

# Combined pitavastatin and dacarbazine treatment activates apoptosis and autophagy resulting in synergistic cytotoxicity in melanoma cells

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**Abstract.** Melanoma is an aggressive skin cancer and its incidence is increasing faster than any other type of cancer. Whilst dacarbazine (DTIC) is the standard chemotherapy for metastatic melanoma, it has limited success. Statins, including pitavastatin, have been demonstrated to have a range of anti-cancer effects in a number of human cancer cell lines. The present study therefore explored the anti-cancer activity of combined DTIC and pitavastatin in A375 and WM115 human melanoma cells. Cell survival assays demonstrated that combined DTIC and pitavastatin treatment resulted in synergistic cell death. Cell cycle analyses further revealed that this combined treatment resulted in a G1 cell cycle arrest, as well as a sub-G1 population, indicative of apoptosis. Activation of apoptosis was confirmed by Annexin V-fluorescein isothiocyanate/propidium iodide double-staining and an increase in the levels of active caspase 3 and cleaved poly (ADP-ribose) polymerase. Furthermore, it was demonstrated that apoptosis occurs through the intrinsic pathway, evident from the release of cytochrome c. Finally, combined DTIC and pitavastatin treatment was demonstrated to also activate autophagy as part of a cell death mechanism. The present study provides novel evidence to suggest that the combined treatment of DTIC and pitavastatin may be effective in the treatment of melanoma.

## Introduction

Melanoma is a particularly aggressive skin cancer and its incidence is increasing faster than any other cancer worldwide (1). At present, the 10-year survival rate of patients diagnosed with advanced (stage IV) metastatic melanoma is <10% (2), which

highlights the importance of early detection and treatment. The Food and Drug Administration approved the chemotherapeutic agent dacarbazine (DTIC) for the treatment of metastatic melanoma in 1975, and it remains the only licensed chemotherapeutic agent in use today (1). DTIC is a methylating agent which causes DNA damage, cell cycle arrest and apoptosis. Despite this, only 2% of all patients with metastatic melanoma receiving this treatment demonstrate a significant response and only 11.2% demonstrate a partial response (3). Resistance to DTIC has been associated with the upregulation of pro-survival signals and anti-apoptotic molecules in cancer cells. Despite its moderate effects, DTIC continues to be the standard treatment for metastatic melanoma as no other chemotherapeutic treatment has been demonstrated to have a significantly increased chance of survival when compared with DTIC (4,5). Provided the limited efficacy of the current metastatic melanoma chemotherapies in addition to the increasing incidence of melanoma cases, there appears to be a need for the development of more effective treatment strategies.

Statins are a group of drugs commonly used for the reduction of cholesterol levels (6). They work by inhibiting 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase, a critical enzyme in the mevalonate pathway, which is responsible for cholesterol synthesis (6,7). In addition, statins have been demonstrated to serve a function in immune regulation and cancer prevention (8). Evidence from *in vitro* and *in vivo* studies has revealed that statins have a wide range of anticancer activities in various types of cancer (6,9,10). In multiple myeloma cells, simvastatin has been demonstrated to induce S phase cell cycle arrest through the downregulation of cell division cycle 25a, cyclin A and cyclin dependent kinase expression and the activation of checkpoint kinase 1 (9). This cell cycle arrest was accompanied by intrinsic apoptosis as demonstrated by diminished B-cell lymphoma 2 (Bcl-2) protein levels, increased cytosolic cytochrome c and active caspase 9 and caspase 3 levels. In human glioblastoma cells, previous studies have revealed that erivastatin, pitavastatin and fluvastatin are potent anti-proliferative agents (10,11). In addition, one clinical trial revealed that fluvastatin reduced tumour proliferation and increased apoptotic activity in high-grade, stage 0/1 breast cancer (12). Pitavastatin has been demonstrated to exert a cytotoxic effect on U87 glioblastoma tumour growth *in vivo* (10).

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On a molecular level, pitavastatin treatment has been demonstrated to upregulate the cell cycle regulator p21 and to inhibit nuclear factor- $\kappa$ B (NF- $\kappa$ B) in different tumour cells, which resulted in cell cycle arrest and apoptosis (13,14). Finally, in glioma cells, autophagic cell death was demonstrated to be a potential mechanism of pitavastatin-induced cytotoxicity by also resulting in the inhibition of NF- $\kappa$ B (15). However, the exact molecular mechanisms underpinning the anticancer activity of pitavastatin remain mostly unknown.

Given the shortage of treatments for metastatic melanoma, the present study therefore aimed to explore the effects of combined pitavastatin and DTIC treatment in human melanoma cells. The present study demonstrated that this combined treatment results in the synergistic inhibition of cell survival and further demonstrated that this occurs through the induction of intrinsic apoptosis and autophagy cell death pathways.

