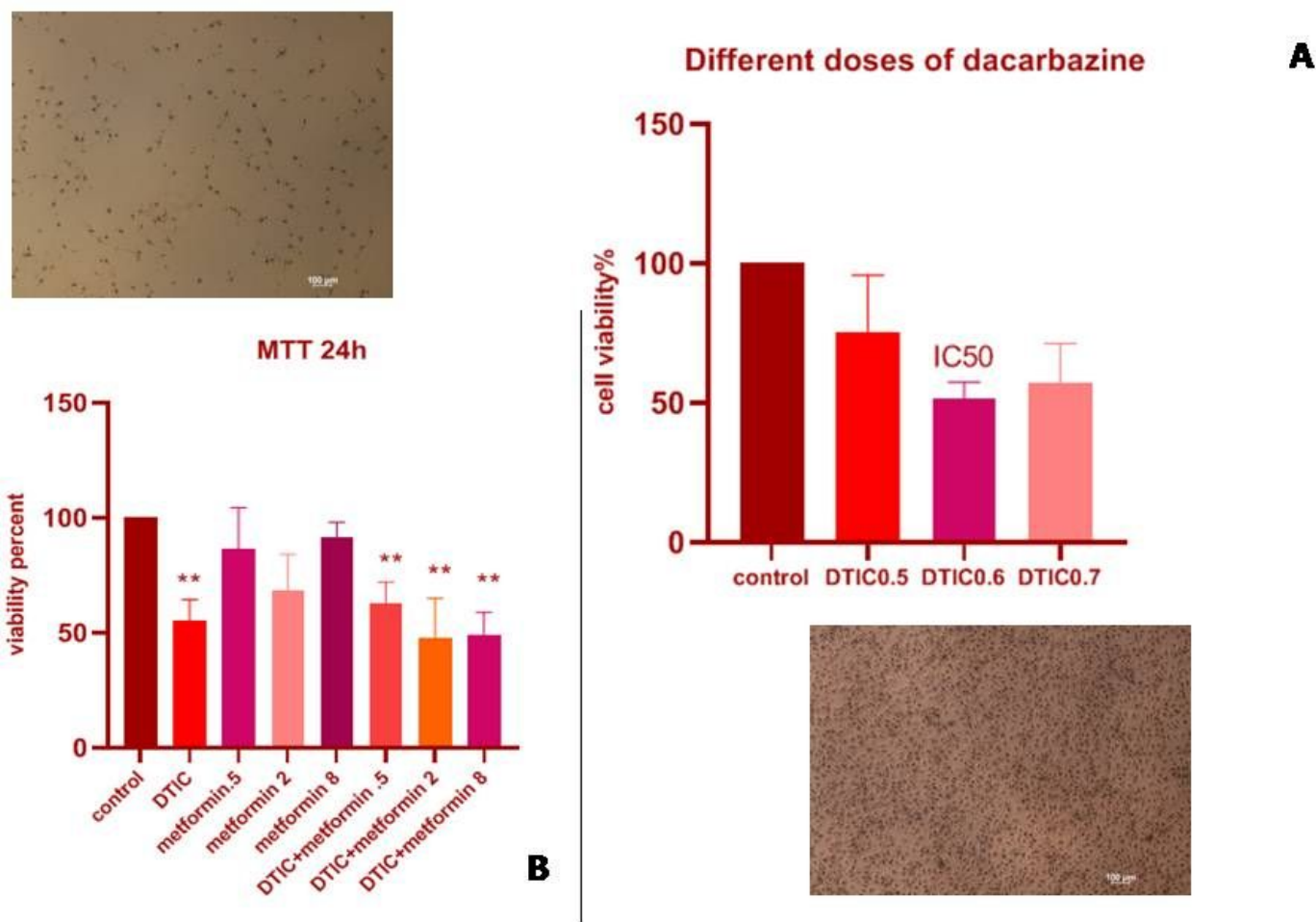
