In Vitro Inhibition of Melanoma (B16f10) Viability and Colonization through Combining Metformin and Dacarbazine

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Abstract

Background: Dacarbazine is considered as a standard treatment for melanoma, but resistance to anticancer therapy is a major cause of cancer stem cells invasion. In vitro assays have shown that metformin interferes with cell viability, proliferation, and apoptosis.

Method: Melanoma cell line B16f10 was treated with dacarbazine IC50, metformin in different doses (0.5, 2 and 8 mM) and combination therapy. The influence of treating and cell viability was determined with MTT assay, and the effect of treat on colonization was quantified. Changes in cleaved PARP were investigated using immunoblotting. The cytotoxicity effect of Dacarbazine was further analyzed.

Result: Metformin induced cytotoxicity on B16-F10 cells; cell viability, determined at various time intervals (24 and 48 h) and in the presence of different drug concentrations (≥0.7μM), was reduced by ~50% following 24 h. The proliferation rate was evaluated over 24-48 hours and 12 days using varying subcytotoxic and cytotoxic concentrations of metformin (2-8μM), which was reduced in a dose-dependent manner. Resistance cells resulted in slender spindles and better colonization. Finally, metformin decreased the cytotoxicity of dacarbazine and increased apoptosis.

Conclusion: A study with B16-F10 cells showed that the drug combination induces significantly more apoptosis compared with when each drug is individually used. B16F10 was the most sensitive and resistant at a normal dose of metformin and dacarbazine, which is a very encouraging result with regards to the possibility of metformin becoming a new tool for melanoma research and treatment.

Keywords: Melanoma, Combination therapy, Metformin, Colonization

Introduction

Melanoma, with increased incidence over the recent years, is the deadliest type of skin cancer with a high potency and rapid metastasis to other organs. Such cancers develop when unrepaired DNA damage to skin cells triggers mutations, which
leads to skin cells multiplying rapidly and forming malignant tumors.\(^1\) Metastasis is a major cause of mortality in patients with cancer, although surgical removal of tumors can prolong survival.\(^2\) Much work has been done to discover the underlying processes of tumor metastasis; moreover, many of the related factors and mechanisms have been uncovered and investigated. Despite the dramatic effects in a subgroup of patients, most patients with metastatic melanoma do not obtain long lasting clinical benefits from dacarbazine, suggesting the development of resistance.\(^3\) Some chemotherapy drugs such as dacarbazine were administered and found to be able to interact with tumor cells at colonization sites, where the microenviromental constraints are distinct from those found at primary tumors. Dacarbazine is a unique alkylation agent, which is generally considered as the most active agent for remediing malignant melanoma as approved by U.S. Food and Drug Administration.\(^4,\)\(^5\) Melanoma is known for its notorious resistance to chemotherapy, a major obstacle to successful treatment. The basis for drug resistance in melanoma is not well understood and various mechanisms have been postulated, including dysregulation in apoptotic pathways, defects in drug transport, detoxification, changes in enzymatic systems that mediate cellular metabolic machinery, and enhanced DNA repair.\(^6\) Therefore, overcoming drug resistance may have a significant impact on the survival rate. One of the various factors contributing to the sensitivity of drugs is the limited amount of drug reaching the tumor and affecting the tumor microenvironment. Employed as the first line therapy for diabetic mellitus type 2, metformin (1,1-dimethylbiguanide hydrochloride) decreases blood glucose levels and gluconeogenesis in the liver, augments glucose uptake and utilization by the skeletal muscle, reduces insulin\(^7\) and dacarbazine resistance, and increases cytotoxicity effects of chemotherapy.

These results have significant clinical implications. It is possible that the implementation of dacarbazine for the treatment of melanoma is not only ineffective, but also perilous and counter-productive if not combined with other modalities that mitigate the promalignant. Apoptosis plays an important role in development and homeostasis. It is characterized by marked changes such as cellular morphology and cleavage of poly (ADP-ribose) polymerase (PARP). PARP was subsequently shown to be cleaved into 89- and 24-kDa fragments that respectively contain the active site and the DNA-binding domain of the enzyme during drug-induced apoptosis in a variety of cells.\(^8\) Such cleavage essentially inactivates the enzyme by destroying its ability to respond to DNA strand breaks.

The present study aimed to demonstrate that
the treatment of B16 mouse melanoma cells with metformin and chemotherapy drug causes proliferation block and apoptosis, while combination therapy with dacarbazine reduces colonization. Moreover, cPARP change was analyzed and tested to determine whether or not cPARP level could be affected by metformin and DTIC. This study further showed that metformin exerts potent antimelanoma effects in vitro and can be used with chemotherapy drugs.