## Materials and methods

**Cell culture and treatments.** The human melanoma cell lines A375 and WM115, sourced from the Department of Human Biology, University of Cape Town (Cape Town, South Africa), were maintained in Dulbecco's modified Eagle's medium, Biological Industries Israel Beit-Haemek (Kibbutz Beit-Haemek, Israel) supplemented with 10% foetal bovine serum, Biological Industries Israel Beit-Haemek in a humidified 5% CO<sub>2</sub> balanced air incubator at 37°C. Pitavastatin (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and DTIC (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) were dissolved in dimethyl sulfoxide (DMSO) to give stock concentrations of 5 mM, which were stored for no more than 5 days. Control cells were treated with equivalent concentrations of DMSO (vehicle). Autophagy inhibitor 3-methyl adenine (10 mM) (3MA; Sigma-Aldrich; Merck KGaA) was added at 37°C for 1 h prior to pitavastatin/DTIC treatment.

**Cytotoxicity assays.** Cells were seeded in 96-well plates at a density of 4x10<sup>3</sup>-5x10<sup>3</sup> cells per well and allowed to settle for 48 h at 37°C. Cells were treated with a range of pitavastatin (0-5.0  $\mu$ M) and/or DTIC (0.0-100  $\mu$ M) concentrations or vehicle for 48 h at 37°C. Cytotoxicity was assessed using an MTT assay kit as per the manufacturer's protocol (Roche Diagnostics GmbH, Mannheim, Germany) (16). Briefly, 10  $\mu$ l MTT solution was added to each well and cells were incubated at 37°C for 4 h, followed by addition of 100  $\mu$ l solubilisation buffer [10% SDS; Sigma-Aldrich; Merck KGaA] in 0.01 M hydrochloric acid (HCl) (Sigma-Aldrich, Israel)] and incubation for 16 h at 37°C. Absorbance at 585 nm was determined for each well and the mean cell viability was calculated as a percentage of the mean vehicle control.

**Cell cycle analysis.** Cells were plated at a density of 3x10<sup>5</sup>-4x10<sup>5</sup> cells per 6-cm dish and allowed to settle for 24 h at 37°C. Log-phase cultures were exposed to drugs or vehicle for 48 h at 37°C. Cells were then trypsinised, washed with PBS and fixed in 95% ethanol at 4°C overnight, followed by RNase A (50  $\mu$ g/ml; Sigma-Aldrich; Merck KGaA) treatment for 15 min at 37°C and immediately stained for 30 min at room temperature with propidium iodide (PI; Sigma-Aldrich; Merck KGaA). Cellular DNA content was determined using

flow cytometry with individual samples subjected to a FACSCalibur flow cytometer with a 488 nm coherent laser (BD Biosciences, San Jose, CA, USA). Cellquest Pro version 5.2.1 software (BD Biosciences) was used for data acquisition and analyses were performed using Modfit version 2.0 software (BD Biosciences).

**Apoptosis detection.** Log-phase melanoma cultures were treated with 1.0  $\mu$ M pitavastatin, 40.0  $\mu$ M DTIC, combined pitavastatin (1.0  $\mu$ M)-DTIC (40.0  $\mu$ M) or vehicle for 48 h at 37°C. Adherent and floating cells were collected and double-labelled with Annexin V-Fluorescein isothiocyanate (FITC) and PI using Annexin V-FITC Apoptosis Detection Kit (Sigma-Aldrich; Merck KGaA) as per the manufacturer's protocol. Annexin V-FITC was used to quantitatively determine the percentage of apoptotic cells while PI was used to stain all dead cells. Cells were analysed by flow cytometry with a 488 nm coherent laser equipped with FACStation running version 3.3 Cell Quest software (BD Biosciences).

**Cytochrome c release.** Melanoma cells treated as aforementioned with vehicle or pitavastatin-DTIC for 48 h at 37°C were trypsinized, re-suspended in HB-7S buffer [1 mmol/l EGTA Na-free, 5 mmol/l Tris-HCl (pH 7.4), 1 mmol/l DTT, and 11% sucrose] (Sigma-Aldrich; Merck KGaA) and subcellular fractions were collected as described previously (17).