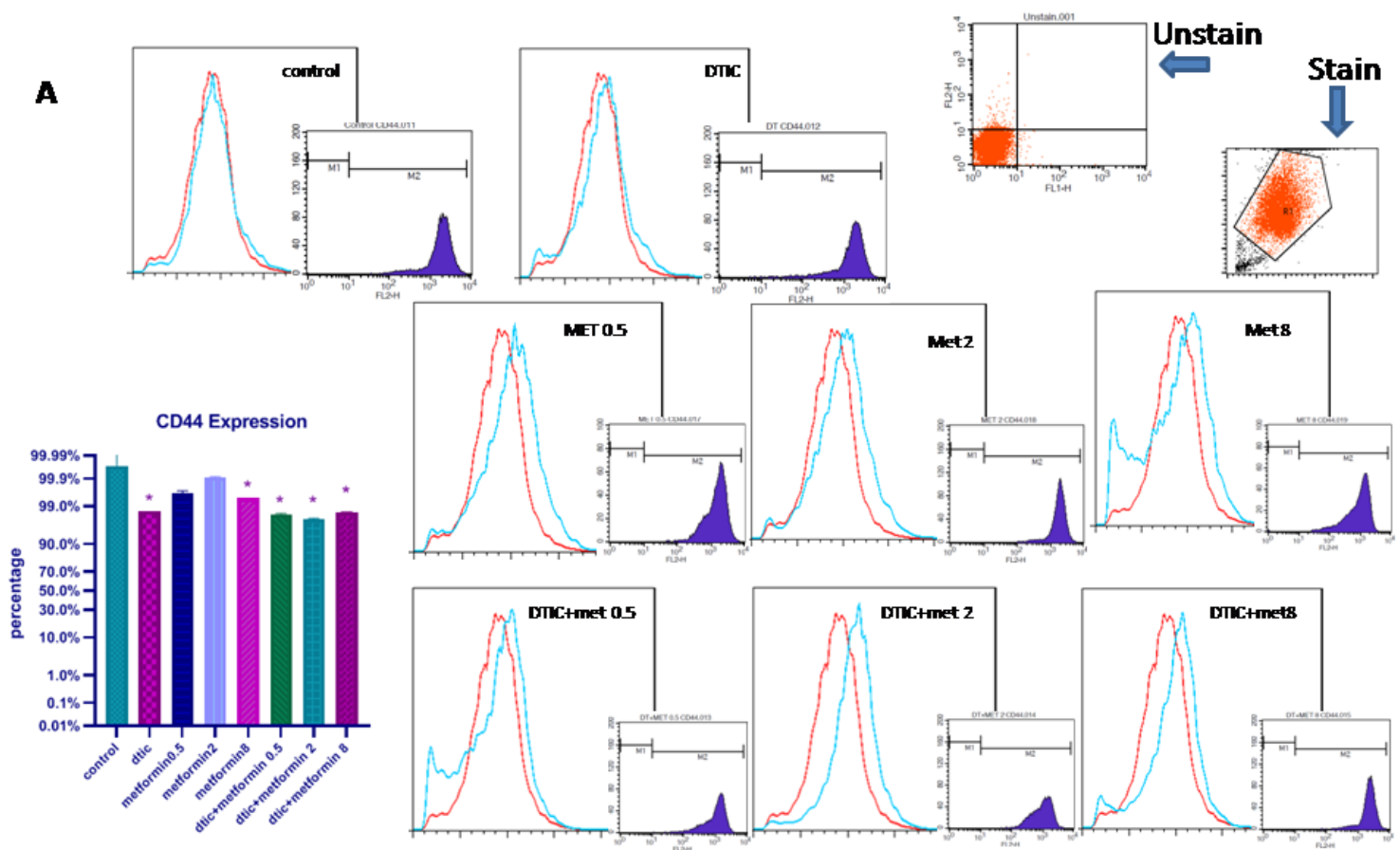