Materials and Methods

This experimental study is an inductive quantity content analysis of B16f10 cancer cell line. B16f10 melanoma cell was purchased from Pastor Institute (Tehran, Iran) and maintained in DMEM with high glucose and 10% fetal bovine serum (FBS). Eight groups were tested. The first, control group that not treated (negative control), groups 2-4 that treated with different concentrations of metformin (0.5, 2 and 8 mM), group 5 that treated with concentration of 134 μg/mL dacarbazine (positive control), the appropriate dose of dacarbazine + different doses of metformin tested (groups 6-8). The following tests were performed on these groups:

Cell culture

B16f10 cells were obtained from Pastor Institute. In order to achieve ~80% confluence on the culture plate, 5×10^5 cells were plated in DMEM (10% fetal bovine serum and 1% penicillin/streptomycin) at 37°C in a humidified atmosphere with 5% CO₂ and rested for 48 h. The cells were prepared for experiments using conventional trypsinization procedure with trypsin/EDTA and 6 well flat-bottom plates (200 cells/well) were incubated for tumor colony form assay and 96-well flat-bottom plates (5×10^3 cells/well) were incubated for cell viability assessment. Cells were rested for 24 h and then treated with appropriate concentrations of metformin hydrochloride (0.5-2-8 mM) and/or lethal dose of 50% dacarbazine (134μg/mL).

Cell viability

The cell number and mitochondrial dehydrogenize activity, as the index of cell viability, were determined with MTT (3,4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay (Mijatovic, E). The MTT absorbance, proportionate to the number of viable cells, was measured in ELISA (Enzyme-linked immune asorbent assay) reader at 570 nm.

Establishment of dacarbazine-resistant and tumor colony form assays

To ensure that the viability of the cells was enhanced after 48 h as the result of dacarbazine resistance; the cells became drug-resistant at first
and then the dacarbazine-resistant cells colonization was investigated. B16f10 cells were plated at a culture plate. After 24 hours, the medium was changed, 500 μg/mL of dacarbazine was added and the cells confluence (the number of cells) was reduced to less than 20% because of drug influence. Regular medium was changed approximately every 2 to 3 days as the cells repopulated. If the cells survived in this media, treating was repeated four times with a gradual increase in the dacarbazine concentration (up to 1000/mL) in the last cycle. The resultant cells continued to grow in regular medium with no drug. After that, resistant cells and normal cells were treated with a lethal dose of 50% of dacarbazin (134 μg/mL, 0.66±0.2 μM) and/or combination with metformin (2-8 mM) cells sensitive to treatment or media alone. The culture medium was then removed and fresh DMEM supplemented with 10% FBS was added. Two hundred cells incubated in 6-well flat-bottom plates were plated in 2.5 mL. The cells were maintained in fresh culture medium for the next 10 days at 37°C in a 5% CO₂ atmosphere and humidified incubator to form colonies. Media were changed every two days. After 12 days, cultures were fixed using 3.7% formaldehyde in PBS and the colonies were then stained with Giemsa. Cultures were visualized under an inverted microscope and colonies larger than 100 μm were manually counted from 5 random fields per dish.

**Western Blot**

Cell pellets were lysed and separated on a 12% sodium dodecyl sulfate-polyacrylamide gel and transferred to nitrocellulose membranes. The antibody for total PARP and cPARP-1 (Cell Signaling Technology, Boston, USA) was added and incubated at 4°C overnight. Having the membrane washed, it was incubated with secondary rabbit antibody at room temperature for 1.5 hours. The bands appeared using ECL (BIO RAD) and were analyzed using an image J analyzer.

**Statistical analysis**

The statistical significance of the differences was analyzed by student t-test or one-way analysis of variance (ANOVA). The value of \( P < 0.05 \) was considered statistically significant.