**Western blotting.** Cells were harvested and the protein was prepared as described previously (16). Primary antibodies used were as follows: Anti-PARP1/2 (cat no. sc-7150), anti-p53 (cat no. sc-126), anti-p21 (cat no. sc-756), anti-Bcl2-associated X, apoptosis regulator (Bax; cat no. sc-7480), anti-cyclin D (cat no. sc-753), anti-cytochrome c (cat no. sc-65396), anti-COXIV (cat no. sc-69359) and anti-cyclin E (cat no. sc-247; Santa Cruz Biotechnology, Inc.), anti-actin (cat. no., A4700; Sigma-Aldrich; Merck KGaA), anti-LC3-phosphatidylethanolamine conjugate (cat no. 2775), anti-BCL-2 (cat no. 2876), anti-Caspase-3 (cat no. 9661) and anti-Caspase-8 (cat no. 9746; Cell Signaling Technology, Inc., Danvers, MA, USA). Following primary antibody incubation, membranes were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (1:5,000; Bio-Rad Laboratories, Inc.) and antibody-reactive proteins were visualized using the electrochemiluminescence reaction detection system as previously described (Thermo Fisher Scientific, Inc., Waltham, MA, USA) (18).

**Autophagy assays.** Autophagy was confirmed by the presence of fluorescent puncta in cells transfected with a green fluorescence protein (GFP)-light chain 3 LC3) expression vector (cat. no., 24920; Addgene, Inc., Cambridge, MA, USA), as previously described (16).

**Statistical analysis.** Results are presented as the mean  $\pm$  standard error of the mean (SEM) of the three independent experiments. Statistical analysis of data was performed using the two-sample t-test in Microsoft Excel 2013 (Microsoft Corporation, Redmond, WA, USA) or a one-way ANOVA with Tukey's post hoc test in Graph Pad Prism v.5. (GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

## Results

**Pitavastatin and DTIC synergistically inhibit melanoma cell survival.** To investigate the anti-cancer effect of pitavastatin on melanoma, human A375 and WM115 melanoma cells were treated with increasing concentrations (0-5  $\mu\text{M}$ ) of pitavastatin for 48 h. An MTT assay was used to determine cell viability and a dose-dependent decrease in cell survival was observed in A375 and WM115 cells (Fig. 1A). The results revealed that 4  $\mu\text{M}$  pitavastatin treatment resulted in the death of >50% of cells, suggesting that it exerts potent cytotoxic effects against melanoma cells specifically at high concentrations. DTIC has previously been demonstrated to inhibit A375 cell survival but only at a high concentrations of 25-100  $\mu\text{M}$  (19). Therefore, the present study aimed to determine whether the combined treatment of pitavastatin and DTIC may have a greater anti-cancer effect on A375 and WM115 cells. To this end, cells were treated with 1  $\mu\text{M}$  pitavastatin for 1 h followed by treatment with increasing concentrations (10-100  $\mu\text{M}$ ) of DTIC for 48 h. Fig. 1B and C demonstrate that the combined treatment resulted in enhanced anti-cytotoxic activity compared with DTIC treatment alone. Whilst 40  $\mu\text{M}$  DTIC resulted in the death of <15% of melanoma cells, when cells were pre-treated with 1  $\mu\text{M}$  pitavastatin, it resulted in the death of ~50% of melanoma cells at the same DTIC concentration. The results of the present study demonstrate that combined pitavastatin and DTIC treatment results in a synergistic cytotoxic effect in melanoma cells.

**Combined pitavastatin and DTIC treatment induces G1 cell cycle arrest in melanoma cells.** The present study aimed to investigate the mechanism by which combined pitavastatin-DTIC treatment synergistically inhibits melanoma cell survival. To this end, A375 and WM115 cells were treated with vehicle, pitavastatin (1.0  $\mu\text{M}$ ), DTIC (40.0  $\mu\text{M}$ ) or pitavastatin-DTIC (1.0 and 40.0  $\mu\text{M}$ , respectively) and the effect on the cell cycle profile was determined by flow cytometry. Fig. 2A demonstrates that combined pitavastatin-DTIC treatment induced a significantly greater G1 cell cycle arrest than either treatment alone. To further explore this, A375 and WM115 cells were treated with pitavastatin and DTIC and markers of cell cycle arrest were analysed by the use of western blotting. Fig. 2B demonstrates the p53 response elicited by combined pitavastatin-DTIC treatment. In the two cell lines, treatment with only pitavastatin resulted in an increase in p21 levels, however, when WM115 cells were treated with pitavastatin-DTIC, p21 levels were even further increased. For A375 and WM115 cells pitavastatin-DTIC treatment corresponded with a decrease in cyclin D1 and cyclin E1, which are required for the transition from G1 to S phase. These results provide compelling evidence that combined pitavastatin-DTIC treatment results in G1 cell cycle arrest in melanoma cells.