Figure 1

A, Different doses of dacarbazine were examined (0.5, 0.6 and 0.7 mM) and the IC₅₀ dose specified. Dose dependent toxicity of treated cells was determined by MTT assay 24 h.*p<0.05-**p<0.01 B. B16 melanoma cells were incubated with different concentrations of metformin and dacarbazine IC₅₀. All doses of metformin, especially at 2 mM, in combination with dacarbazine (IC₅₀) inhibited the growth of B16F10 melanoma cells after 24 h. Hajimoradi et al,2020.



B-CD44 expression after 24 h treatment

Figure 2

A: Flow cytometric quantitative analysis of the expression of CD44 in B16-F10 cells without dacarbazine treatment and in cells treated with 134 $\mu\text{g}/\text{mL}$ dacarbazine, different doses (0.5, 2, and 8 mM) of metformin and combination therapy for 24 hours. B; FACS analysis of CD44 biomarker in B16F10 as a column graph. The experiment is representative of 3 independent experiments. * $p < 0.05$

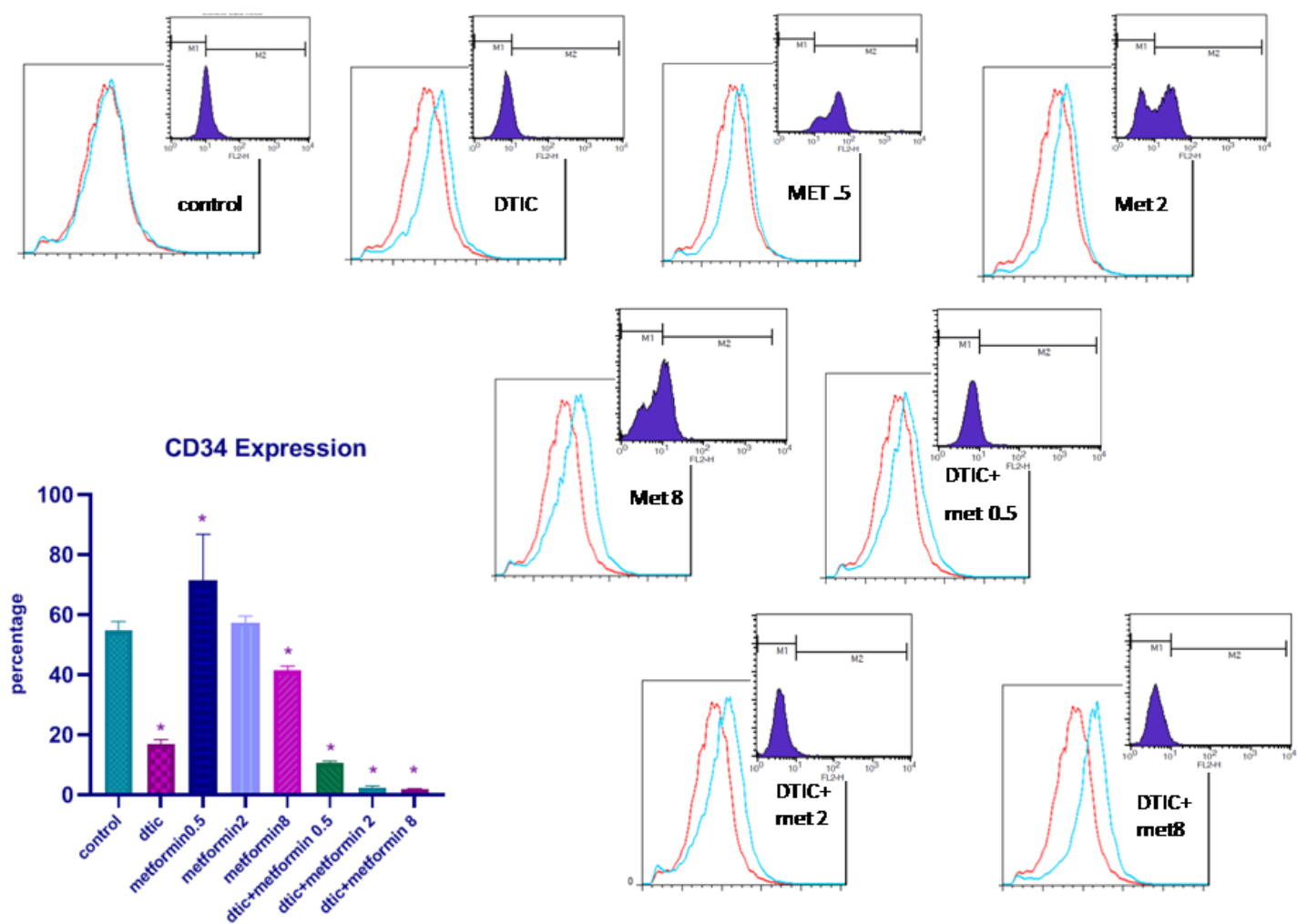


Figure 3

A: Flow cytometric quantitative analysis of the expression of CD34 in B16-F10 cells without dacarbazine treatment and in cells treated with 134 $\mu\text{g}/\text{mL}$ dacarbazine, different doses (0.5, 2, and 8 mM) of metformin and combination therapy for 24 hours. B: FACS analysis of CD34 biomarker in B16F10 as a column graph. The experiment is representative of 3 independent experiments. * $p < 0.05$

