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The Pan-Aurora Kinase Inhibitor, PHA-739358, Induces Apoptosis and Inhibits Migration in Melanoma Cell Lines

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Abstract

Treatment of metastatic melanoma has long been a challenge due to its resistance to traditional chemotherapeutics leading to the search for alternative strategies. Aurora kinases are key mitotic regulators that are frequently overexpressed in various cancers including melanoma, making them ideal targets for anticancer therapeutics. Several Aurora kinase inhibitors have been developed and tested pre-clinically and clinically. PHA-739358 is currently the most advanced clinical compound; however its antitumor effect has not been tested in melanoma. In this study, the anti-proliferative and anti-invasive effects of PHA-739358 were investigated in melanoma cell lines. The results demonstrated that PHA-739358 produces a time and dose dependent inhibition of cell proliferation, induction of apoptosis, and inhibition of cell migration. Downregulation of MMP-2 via inhibition of NFκB signaling pathway may contribute to PHA-739358-induced migration inhibition. Furthermore, PHA-739358 enhanced temozolomide-induced caspase activation. This study provides a promising new strategy for the treatment of advanced melanoma.

Keywords

Cutaneous melanoma; Aurora kinase inhibitor; PHA-739358; Apoptosis; Migration; MMP-2; and NFκB

Introduction

Melanoma is the least common but the most deadly form of skin cancer, and is highly invasive and markedly resistant to conventional therapy. It has been estimated that there will be 76,250 new cases of invasive melanoma and 9,180 deaths from melanoma in the United States in 2012 [1]. Melanoma is easily curable by surgical excision when detected early, but it is nearly incurable when discovered in its later stages owing to both initial and rapidly acquired resistance to treatment. The 5-year survival rate for metastatic melanoma is dismal, ranging from 5% to 10% with a median survival rate of less than 8 months with treatment

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[2]. Standard therapy with agents such as dacarbazine, temozolomide (TMZ), and IL-2 is associated with notoriously low response rates (reviewed in [3]). Therefore, there is an urgent need for the identification of novel agents for this deadly disease.

Recent progress in understanding the molecular mechanisms involved in the initiation and progression of melanoma has created new opportunities for developing novel therapeutic modalities. The discovery of BRAF mutations in melanoma created the first opportunity to develop oncogene-directed therapy in this disease and led to the development of compounds that inhibit aberrant BRAF activity. A decade later, vemurafenib, an orally available and well-tolerated selective $BRAF^{V600E}$ inhibitor, ushered in a new era of molecular treatments for advanced disease [4–5]. Additional targets have been identified, and novel agents that impact on various signaling pathways or modulate the immune system hold the promise of a whole new therapeutic landscape for patients with metastatic melanoma (reviewed in [6]). Of the drugs tested to date in patients with metastatic melanoma, those that have yielded the best results are $BRAF^{V600E}$ inhibitors in melanomas carrying the V600E mutation [5]; c-kit tyrosine kinase inhibitors in melanomas carrying c-kit mutations; and anti-cytotoxic T lymphocyte antigen 4 (CTLA-4) antibodies, which block the mechanisms involved in immune tolerance [7]. However, the rapid development of resistance to BRAF and c-kit inhibitors and the lack of biomarkers that predict treatment response in the case of CTLA-4 blockers are significant clinical problems emphasizing the demand for additional and/or alternative treatment strategies [6, 8].

Mitosis is a critical step in the proliferation of cancer cells and involves many redundant checkpoint systems controlling key steps of the process. Drugs that interfere with the normal progression of mitosis belong to the most successful cytotoxic agents currently used for anticancer therapy. The family of Aurora kinases (including members A, B, and C) plays an important role in maintaining the fidelity of mitosis, making them attractive novel targets for anticancer treatment [9].

Aurora kinases are frequently over-expressed in various human cancers including cutaneous melanoma [10–11]. Both Aurora kinases A and B have been shown upregulated in melanoma [10]. Furthermore their levels correspond to the progression of melanoma, increasing from *in situ* to primary and metastatic melanoma [10]. Their fundamental role in cell cycle regulation and observed aberrant expression in a broad range of malignancies prompted the development of small molecules that selectively inhibit their activity. Currently, there are about 30 Aurora kinase inhibitors (AKIs) in different stages of pre-clinical and clinical development. Several of them, such as VE-465, ENMD-2076, and PF-03814735, have been tested in pre-clinical melanoma models [10–12], indicating the promising anti-melanoma effect of AKIs.

PHA-739358 is currently the most advanced clinical compound, which potently inhibits all Aurora kinase family members (A, B, and C), with a dominant inhibition of Aurora Kinase B [13]. PHA-739358 is one of the first AKIs to enter the clinic and has been studied in Phase I and II trials, showing great therapeutic potential in anticancer therapy in a wide range of cancers, including both advanced solid tumors and leukemias [13–14]. The clinical activity of PHA-739358 has largely been consistent with cytostatic effects, with the best

response so far being stable disease in about 23.7% of evaluable patients with advanced or metastatic solid tumors [15]. However, the effect of PHA-739358 on melanoma has not yet been tested. In this study, we investigated the anti-proliferative and anti-invasive effects of PHA-739358 on melanoma *in vitro*. In addition the potential of combination therapy of TMZ and PHA-739358 for melanoma treatment was also examined.