**Combined pitavastatin-DTIC treatment activates intrinsic apoptosis.** The presence of sub-G1 peaks on DNA content histograms is generally accepted to represent apoptotic cells (20). As cell cycle analysis demonstrated that combined pitavastatin-DTIC treatment resulted in sub-G1 peaks (Fig. 2A), the present study aimed to determine whether pitavastatin-DTIC treatment induced cell death by apoptosis. To this end, A375 and WM115 cells were treated with pitavastatin-DTIC and

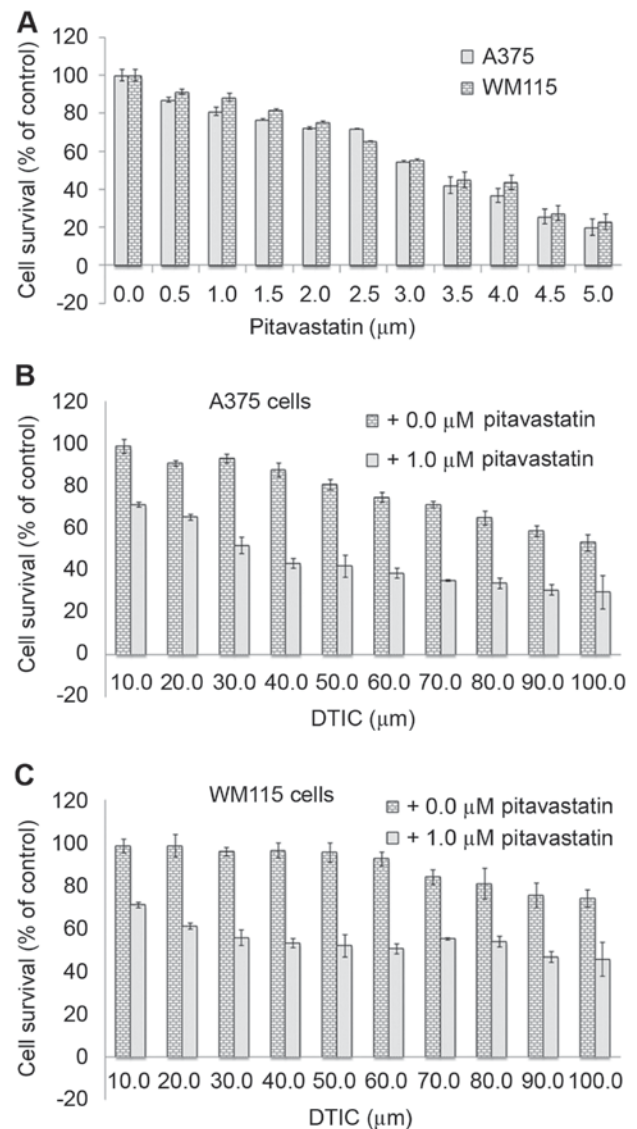


Figure 1. Pitavastatin and DTIC synergistically inhibit melanoma cell survival. (A) Cell survival rate as measured by an MTT assay of A375 and WM115 cells treated with increasing concentrations of pitavastatin (0.0-5.0  $\mu\text{M}$ ) or vehicle. (B) A375 and (C) WM115 cells treated with 1.0  $\mu\text{M}$  pitavastatin and then treated with increasing concentrations (10.0-100.0  $\mu\text{M}$ ) of DTIC. Results are presented as the mean percentage  $\pm$  standard error of the mean of untreated cells and represent the pooled results of at least three experiments performed in quadruplicate. DTIC, dacarbazine.

western blotting was performed to assess PARP cleavage and active caspase 3 levels, which are molecular markers of apoptosis. Fig. 3A demonstrates that levels of the active cleaved PARP and caspase 3 proteins notably increased in response to combined pitavastatin-DTIC treatment, suggesting that apoptosis is indeed activated.

Apoptosis may be activated through two main pathways, namely the extrinsic and intrinsic pathways (21). Whilst extrinsic apoptosis is mediated by death receptors and characterized by caspase 8 activation (22), intrinsic apoptosis is mitochondria mediated and usually triggered by intracellular signals including hypoxia and DNA damage (23). During intrinsic apoptosis, the overexpression of pro-apoptotic Bcl-2 proteins disrupts the mitochondrial membrane and eventually leads to the release of cytochrome c (24). In order to determine