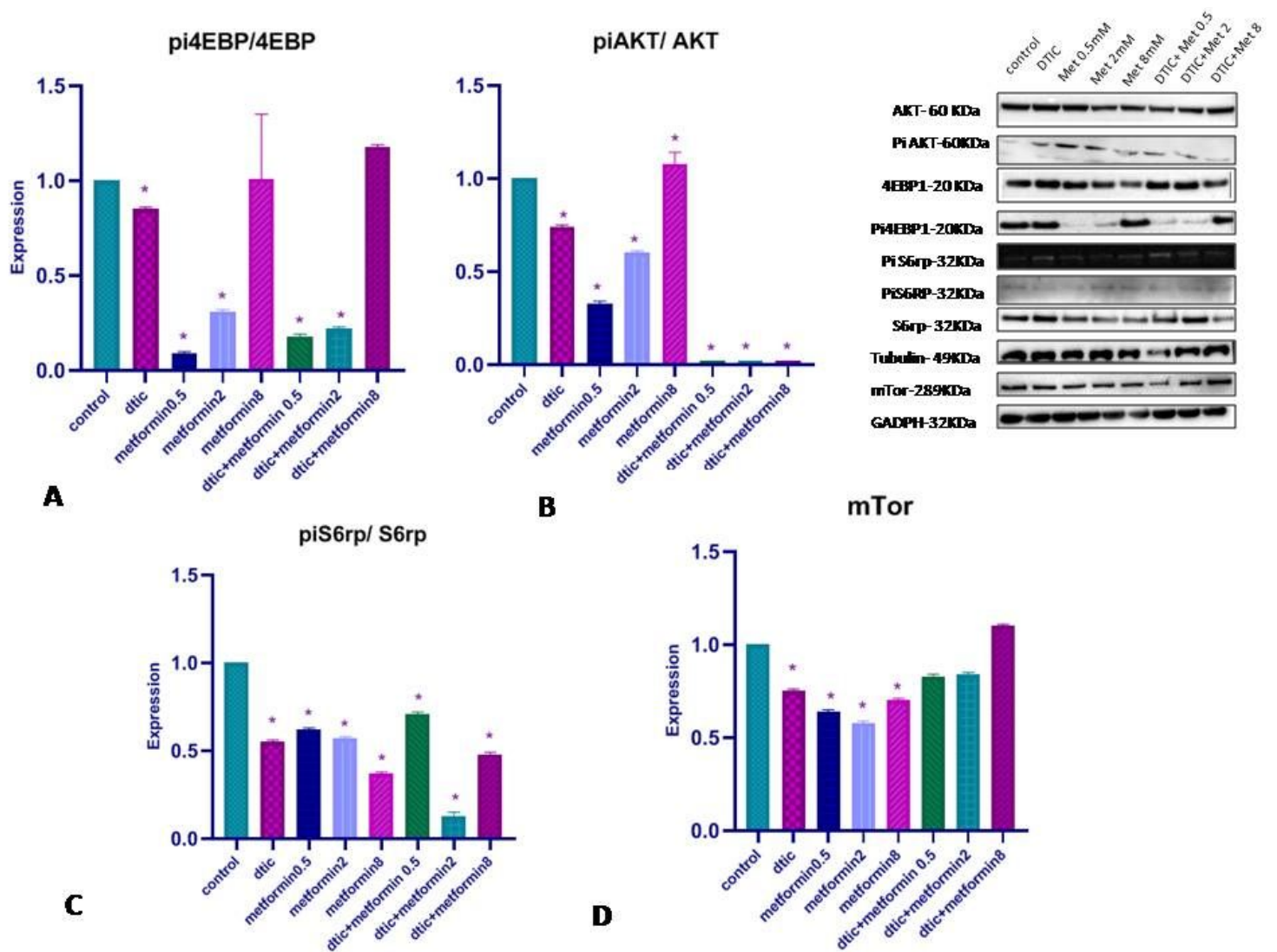


Figure 4

B16F10 cells were treated with dacarbazine 134 $\mu\text{g/ml}$ and different doses of metformin (0.5, 2, and 8 mM) for 24 h. Then, the cell lysates were extracted for Western blot analysis using antibodies specific to some intracellular intracellular pathways, such as A; pi4EBP/4EBP, B; piAKT/AKT, C; piS6rp/S6rp and D; mTOR. The experiment is representative of 3 independent experiments. * $p < 0.05$

