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Combination treatment with ornidazole and dacarbazine inhibits proliferation, cell migration and induces DNA damage in melanoma cells

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Metastatic cancers are responsible for 90% of cancer related deaths and melanoma is known as one of the deadliest cancers. Dacarbazine (DTIC) for metastatic melanoma has become an approved first-line treatment in routine clinical practice. However, response rates to single-drug therapy with dacarbazine are quite low. Therefore, combination drug therapy as a method of treatment that combines two or more therapeutic agents is the cornerstone of cancer therapy. Here, we examined the effects of the combination of ornidazole, a derivative of 5-nitroimidazole which is active against protozoa and anaerobic bacteria, and DTIC on melanoma cells to investigate novel advanced combination therapies for melanoma. Doses in this study are 0, 800 and 1200µg/mL for ornidazole, 0, 5, 25, 50, 100, 200, 300, 600, 1200µM/L for DTIC and 800µg/mL+100µM/L, 800µg/mL+200µM/L, 1200µg/mL+100µM/L, 1200µg/mL+200µM/L for ornidazole+DTIC combination. Treatment effect of ornidazole and DTIC as a single-agents and in combination on cell viability was investigated with crystal violet and MTT assays. As well as the effect of them on migration ability was assessed by wound healing assay, the effect of them on DNA damage induction was evaluated by comet assay in B16F10 melanoma cells *in vitro*. Our data showed that combination treatment with ornidazole and DTIC markedly inhibited cancer cell proliferation and migration. DNA damage was also significantly induced by this combination treatment. Our study showed that ornidazole/DTIC combination drug therapy has more effective therapeutic potential for melanoma compared to DTIC therapy alone. In conclusion, our findings suggest that combination therapy with ornidazole/ DTIC could serve as a new and valuable approach for melanoma treatment.

Keywords: Cancer cells, Combined therapy, Tumour targeting

Melanoma has the highest mortality rate of all skin cancers[1]. Melanoma has a poor prognosis and its incidence is increasing rapidly worldwide[2,3]. Melanoma develops in the melanocyte cells that produce the dark pigment (melanin) that gives skin its color[4]. Chemotherapy drugs and radiotherapy are clinically used to treat melanoma. Intravenous administration of single-agent dacarbazine (DTIC) that is a cell cycle nonspecific antineoplastic alkylating drug[5] is commonly used for the treatment of metastatic malignant melanoma[6,7]. However, response to treatment with DTIC alone were observed in 5% of patients, while frustration was observed in 10 to 25% of patients[8,9]. The biggest obstacle to successful treatment of metastatic melanoma is its resistance to chemotherapy[10]. Therefore, the importance of DTIC single-agent and DTIC based combination chemotherapy is increasing[11,12] and a new treatment approach is needed to be developed. Tumour heterogeneity requires development of new treatment strategies and new drugs targeting multiple pathways. Some scientific research points to natural agents and its bioactive compounds as they have fewer side effects compared to existing synthetic drugs used for chemotherapy[13,14]. Because metastatic melanoma shows a poor response to single chemotherapeutic agents[15], this has led to further investigation of combinations of these agents to achieve better results. Studies have shown that combination chemotherapy can increase response and survival rate[16-18]. Many studies have also shown that antitumour activity of cancer drugs can be improved by using cytotoxic agents in combination[19].

Ornidazole is a nitroimidazole antiprotozoal agent used in amoeba and trichomonas infections. It enters the cell by passive diffusion of protozoal and

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anaerobes, binds to cell DNA and inhibits DNA synthesis[20]. In the literature, there is no study on melanoma of ornidazole and DTIC to be administered in combination. At the same time, it is thought that our study will contribute to reducing the cost of drug development. To determine the potential therapeutic efficacy of ornidazole and DTIC combination for the treatment of melanoma, we used B16F10 melanoma cells in this study because our previous study had demonstrated the protective effect of ornidazole on cell death in melanoma cells through the hedgehog signaling pathway *in vivo* and *in vitro*. Also it was found in our previous study that ornidazole significantly induces apoptosis and ER stress-mediated apoptosis in B16F10 cells both *in vitro* and *in vivo*[21]. Given the lack of treatments for metastatic melanoma, therefore in this study it was aimed to investigate the combination efficacy of DTIC and ornidazole in the treatment of malignant melanoma.