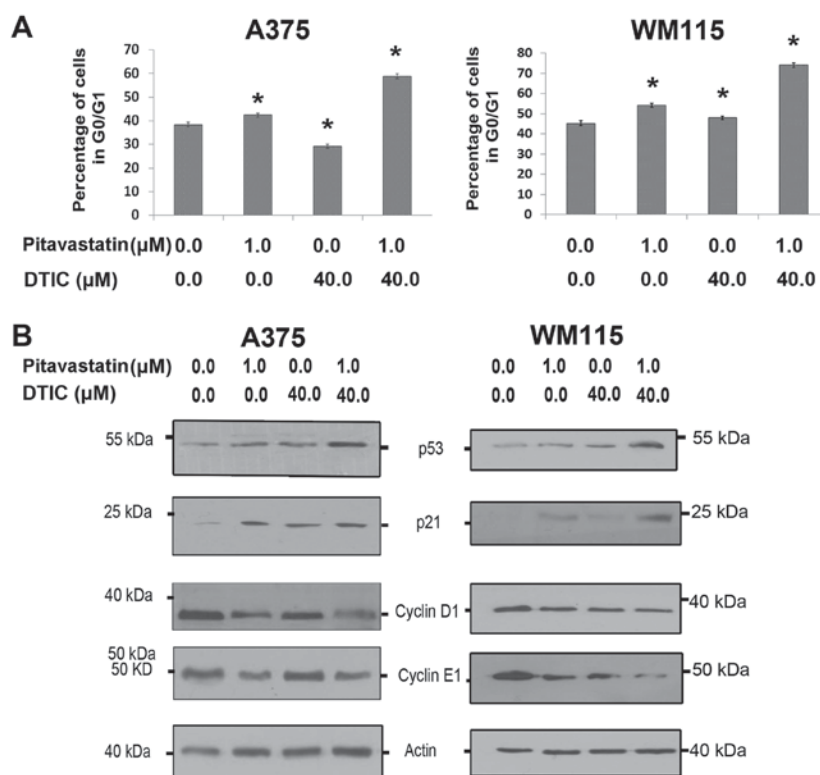


Figure 2. Combined pitavastatin and DTIC treatment induces G1 cell cycle arrest in melanoma cells. (A) Cell cycle analysis of A375 and WM115 cells treated with vehicle, pitavastatin (1.0  $\mu\text{M}$ ), DTIC (40.0  $\mu\text{M}$ ) or pitavastatin-DTIC (1.0 and 40.0  $\mu\text{M}$ , respectively). The proportion of cells at G0/G1 phase was expressed as a percentage of the total number of cells analysed and presented in the graphs as the mean  $\pm$  standard error of the mean of three independent experiments (\* $P < 0.01$  as compared with vehicle, one-way ANOVA with Tukey's post hoc test). (B) A375 and WM115 cells were treated with 1.0  $\mu\text{M}$  pitavastatin, 40.0  $\mu\text{M}$  DTIC, pitavastatin-DTIC (1.0 and 40.0  $\mu\text{M}$ , respectively) or vehicle. Protein extracts were analysed by SDS-PAGE (8-15%) and western blotting using antibodies against p53, p21, Cyclin E1 and Cyclin D1. Actin was detected as a loading control. DTIC, dacarbazine.

whether combined Pitavastatin-DTIC treatment activated either one of the apoptotic pathways, levels of intrinsic and extrinsic apoptotic molecular markers were measured by western blotting. The results of the present study demonstrate that combined pitavastatin-DTIC treatment did not activate the pro-apoptotic factor caspase 8, suggesting that the extrinsic apoptotic pathway is not activated (data not shown). On the other hand, combined pitavastatin-DTIC treatment induced expression of the intrinsic pro-apoptotic factor Bax and decreased expression of the anti-apoptotic protein Bcl-2. Furthermore, Fig. 3B demonstrates that pitavastatin-DTIC treatment led to a notable increase in cytoplasmic cytochrome c protein levels and a notable decrease in mitochondrial cytochrome c protein level in A375 cells. Cytochrome oxidase IV was used as a mitochondrial marker and actin was detected as a marker for cytoplasmic fraction. Collectively, the results of the present study demonstrate that combined pitavastatin-DTIC treatment induces cell death through intrinsic apoptosis in melanoma cells.

#### Combined pitavastatin-DTIC treatment induces autophagy.

Increasing evidence has revealed that autophagy is activated in response to statins (25) and large vacuoles indicative of autophagy were observed in A375 and WM115 cells treated with pitavastatin alone or and pitavastatin-DTIC combined (data not shown). In order to explore this, protein extracts from cells treated with pitavastatin, DTIC, pitavastatin-DTIC or vehicle were assessed for LC3II, a molecular marker of autophagy, by

western blotting. Fig. 4A demonstrated that pitavastatin alone or in combination with DTIC induces high levels of LC3II. In order to further confirm that combined pitavastatin-DTIC treatment induces autophagy, A375 and WM115 cells were transiently transfected with a GFP-LC3 expression vector and autophagosome formation was monitored by confocal microscopy. As a result, the combined treatment of pitavastatin-DTIC led to a significant accumulation of GFP-LC3 puncta (Fig. 4B).