**Results**

Treatment of metastatic melanoma remains challenging because it is difficult to obtain long-term clinical benefits, even with the novel approved drugs. Metformin therapy inhibited the proliferation and colonization of B16f10 at doses of 2 mM and higher (Table 1). Two of the analyzed doses are commonly used in patients with standard dosing of metformin (under 3 mM) and 8 mM. At first, IC50 of dacarbazine on cell line was examined. IC50 without metformin was 134 μg/mL. Different doses of dacarbazine were examined (0.6, 0.7, 0.8 and 3 mM) and IC50 dose

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**Figure 3.** The B16F10 cells were plated in normal media conditions and media with dacarbazine. The morphology of normal melanoma and resistant melanoma. Images were taken with inverted microscope.
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was specified. In 3mM treated, cell death was very high; thus, no significant difference was observed in the control group. Therefore, the experiment was continued with IC50 dose. With a fixed dose of dacarbazine, a significant increase in cytotoxicity was observed with the increasing doses of metformin. MTT assay performed after 24 (Figure 1) and 48 hours (Figure 2) demonstrated that after 24 h metformin was able to reduce cell viability with or without dacarbazine (Table 1 and 2). The result of 48h did not show any significant statistical changes, which is because of the prolonged exposure of melanoma cells to dacarbazine resistant cell lines. Dacarbazine increased the viability for a long-term treatment, while combination therapy with metformin decreased the viability.

The use of metformin in normal doses after 24 hours reduced the survival of cells by 10%, while the combination of metformin and dacarbazine resulted in a 50% reduction in cell viability (Table 1). Following 24 hours of treatment (Figure 1), metformin with concentrations of 2 and 8 mM in combination with dacarbazine significantly impaired the viability of B16f10 cells by 52.50% and 50.66%, respectively. Treatment of dacarbazine with normal doses (2mM) and high doses (8mM) of metformin had significance differences compared to control of 0.05 and 0.01, respectively. Other doses of metformin without dacarbazine did not have any significant effects on the viability of cells compared to the control group. Only dacarbazine with the concentration of higher than IC50 (0.7 mM), significantly reduced the viability of B16f10 cell compared to the control group. No significant difference was observed between the viability of treated cell and other doses of metformin (0.5, 2 and 8 mM). The viability of cells treated with dacarbazine increased after 48 hours, in comparison with the control; however, combination treatment with metformin reduced cell viability, though not significantly (Figure 2).

Changes in the morphology of a single B16f10 cell following dacarbazine treatment

Changes were investigated in the surface morphology of B16f10 cells treated with different concentrations of dacarbazine at different times. In the control group, the cells had a regular shape and the surface was relatively smooth and intact. Following treatment by dacarbazine, significant

| Table 1. Dose-dependent toxicity of metformin alone and combined with chemotherapy dacarbazine |
|----------------------------------|-----------|---------|---------|---------|
| **Case**                        | **Mean**  | **N**   | **SD**  | **SEM** |
| Control                         | 100       | 4       | 00.0    | 4       |
| Metformin .5 mM                 | 82.00     | 3       | 19.07   | 11.01   |
| Metformin 2 mM                  | 93.75     | 4       | 6.70    | 3.35    |
| Metformin 8 mM                  | 85.33     | 3       | 9.07    | 5.23    |
| DTIC IC50                       | 68.66     | 3       | 15.37   | 8.87    |
| DTIC+Metformin .5 mM            | 62.75     | 4       | 9.322   | 4.66    |
| DTIC+Metformin 2 mM             | 47.50     | 4       | 17.59   | 8.79    |
| DTIC+Metformin 8 mM             | 80.33     | 3       | 15.04   | 8.68    |

SD= Standard deviation; SEM= Standard error of the mean

Figure 4. Representative images (10×) of the formed tumor colony. Images are representative of 10-day old cultures.
changes were observed in cell morphology. The cell gradually shrank into slender spindles and more melanin. The image shows that the average cell height increased with dacarbazine concentration, but was not significantly correlated with the dacarbazine treatment time (Figure 3).

Colonies with more than five cells were counted in 20 randomly-selected fields (×100) after 10 days using an inverted microscope (Figure 4); the percentage of colony formation was further calculated to investigate whether metformin could prevent melanoma colonization in vitro. In the presence of metformin, an average two-fold reduction was observed in the number of tumor colonies (Figure 5). The test was repeated with different doses of metformin and in the absence of metformin. The cells treated by metformin and dacarbazine generated fewer colonies in comparison with treatment by only metformin. No significant difference was found between 2 and 8 mM metformin in colonization, yet Metformin in normal dose (2 mM) was able to cause a two-fold reduction in colonization with dacarbazine.

PARP is a terminal proapoptosis protein cleaved to produce the active forms. To determine the effect of the drug on apoptosis, the amount of total PARP and cleaved PARP was measured by Western blot. Metformin treatment alone (especially at 2 mM) and in combination with dacarbazine for 24 hours increased apoptosis in melanoma cell B16f10, as compared with the control group (Figure 6A and 6B). As shown in figure 6, a decrease in cleaved PARP was observed in cells treated with DTIC and dacarbazine with metformin in normal dose (2 mM), indicative of DNA fragmentation and typical of apoptosis. The induction of apoptosis was further corroborated by WB analyses showing PARP cleavage in metformin-treated cells.