Materials and Methods

Cell line and reagents

Ornidazole (cat. no: 16773-42-5, Sigma, Germany), Dacarbazine (DTIC, cat. no: CAS 4342-03-4, Sigma, Germany) were purchased from Sigma (Sigma, Germany) and B16F10 mouse melanoma cell line was purchased from the American Type Culture Collection (ATCC, CRL-6475, Manassas, VA, USA).

Cell culture

Melanoma cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, cat. no: 11965084, Thermo) supplemented with 100 mg/mL streptomycin (cat. no: 3810-74-0 Sigma), 100U/mL penicillin (cat. no: P4443, Sigma) and 10% fetal bovine serum (Sigma). All cell cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2.

Determination of Cell viability by MTT assay

The effects of DTIC and ornidazole on melanoma cell growth were assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) assay. Cells were seeded in 96-well plates (densities of 10^4 cells per well) for 24h. Then, the cells were treated with only ornidazole (0, 800 and 1200µg/mL) or only DTIC (0, 5, 25, 50, 100, 200, 300, 600, 1200µM/L) or combination of them (800µg/mL+100µM/L, 800µg/mL+200µM/L and 1200µg/mL+100µM/L, 1200µg/mL+200µM/L) for various periods of time (24,48 and 72h). For combination treatment, the cells were treated with firstly ornidazole and 1h later DTIC at different doses. We performed MTT test to determine IC50 values of DITC. So, we tested more different doses of DTIC. Beacuse in our previous work, we have already obtained IC50 values of ornidazole[21], we did not test for ornidazole again. So, only DITC doses in MTT test are different from crystal violet. They were incubated with 100 µL/well MTT solution and solubilizing buffer (20% sodium dodecyl sulfate, Tris-Hydrochloride 1M Solution, ddH_2O) for 4h. After overnight incubation, the absorbance of each plates values were measured at 590nm to determine cell viability. Each experiment was repeated for at least three times.

Crystal violet assay

Cells were plated at 70,000 cells/well in 96-well microplate, and allowed to adhere overnight under humidified atmosphere containing 5% CO_2 at 37°C for 48h. The cells were treated with only ornidazole (0, 800 and 1200µg/mL) or only DTIC (0, 100, 200µM/L) or combination of them (800µg/mL+100µM/L, 800µg/mL+200µM/L and 1200µg/mL+100µM/L, 1200µg/mL+ 200µM/L) for 48h. For combination treatment, the cells were treated with firstly ornidazole and 1h later DTIC at different doses. Dead cells and media were washed away after treatment and the remaining cells stained with 0.5% crystal violet solution (Sigma-Aldrich) at 25°C for 30min. The plates were rinsed with water and dried. The amount of purple dye retained indicates the number of cells remaining adhered to the well, the absorbance values of each well was determined spectrophotometrically at 570nm.

Wound healing assay

The effect of ornidazole and DTIC combination treatment on the potential of melanoma cell migration was determined by wound healing migration assay. 80,000 cells were seeded into 6-well plates and incubated at 37°C until reaching 80–90% confluence. Cells were washed with PBS and cultured in DMEM-medium containing 0.05% FBS for 16h. The confluent cell monolayer was scratched with a 200µL sterile pipet tip and thoroughly washed with PBS[22]. Then they were incubated with ornidazole (0, 800, 1200µg/mL), DTIC (0, 100, 200µM/L) and ornidazole+ DTIC (800µg/mL+100µM/L, 800µg/mL+200µM/Land 1200µg/mL+100µM/L, 1200µg/mL+200µM/L) for 0, 8 and 24h. Digital camera installed on microscope (Leica, Germany) was

used to photograph the wound closure. Image J software was used to measure the wound area[23].