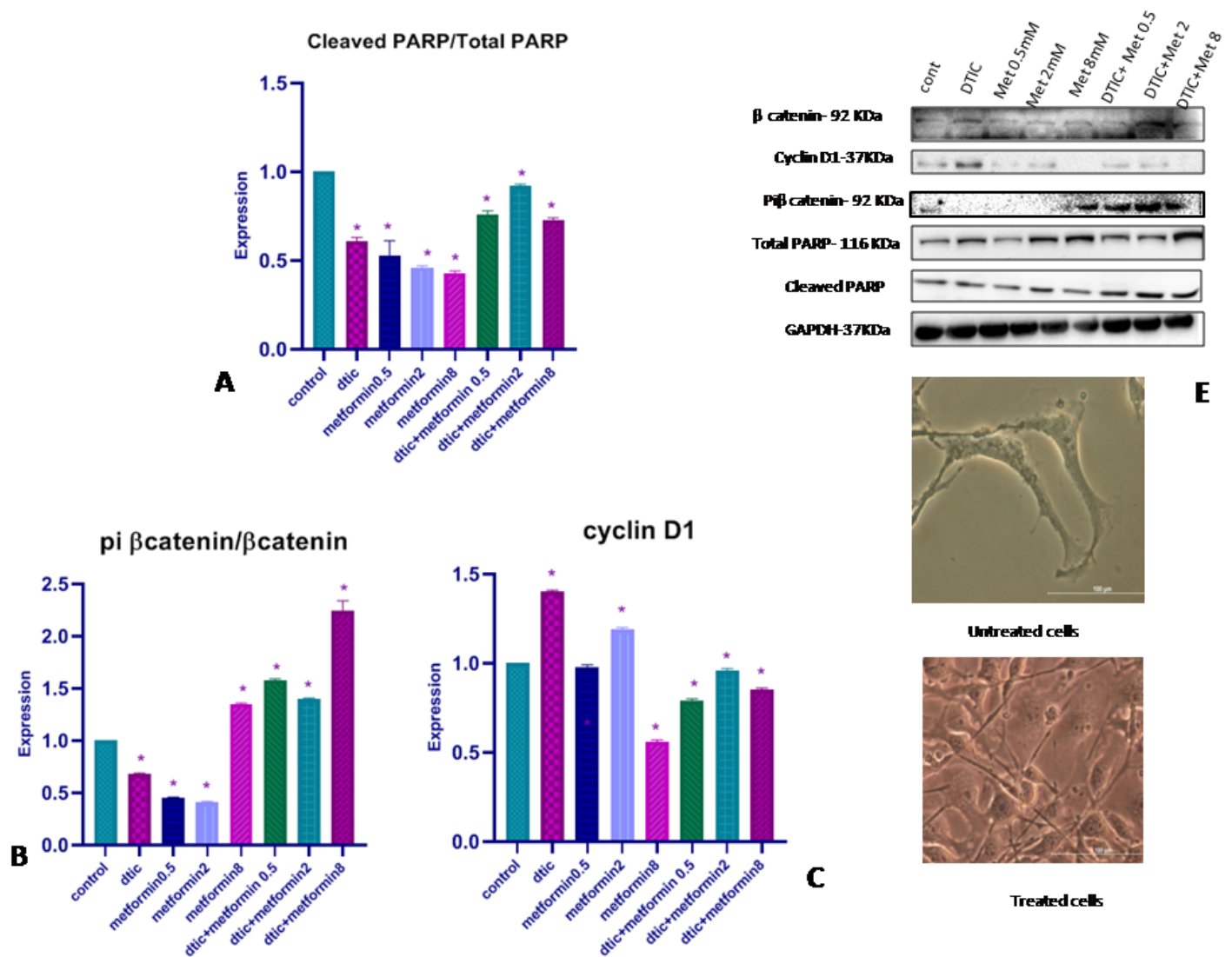


Figure 5

B16F10 cells were treated with dacarbazine 134 μ g/ml and different doses of metformin (0.5, 2, and 8 mM) for 24 h. Then, the cell lysates were extracted for Western blot analysis using antibodies specific to some intracellular pathways, such as A; Cleaved PARP/PARP, B; pi β catenin/ β catenin and C; cyclin D1. The experiment is representative of 3 independent experiments. * $p < 0.05$