Results

Overexpression of Aurora kinases A & B in melanoma

It has been shown that both AURKA and AURKB levels are elevated in melanoma and increasing expression levels correspond to the progression of melanoma, from *in situ* to primary and metastatic melanoma [10]. Our analysis of DNA microarray gene expression profiling datasets of normal skin and cutaneous melanoma human tissues deposited in the oncomine database (www.oncomine.com) also showed overexpression of both AURA ($p < 0.001$) and AURKB ($p < 0.00001$) in melanoma tumor tissues in comparison to normal skin or benign nevi (Supplemental Fig.1A&B). No difference was observed between normal skin and benign nevi for either AURKA or AURKB (Supplemental Fig.1A&B). Consistent with these findings, western blotting analysis demonstrated increased levels of AURKA and AURKB in all six melanoma cell lines tested, including WM3211, A375, Lu1205, SK Mel5, C82-2C, SK Mel28, in comparison to normal human melanocytes (NHMCs) (Supplemental Fig.1C). MDA-MB-231 was used as a positive control for both AURKA and AURKB (Supplemental Fig.1C). β-actin was used as a loading control (Supplemental Fig.1C).

Effects of PHA-739358 on cell viability in melanoma cell lines

WM3211, Lu1205, or SK Mel28 melanoma cells were treated with serial dilutions of PHA-739358. SRB (Sulforhodamine-B) cell viability assay was performed at 24, 48, or 72 hrs after drug treatment for all three cell lines. The results demonstrated a dose and time dependent decrease of cell viability upon PHA-739358 treatment in all three cell lines (Fig. 1A~C), with low IC50s against WM3211 (1.76±0.04 μM) and Lu1205 (3.34±0.05 μM) cell lines, and a relatively higher IC50 against SK Mel28 cell line (12.45±0.27 μM) after 72 hr of drug treatment (Fig.1D).

Effect of PHA-739358 on cell cycle progression in melanoma cell lines

Propidium iodide staining combined with FACScan flow cytometry analysis was performed to investigate the effect of PHA-739358 on cell cycle progression in three melanoma cell lines (WM3211, Lu1205, and SK Mel28.). The results shown in Fig.2(A~J) indicate that PHA-739358-treated cells present an increased G2/M-phase population in all three cell lines in comparison to untreated control cells (2-fold induction at 1 μM and 3-fold induction at 5 μM were observed in WM3211 cell line; 7-fold induction was observed at both 1&5 μM treatment in Lu1205 cell line; 12-fold induction at 5 μM and 7-fold induction at 10 μM were observed in SK Mel28 cell line) (Fig.2J). A dramatically increased polyploidy population was observed in WM3211 (1.9~3.0 fold) and Lu1205 (1.5~1.9 fold) cell lines; only a slight induction (from 6.62% to 8.15% @ 5 μM) was observed in SK Mel28 cell line (Fig.2J). The data also demonstrated increased SubG1-phase population (Debris) upon PHA-739358 treatment in both WM3211 (4.8-fold @ 1 μM and 3.9-fold @ 5 μM) and SK Mel28 (1.3-fold

@ 5 μM and 1.5-fold @ 10 μM) cell lines, and a 1.2 fold induction (from 6.86% to 8.43%) in Lu1205 at 5μM of PHA-739358 treatment (Fig.2J).

PHA-739358-induces caspase activation which can be further enhanced by the addition of TMZ

Caspase 3/7 activity assays indicated that PHA-739358 treatment resulted in a dose-dependent increase of caspase 3/7 activity in all three cell lines, including WM3211, Lu1205, and SK Mel28 (Fig.3A~C). Since SK Mel28 is the most resistant cell line (IC50=12.45±0.27 μM) (Fig.1D), we investigated whether addition of PHA-739358 could sensitize SK Mel28 cells to TMZ treatment. SK Mel28 cells were treated with 0, 5 or 10 μM of PHA-739358 with or without 200μM of TMZ treatment. Caspase activity assay was performed at 72 hrs after drug-treatment. From Fig.4 we can see that, TMZ alone slightly decreased caspase activity in comparison to untreated control; however, when combined with 5 μM of PHA-739358, the caspase activity was significantly enhanced over either drug alone. Increased caspase activity was also observed when TMZ was added to 10 μM of PHA-739358 in comparison to either TMZ or PHA alone (Fig.4).