Comet assay

Melanoma cells that were treated with Ornidazole and DTIC (Ornidazole; 0, 800 and 1200µg/mL, DTIC; 100, 200µM/L and ornidazole+DTIC; 800µg/mL+100µM/L, 800µg/mL+200µM/L and 1200µg/mL+100µM/L, 1200µg/mL+200µM/L) for various periods of time (24 ,48, 72h) were embedded in low-melting agarose at a ratio of 1:10(v/v) on entirely frosted microscope slides previously coated with normal melting agarose layer. The agarose gel with embedded cells was covered with a slide. After the gel solidified, the slide was removed and LMA was poured on the sample. After solidification of LMA, cells were exposed to lysis buffer (1% Triton X-100, 2.5M NaCl, 10mM Tris-HCI, 100mM EDTA, pH-10) at 4°C for 1h. Following lysis for 1h at 4°C in lysing, slides were placed in gel electrophoresis unit filled with electrophoresis buffer (1mM EDTA, 300mM NaOH, pH 13) for 40min to allow the unwinding of the DNA. Electrophoresis was then performed at 25V for 20min. Subsequently slides were neutralized with 0.4M Tris-HCl buffer (pH 7.5) for 15min and then with 96% ethanol for 10min. After staining the slides with ethidium bromide for 10min in the dark[24], the slides were viewed at ×40 magnification using fluorescence microscope (Leica, Germany). Measured Olive Tail Moment (OTM) was derived from 100 melanoma cells per sample group. Data were analysed with Comet Score software (CaspLab).

Statistical analysis

Statistical analysis was performed using Graph Pad Prism 5.0 (GraphPad Software, Inc.) and data are presented as the mean±SD (standard deviation) of three independent experiments. Data of crystal violet and MTT assays were analysed one-way ANOVA with multiple comparisons were performed by Dunnet's testing post hoc test. For scratch assay (wound healing assay) and comet assay, two-way ANOVA test followed by Bonferroni post hoc test was performed. $P < 0.05$ was regarded as statistically significant.

Results

In this study B16F10 cells were used, we demonstrate that combined therapy of DTIC and ornidazole could be more effective.

Combination treatment of ornidazole and DTIC reduced cell viability in B16F10 cells

MTT assay results

To evaluate the efficacy of DTIC with ornidazole against melanoma, we investigated the viability of melanoma cell lines. Firstly IC_{50} values (the concentration of drug which results in 50% cell viability) of DTIC on B16F10 cells was determined as 600µM/L for 24h, 600µM/L for 48h and 300µM/L for 72h by MTT assay. DTIC decreased cell viability of B16F10 cells at concentrations more than 100µM/L for 24h, 25µM/L for 48h and 5µM/L for 72h. We found that this decreases were statistically significant ($P<0.05$) (Fig. 1). B16F10 cells were treated with ornidazole (800, 1200µg/mL) in combination with DTIC (100, 200µM/L) for various times. We