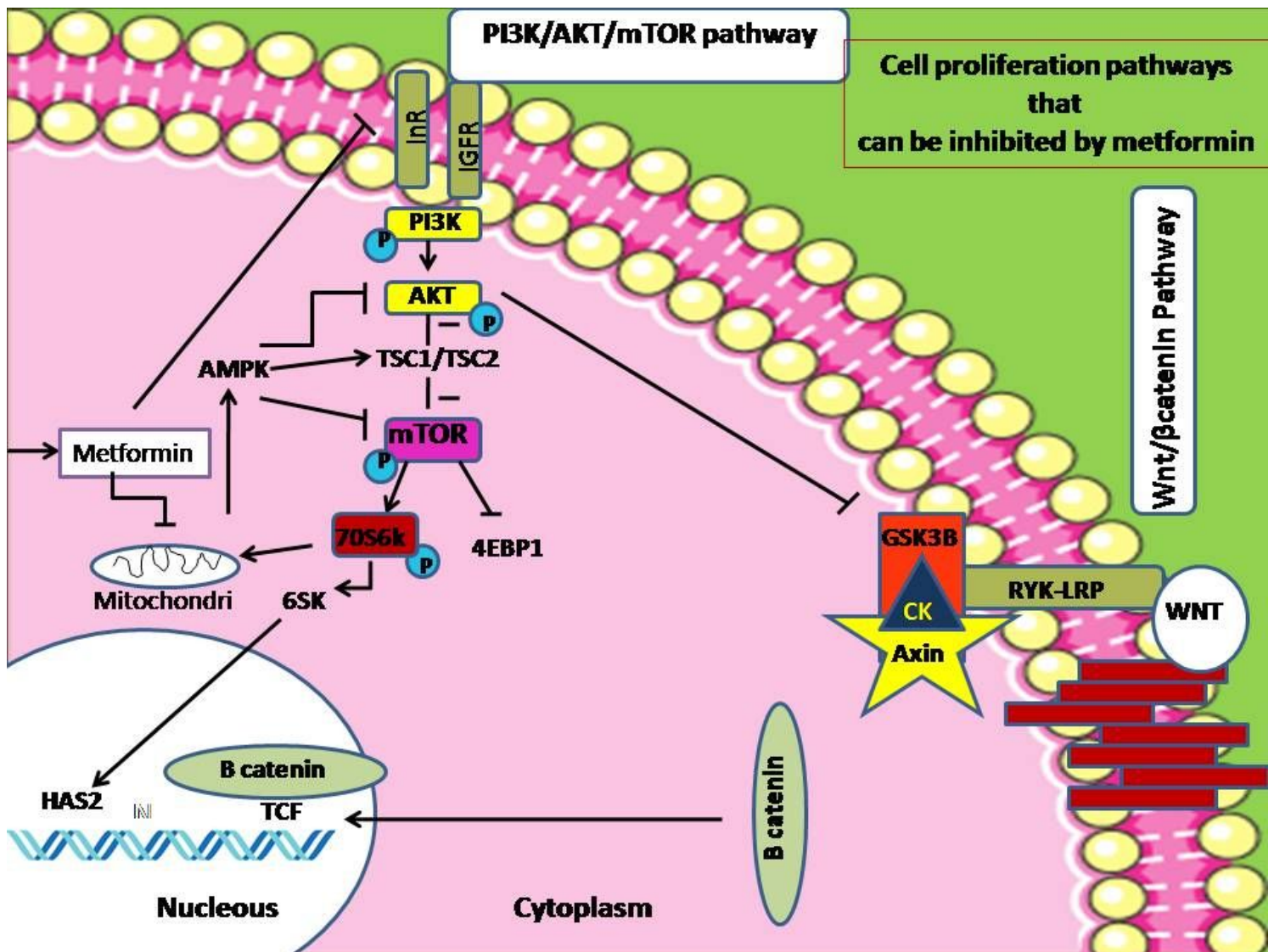


Figure 6

Graphical representation of the mechanistic effect of metformin via modulation of PI3k/AKT/mTOR and Wnt signaling pathways.