Discussion

In the present study, the expression level of cleaved PARP was investigated. The results showed that metformin therapy increased the amount of cleaved PARP; thereby, slowing the growth of melanoma cells and colonization. According these data, metformin was demonstrated to have anticancer activities on melanoma cell line.

This study also showed that metformin alone in normal doses and combined with dacarbazine were able to inhibit the highest colonogenicity. The data exclusively revealed that metformin destroyed cancer cells by interfering with specific molecules that drive the growth and spreading of the tumor. Tumor differentiation could be consistent with ability of colonization.6

![Figure 5](image)

**Figure 5.** The percentage of colony forming. Metformin in normal dose inhibited the colony forming better than high dose and dacarbazine alone.
Administration of metformin with chemotherapy drugs can increase the cytotoxicity effects of the drug and prevent cellular resistance. Metformin also prevents metastasis and invasive cancer cells by diminishing stem cell populations. In this study, exploring the effects of a combination of metformin and cytotoxicity effects on a subpopulation of resistant cells for colonogenic properties, the antimelanoma activities of metformin were primarily examined. MTT data demonstrated that dacarbazine exerted cytotoxicity to cells in a dose-dependent manner, while metformin treatment alone and in combination, only showed minor cytotoxicity. This data showed that changes in some fundamental properties is necessary for cancer cells to clone in vitro and suggested that metformin might be a novel antimelanoma agent with low cytotoxicity to normal cells. The exposure time for viability was short (24 h) and cloning (12 days) suggested that metformin induced a change in the proliferative capacity of melanoma cell population. Dacarbazine through DNA methylation caused high MGMT expression, Bcl expression, AKT activation and NF-kB activation, creating resistance to the drug and increasing the proliferation of cancer cells after the initial response. Metformin combined with MGMT, PI3K/AKT/mTOR, and proteasomal inhibition prevented the proliferation of cancer cells.

The presence of cleaved PARP might be related to the activation of DNA repairing pathways and could be interpreted as a DNA damage during treatment. This must be due to the activation of apoptotic pathways. PARP-1 is a protein of 113 kDa cleaved to produce fragments of 89 and 24 kDa in a wide variety of cells undergoing apoptosis, hence a hallmark of apoptosis. It is also thought that PARP cleavage serves to prevent the futile repair of DNA strand breaks during the

![Cleaved PARP](image)

**Figure 6.** Effect of DTIC and metformin was measured by Western blot analysis. The expression of cPARP enhanced in alone and combination therapy.
apoptotic program. The presence of an active and uncleavable PARP might interfere with the onset of apoptosis. In this study, it was shown that interference with PARP cleavage actually enhanced apoptosis. In conclusion, combination treatment with dacarbazine and metformin in normal dose can induce apoptotic pathways.

Since metastatic melanoma is unresponsive to therapy, that is currently available, research is now focused on different treatment strategies such as combining surgery, chemotherapy, and radiotherapy. The molecular basis of resistance to chemotherapy seen in melanoma is multifactorial, comprising defective drug transport system, altered apoptotic pathway, deregulation of apoptosis and/or changes in enzymatic systems mediating cellular metabolic machinery.

Understanding the alterations in molecular processes involved in drug resistance may be conducive to develop novel therapeutic approaches to treat malignant melanoma. It is further desired to overcome chemoresistance in melanoma by a deeper understanding of the chemical nature approach (chemotherapy) and apoptotic pathways. Further in vitro, in vivo and clinical controlled studies are required to establish this hypothesis.

We believe that metformin cotreatment with chemotherapy may prevent melanoma resistance and improve long-term survival. More studies are proposed to show the evidence of the mechanism underling the above effects of metformin.

Finding

A study with B16-F10 cells showed that the drug combination induced significantly more apoptosis than each individually used drug. B16F10 was the most sensitive and resistant at normal doses of metformin with dacarbazine, which is a promising result for the possibility of metformin becoming a recent tool for melanoma investigation and treatment.

We believe that metformin cotreatment with chemotherapy may prevent melanoma resistance and improve long-term survival. Further studies are required to provide evidence as to the mechanisms underling the above effects of metformin.

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Conflict of Interest

None declared.

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