Whilst previous studies have suggested that chemotherapy may induce cell death via apoptosis and autophagy (26), other studies have demonstrated that this may induce autophagy, which attenuates apoptosis leading to drug resistance (27). Therefore, the present study examined, using Annexin V assays, whether the pitavastatin-DTIC-induced autophagy is a cell death or a cell survival mechanism. Fig. 4C demonstrates that pharmacological inhibition of autophagy, through the use of 3MA, significantly reduced the cytotoxicity and the total cell death induced by combined pitavastatin-DTIC treatment. Taken together these observations suggest that pitavastatin-DTIC induced autophagy favours cell death and contributes to cytotoxicity.

#### Discussion

Malignant melanoma incidence is rapidly increasing and has almost doubled in the previous decade (2). Whilst DTIC chemotherapy is the standard treatment, <20% of patients respond at all to the treatment and <5% continue to respond to long-term treatment (28,29). Previous studies have demonstrated that, in

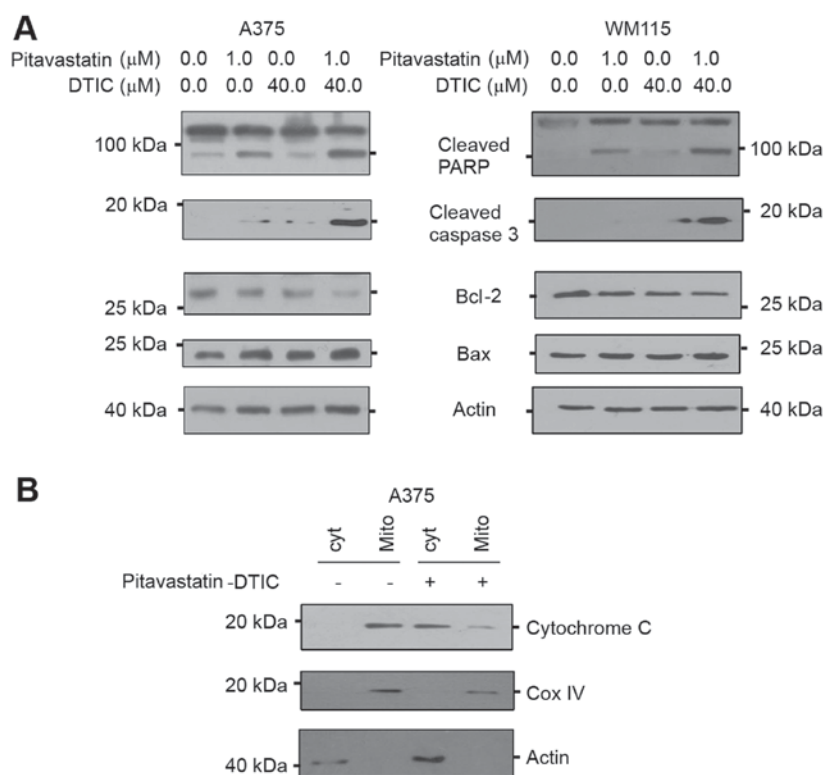


Figure 3. Combined treatment of pitavastatin-DTIC activates intrinsic apoptosis. (A) A375 and WM115 cells were treated with either vehicle, 1.0  $\mu$ M pitavastatin, 40.0  $\mu$ M DTIC or pitavastatin-DTIC (1.0 and 40.0  $\mu$ M, respectively). Protein extracts were analysed by SDS-PAGE (8-15%) and western blotting using antibodies to cleaved PARP, active caspase 3, Bax and Bcl-2. Actin was detected as a loading control. (B) Protein extracts from mitochondrial and cytoplasmic fractions were prepared from A375 cells treated with vehicle or Pitavastatin-DTIC (1.0 and 40.0  $\mu$ M, respectively), resolved by SDS-PAGE (8-15%) and probed with the anti-cytochrome c, Cox IV and actin antibodies. DTIC, dacarbazine; CoxIV, cyclooxygenase IV; Bax, Bcl-2 associated X, apoptosis regulator; Bcl-2, B-cell lymphoma 2; PARP, poly (ADP-ribose) polymerase.

the case of melanoma, resistance to DTIC is associated with low levels of apoptosis and increasing levels of anti-apoptotic proteins (19,29,30).