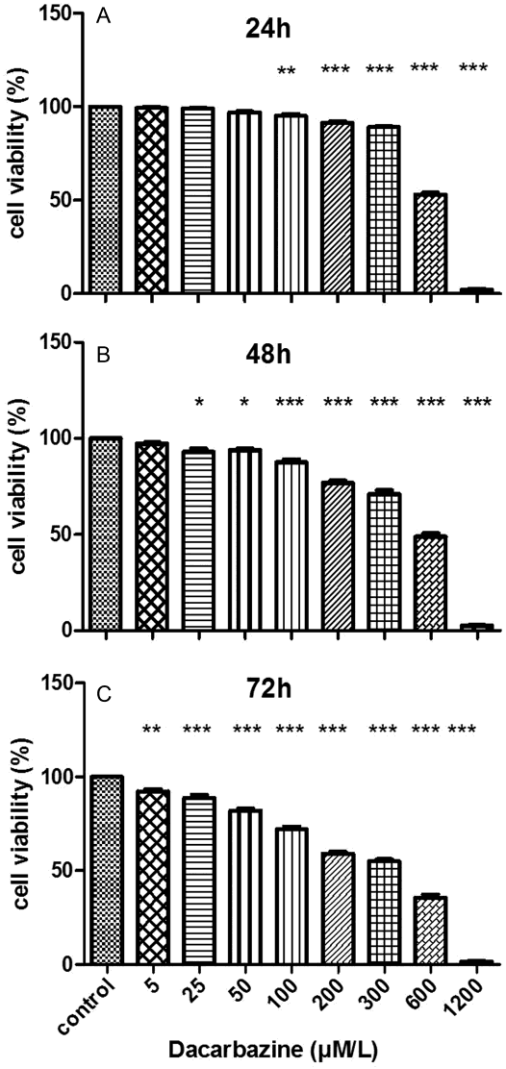

Fig. 1 — Evaluation by the MTT assay of viability of melanoma cells after 24h, 48h and 72h of treatment with different concentrations of DTIC. *P< 0.05, **P< 0.01, ***P< 0.001.

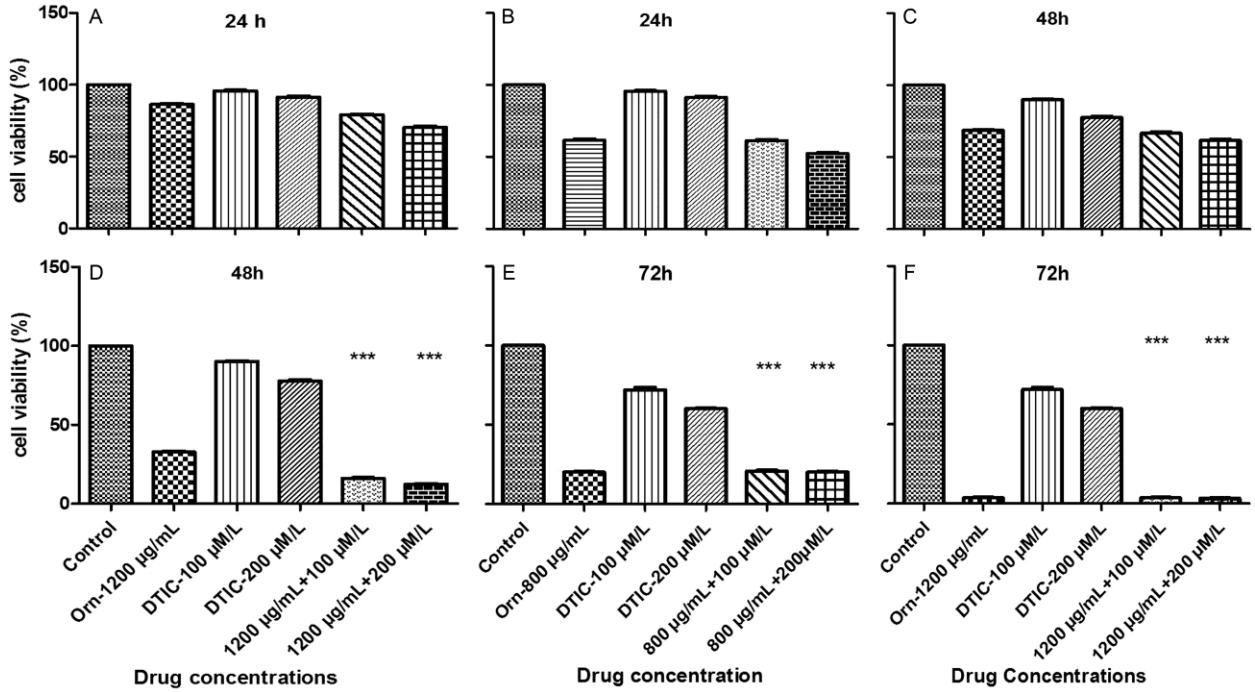

Fig. 2 — Cells were exposed to different doses of ornidazole (Orn), DTIC, and combination therapy and then cell viability was determined 24, 48 and 72h after incubation at 37°C by MTT assay. Data shown are the mean ± SD of 3 independent experiments. ***indicates statistical significance compared to the respective control condition ($P< 0.001$).