Statins, which are anti-lipid agents, have been demonstrated to exert anti-proliferative and anti-cancer effect against a range of types of tumour (9,15,31). The present study therefore explored the *in vitro* efficacy of a combined treatment of DTIC with pitavastatin in human melanoma. The results of the present study provide several lines of evidence to suggest that pitavastatin may synergistically enhance the anti-cancer effects of DTIC. The half-maximal inhibitory concentration ( $IC_{50}$ ) of DTIC in several melanoma cell lines, including A375, A875, SB2, MeWo, B16-F1 and SK-MEL-5, has been demonstrated to be relatively high at  $>100 \mu$ M (5,32). Similar results were also obtained from the present study, where the  $IC_{50}$  of DTIC in the A375 and WM115 cell lines was  $>100 \mu$ M. On the other hand, pitavastatin has also been demonstrated to be cytotoxic on glioma, breast, myeloma and colon cancer with  $IC_{50}$  values of  $<10 \mu$ M (9,10,15). Whilst previous studies have not described the cytotoxic effect of pitavastatin on melanoma cells, the present study demonstrates that pitavastatin is cytotoxic in melanoma cells and treatment with 4  $\mu$ M pitavastatin results in  $\sim 50\%$  cell death. The present study further demonstrated that melanoma cells pre-treated with pitavastatin (1  $\mu$ M) are sensitised to DTIC (40  $\mu$ M) resulting in  $\sim 50\%$  cell death.

Furthermore, the present study revealed that the mechanism of action by which pitavastatin-DTIC combined treatment inhibits melanoma growth involves cell cycle arrest, apoptosis

and autophagy. Previous studies have indicated that high doses of DTIC (2 mM) result in the death of cancer cells through apoptosis (33). In addition, in uveal melanoma cell lines, DTIC treatment (5  $\mu$ g/ml) primarily led to cell cycle arrest in the G1 phase (34). Another previous study demonstrated that DTIC (1 mM) induces S-phase cell cycle arrest in A375 cells and results in a slight increase in the sub-G1 peak (35). The same previous study demonstrated that melanoma cells exposed to parthenolide, a sesquiterpene lactone derived from the leaves of feverfew (*Tanacetum parthenium*), combined with 2 mM DTIC accumulated in the G2/M phase. Notably, pitavastatin was also demonstrated to inhibit the proliferation of human U937 monocytic tumour cells in a dose-dependent manner and to induce S-phase cell cycle arrest (14). According to the same previous study, pitavastatin upregulated p21 protein levels but did not affect the expression levels of cyclin D1. The results of the present study reveal that whilst cells treated with DTIC (40  $\mu$ M) demonstrated slight alterations to the cell cycle profile, combined pitavastatin-DTIC treated cells accumulated in the G1 phase as evidenced by flow cytometry and cell cycle regulator analyses. The results of the present study suggest that the induction of cell cycle arrest by DTIC may be concentration dependent and that pitavastatin enhances the ability of DTIC to halt cell cycle progression. Furthermore, the present study demonstrated that the cytotoxic effect of pitavastatin-DTIC includes the induction of apoptosis. Notably, whilst DTIC treatment (40  $\mu$ M) did not induce apoptosis, combined pitavastatin-DTIC treatment resulted in significant levels of apoptosis as evidenced by the

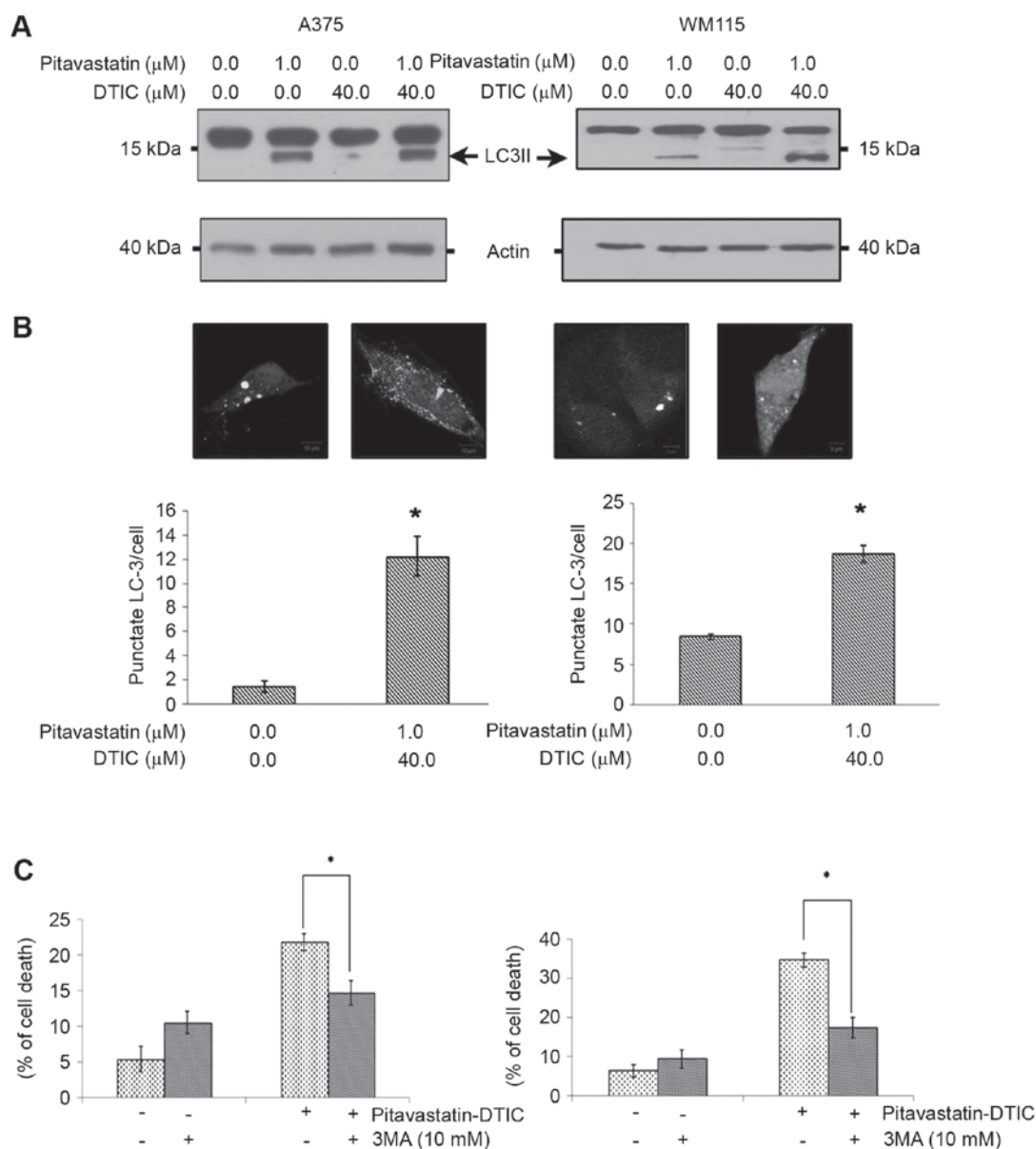


Figure 4. Combined pitavastatin-DTIC treatment induces autophagy. (A) A375 and WM115 cells were treated with either vehicle, 1.0  $\mu\text{M}$  pitavastatin, 40.0  $\mu\text{M}$  DTIC or Pitavastatin-DTIC (1.0 and 40.0  $\mu\text{M}$ , respectively). Protein extracts were analysed using SDS-PAGE (8-15%) and western blotting using antibodies against LC3II and actin was used as a loading control. (B) A375 and WM115 cells were transiently transfected with a GFP-LC3 plasmid and treated with vehicle or pitavastatin-DTIC (1.0 and 40.0  $\mu\text{M}$ , respectively). Autophagy was quantified by counting the number of GFP-LC3 puncta in twenty fields of view at magnification, x400, and dividing by the total number of transfected cells within these fields. The number of GFP-LC3 puncta/cell are presented in the graphs as the mean  $\pm$  standard error of the mean of three independent experiments (\* $P < 0.001$  as compared with vehicle, Student's t-test). (C) Annexin V/propidium iodide double staining assay presenting the percentage of cell death induced by pitavastatin-DTIC when autophagy was inhibited by 10 mM 3-methyladenine and cells treated with pitavastatin-DTIC. (\* $P < 0.05$  with comparisons indicated by lines, Student's t-test). DTIC, dacarbazine; GFP, green fluorescence protein; LC3II, LC3-phosphatidylethanolamine conjugate; LC3, light chain 3.

increased level of apoptosis markers, cleaved PARP and active caspase 3, as well as annexin V staining. The results of the present study are in agreement with a number of studies which demonstrate that high concentrations of DTIC are necessary to induce apoptosis in melanoma cells (19,32,36). Whilst there has been insufficient research to determine which type of apoptosis is induced by DTIC, sensitization to the intrinsic apoptotic pathway has been revealed to augment DTIC-induced melanoma tumour cell death (37). In accordance with these observations, the results of the present study suggest that pitavastatin treatment may enhance DTIC cytotoxicity through augmentation of the intrinsic apoptosis pathway.

Furthermore, the results of the present study demonstrated that melanoma cells treated with combined pitavastatin-DTIC expressed a high level of LC3II, a marker of autophagy. Chemical inhibition of autophagy resulted in enhancement of cell viability, suggesting that pitavastatin-DTIC-induced autophagy occurs as a cell death mechanism. In support of this, previous studies have suggested that DTIC and pitavastatin may serve a function in the induction of autophagy and specifically as a mode of cell death (15,19,38).

In summary, results from the present study suggest that the combined treatment of pitavastatin-DTIC provides a synergistic anti-cancer effect through apoptosis and

autophagy, and this should be further examined for melanoma treatment.

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