observed that ornidazole synergistically enhances the effect of DTIC on cell death compared to DTIC treatment alone (Fig. 2). There was a significant relationship between the control and all the treatment groups. Cells exhibited cell viability of less than 80% and 65% when exposed to DTIC and ornidazole concentrations of 800µg/mL+100µM/L and 1200µg/mL+100µM/L for 24h. The viability of the cells was significantly reduced by all tested concentrations when exposed to the drugs for 48 and 72h.

Crystal Violet assay results

Crystal violet staining was performed to observe the effects of the combination of ornidazole and DTIC on cell viability of B16F10 cells. We observed that ornidazale combined with DTIC concentrations greater than 800µg/mL+100µM/L and 800µg/mL+200µM/L reduced cell viability of melanoma cells. This combination treatment reduced cell viability relative to ornidazole or DTIC alone (Fig. 3).

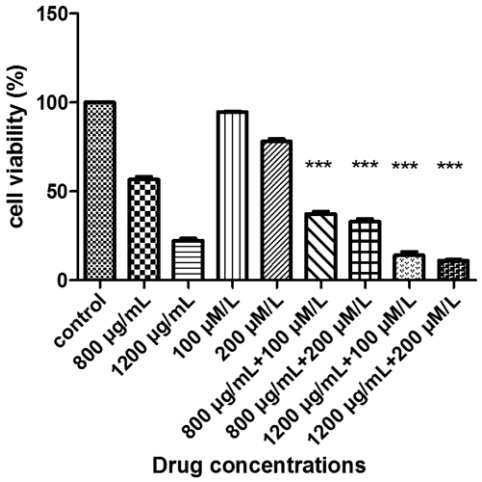

Fig. 3 — Effect of combination therapy on melanoma cell viability by crystal violet assay**.** The cells were treated with Ornidazole (800,1200µg/mL), DTIC (100,200µM/L) and their combination (800µg/ml+100µM/L, 800µg/ml+200µM/L, 1200µg/ml+100µM/L, 1200µg/ml+200µM/L) for 48h. ***$P< 0.001$.

Ornidazole and DTIC combination therapy decreased cell migration in melanoma cells

The weakening of the migration property of cancer cells is very important as it is related to their metastatic potential. Therefore, to test the hypothesis that the combination of ornidazole and DTIC reduces cancer progression, the effect of the combination therapy on melanoma cell migration was evaluated using a wound healing assay. As shown in Fig. 4, the wound was 86% closed within 24h after the scratch in control cells. However, compared with control cells,

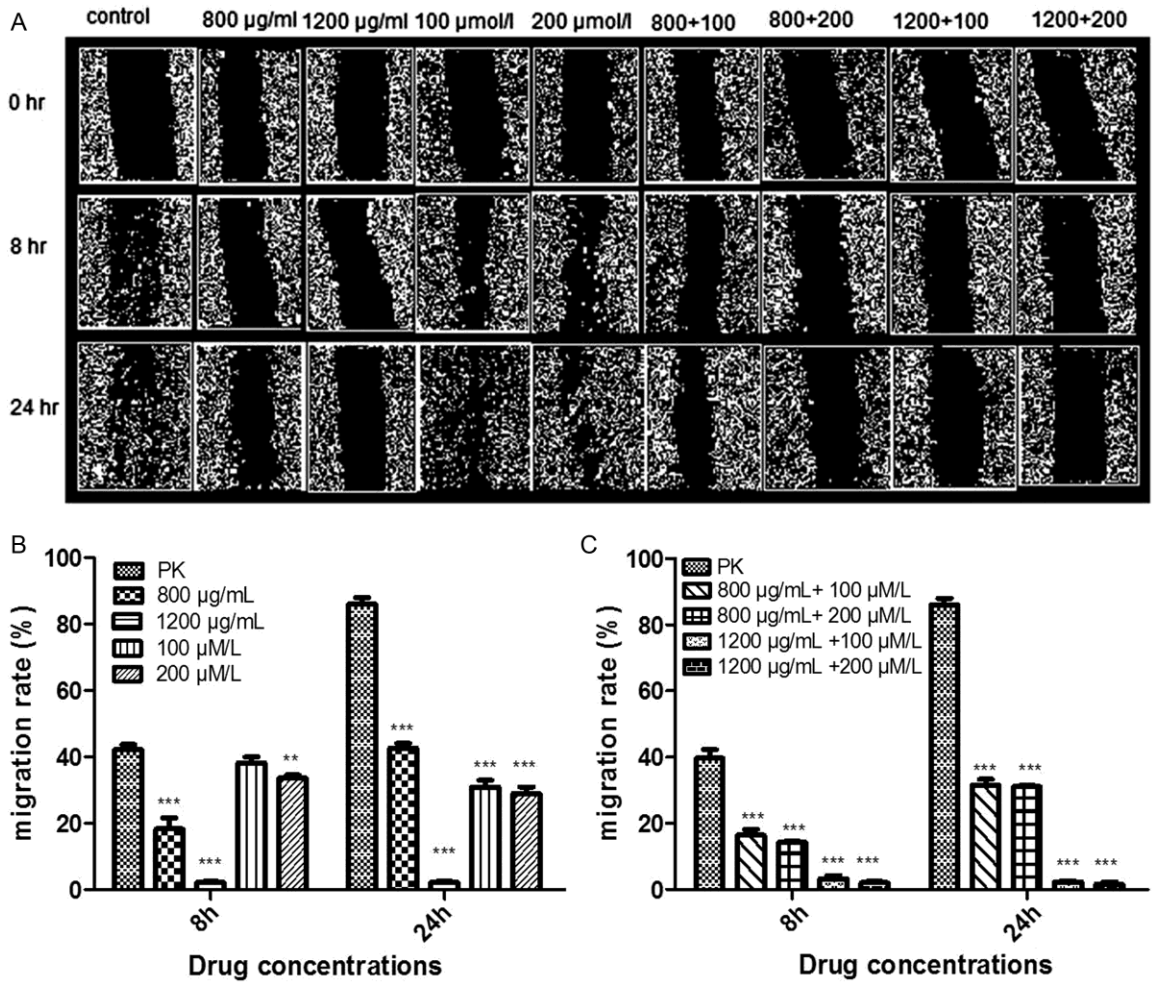

Fig. 4 — Effects of Ornidazole (800, 1200µg/mL) and DTIC (100, 200µM/L) combination treatment on cell migration. (A) Migration of B16F10 cancer cell lines was evaluated by wound-healing assay. Cells images were captured with a microscope at 0, 8 and 24 h. (B-C) The cell wound closure rate. The experiments were performed in triplicate, ***P<0.001 compared the control.

wound closure (after 24h) resulted in approximately 42.7%, 42.6%, 31% at concentrations of 200µM/L (DTIC only), 800µg/mL (ornidazole only) and 800µg/mL+200µM/L (Combination therapy) in melanoma cells, respectively. The wound healing assay results showed that ornidazole and DTIC combination treatment reduced melanoma cell migration compared with control groups.

Enhanced effect on DNA damage ornidazole and DTIC combination therapy

We investigated whether DNA damage of B16F10 cells induced by the single and combination therapy with ornidazole and DTIC. The results showed that combination treatment of ornidazole and DTIC significantly induced DNA damage at 24, 48 and 72h compared to control groups, leading to the development of comet tails in B16F10 cells (Fig. 5). These results suggest that ornidazole and DTIC may have synergistic effects on DNA damage when used in combination in the B16F10 cells.

Discussion

The incidence of melanoma is increasing rapidly worldwide[25,26]. It is the sixth most common type of cancer in women and the fifth most common in men[2]. Current treatments for melanoma, such as radiotherapy, targeted therapy and immunotherapy[27], have many side effects and systemic exposure to drugs that will lead to premature degradation of the drug[28,29]. Early stage melanoma is treatable, but advanced metastatic melanoma develops resistance to treatment and the response to treatment of more advanced cases is low[30]. Although DTIC has been used in the treatment of malignant melanoma for the last 40 years, the patient's response to single drug therapy is limited and temporary[31]. Previous

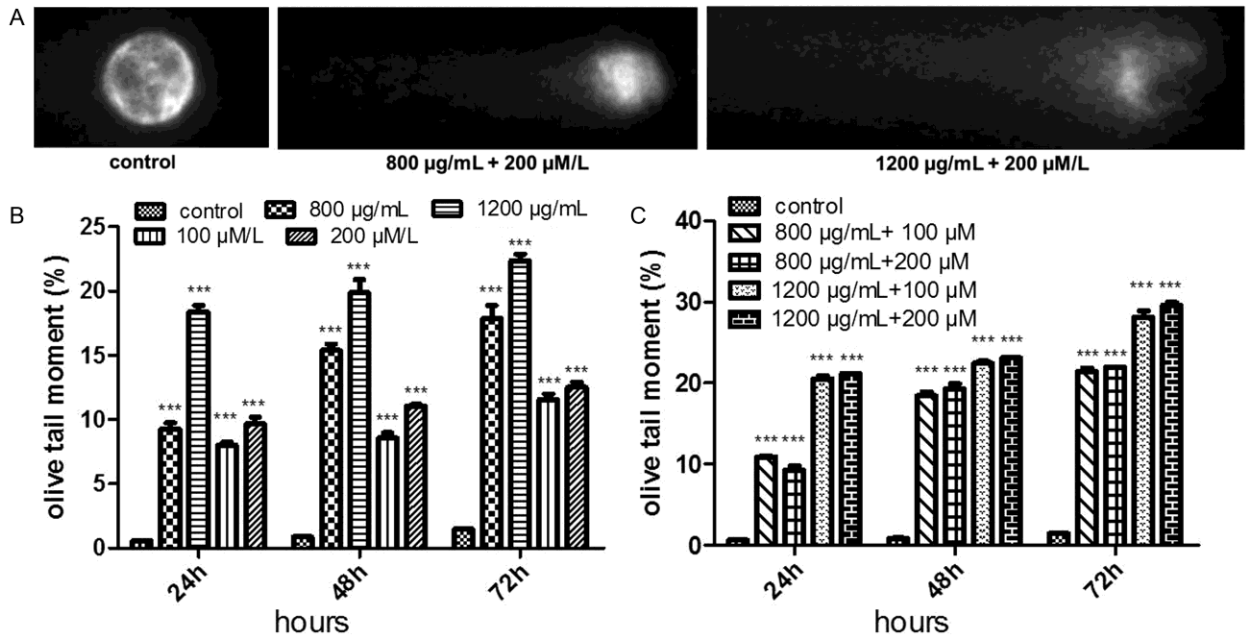

Fig. 5 — DNA damage of B16F10 cells treated with ornidazole (800, 1200µg/mL) and DTIC (100, 200µM/L) at different concentrations for 24h, 48h and 72h was measured by comet assay. (A) Comet images of the B16F10 cells in treatment and control groups were observed under fluorescent microscope. (B) Mean tail moment (µm) represents the damage distribution in the attached cells. [Olive moment = (tail mean-head mean) × % of DNA in the tail]. The experiment was done in triplicates.

researches has shown that resistance to DTIC in melanoma treatment is associated with lower levels of apoptosis and increased levels of antiapoptotic proteins[32]. Therefore, it creates a need for more effective new treatment strategies. To our knowledge, this is the first study to investigate the therapeutic potential of the combination of ornidazole and dacarbazine in the treatment of malignant melanoma. Some studies have shown that combination therapy improves treatment outcomes and results in superior therapeutic effects, especially when a synergistic anticancer activity is achieved[33]. Treatments that combine therapeutic agents may increase the effectiveness of treatment because they target different carcinogenic pathways compared to single-drug therapy[8,34]. Current studies suggest that DTIC combined targeted therapy may improve overall response and one year survival[16,35]. Therefore, the combination of anticancer treatments is clinically attractive. In our previous study, we demonstrated that ornidazole effectively decreased viability and migration ability of CD133+ melanoma cells. We also showed that ornidazole treatment significantly suppressed tumour growth in a mouse melanoma model.[21]. In this study, we investigated the therapeutic potential of the combination of ornidazole, which is an effective drug in the treatment of infections caused by protozoa and anaerobic bacteria, and DTIC used in the treatment of melanoma, *in vitro*. Our MTT and crystal violet assay results showed that single treatment with ornidazole or DTIC reduced the viability of melanoma cells in a concentration dependent manner. In addition, when these drugs are combined at certain concentrations (ornidazole+ DTIC; 800µg/mL+100µM/L, 800µg/mL+200µM/L and 1200µg/mL+100µM/L, 1200µg/mL+200µM/L), it was observed that cell viability decreases more than when used single. These results have shown that ornidazole/DTIC combination decreases more strongly than single drug administration viability of cancer cells and therefore it may be potential treatment for melanoma.

Moderate levels of ROS have been shown to promote cell proliferation and migration and cell survival, thus contributing to tumour development[36]. Accordingly, the use of natural agents to relieve oxidative stress has been proposed as a preventive and therapeutic anticancer strategy. Natural agents have bioactive compounds that promote health, prevent chronic diseases, and microenvironment is regulated by inflammatory cells that participate in the neoplastic process, nurturing tumour cells from their proliferation to their migration to other parts of the body[19]. Cell migration and invasion[37] play an

important role in cancer metastasis, which is the main cause of death in cancer patients[38,39]. The five year survival rates for most metastatic cancers are still quite low, and most are incurable. Additionally, developing a new anticancer drug is very costly and extremely time consuming. ornidazole has been on the market for many years and compared to new drugs, its combination with DTIC makes it a better treatment candidate. Melanoma frequently metastasises and the prognosis is poor with a low disease-free survival rate[40,41]. We evaluated the migration capacity of B16F10 melanoma cells after the combination of ornidazole and DTIC treatment. Our wound healing assay results showed that the combination of ornidazole with DTIC significantly inhibited cell migration *in vitro* compared to ornidazole or DTIC treatment alone. These results suggested that combination therapy with ornidazole/DTIC has an inhibitory effect on migration of cancer cells and therefore may have the potential to improve survival of patients with melanoma. Additionally, studies have shown that some anti-cancer compounds cause DNA damage[42] in cancer cells and stop the protein associated with DNA repair, eventually leading to the death of cancer cells[43,44]. In our previous study, we showed that ornidazole significantly increases DNA damage. In this study, we investigated the effects of ornidazole and DTIC combination therapy on the induction of DNA damage in melanoma cells. Actually B16F10 cells treated with ornidazole/DTIC exhibited significantly decreased cell migration when compared with control groups. Therefore, our results have demonstrated that DTIC may be beneficial in combination with ornidazole.

Conclusion

In conclusion, Our results showed that the DTIC combined targeted therapy group was superior to the DTIC alone group in terms of overall response rate. Due to the prevalence of melanoma, there is undoubtedly a need for the discovery of new anticancer drugs. Combining drugs increases efficacy compared to the monotherapy approach because they characteristically target crucial pathways in a synergistic manner. The findings of our present study suggest the combination of ornidazole and DTIC could exert synergistic effects *in vitro.* Also ornidazole/DTIC combination inhibits proliferation and migration and induces DNA damage in B16F10 cells according to our results. Therefore we suggest that ornidazole can be used with DTIC which is a known cancer drug for melanoma treatment and also ornidazole increases DTIC effects on treatment.

Conflict of Interest

The authors have no conflict of interest for this study.

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