Dose-dependent cell migration inhibition upon PHA-739358 treatment in melanoma cell lines

SK Mel28 and Lu1205 melanoma cells were treated with increasing concentration of PHA-739358 for 72 hrs as indicated in Fig.5A&B, respectively. Representative images of SK Mel28 cells after 72 hr PHA-739358 treatment were shown in Supplemental Fig.2 (A~C). Surviving cells were equally (75,000 cells/500 μl of SFM, confirmed by trypan blue staining) seeded into BioCoat chamber as described in the Materials and Methods and incubated for 24 hrs at 37°C. Supplemental Fig.3 shows representative photographs of SK Mel28 A(a~c) and Lu1205 cells B(d~f) migrating onto the reverse side of the membrane. Fig.5 A&B shows the quantified results for the migration assay for SK Mel28 (A) and Lu1205 (B) cell lines, respectively. The results demonstrated a dose-dependent inhibition of cell migration in both cell lines (35% inhibition @ 0.1μM and 98% inhibition @ 5μM in SKMel28 cell line (Fig.5A); 66% inhibition @ 0.1μM and 100% inhibition @ 3μM in Lu1205 cell line) (Fig.5B). Next, a wound closure assay was performed with SK Mel28 cells and the results showed that PHA-739358 (100nM) significantly reduced cell migration (Supplemental Fig.4).

Time-dependent cell migration inhibition upon PHA-739358 treatment in melanoma cell lines

To further characterize PHA-739358-induced inhibition of cell migration, SK Mel28 cells were treated with 5μM of PHA-739358. Migration assay was performed at 24, 48, and 72hrs after drug treatment. Supplemental Fig.5 A~D shows representative photographs of SK Mel28 migrating onto the reverse side of the membrane after 0, 24, 48, and 72 hr PHA-739358 treatment, respectively. The quantified results showed a 70%, 90%, and 99% migration inhibition after 24, 48, or 72 hr PHA-739358 treatments, respectively, in comparison to Control group (Fig.5C).

Inhibition of NFκB signaling pathway may be involved in PHA-739358-induced MMP-2 downregulation

The effects of PHA-739358 on AURKA autophosphorylation (Thr^{288}) and AURKB-mediated phosphorylation of Histone H3 (Ser^{10}) in SK Mel28 melanoma cell line are shown in Fig.6A&B. Decreased AURKA autophosphorylation and Histone H3 phosphorylation were observed from 0.1μM of PHA-739358 treatment. Matrix Metalloproteinases (MMPs) are a family of proteinases with various substrates and functions, including angiogenesis, tissue invasion, inflammation and tumor metastasis[16–17]. In melanoma, both MMP-2 and MMP-9 were involved in cell invasion [18–19]; however, only MMP-2 overexpression was correlated with progression and survival [20–23]. Here we investigated whether MMP-2 was involved in PHA-739358-induced migration inhibition in melanoma cell lines. SK Mel28 cells were treated with PHA-739358 (0, 0.1, 5, 10 μM) for 72 hrs, and then subjected to immunobloting analysis for MMP-2. As shown in Fig.6C&D, PHA-739358 treatment leads to reduced protein expression of MMP-2 in a dose-dependent manner. Membranes were stripped and reprobed with anti-Phospho-IκBα and anti-Phospho-NFκB-p65 antibodies to evaluate the effect of PHA-739358 on the activities of NFκB signaling pathway. Decreased Phospho-IκBα and Phospho-NFκB-p65 expression was observed after PHA-739358 treatment indicating reduced activity in the NFκB signaling pathway (Fig.6C&D). Consistent with the literature [24], inhibition of NFκB using MG132 (a protease inhibitor that can inhibit NFκB activity) resulted in decreased MMP-2 expression in SK Mel28 cell line (Fig.6E&F)

Discussion

Several Aurora kinase inhibitors have been tested in melanoma pre-clinically in the last couple of years [10–12], indicating Aurora kinases are promising therapeutic targets for melanoma. In this study, we invested the anti-proliferative and anti-invasive effects of PHA-739358 (the most clinically advanced compound that has been entered into Phase II clinical trials) on melanoma cell lines. To the best of our knowledge, this is the first study to show the anti-proliferative and anti-invasive effects of PHA-739358 in melanoma *in vitro*.

The Aurora family comprises three related kinases (Auroras A, B, and C) that share the highest degree of sequence homology in their catalytic domains [25–26]. Expression of aurora A and B is closely linked to the proliferation of many, if not all, cell types, whereas for aurora C, the normal function of which is not clear, expression seems to be restricted to normal testicular tissue [27]. While Aurora A kinase is implicated in regulating mitotic entry, centrosome maturation, and spindle assembly, Aurora B is required for correct chromosome segregation and cytokinesis [26]. Overexpression of Aurora kinases has been demonstrated in a variety of human malignancies [28] including that of cutaneous melanoma [10, 12], and the overexpression has been shown to induce chromosomal instability, leading to aneuploidy [29]. Aneuploidy, which is the salient feature of most cancer cells and is believed responsible for neoplastic transformation, has 100% correlation with mammalian cell transformation [30].

To assess the impact of aurora kinases as a potential molecular target in melanoma, we performed a DNA microarray database search through oncomine (www.oncomine.com), the

world's largest collection of curated cancer genomics data consisting 534 gene expression datasets and 43,000+ samples. Supplemental Fig.1A&B demonstrate the increased mRNA levels of AURKA ($p < 0.001$) and AURKB ($p < 0.00001$) in melanoma tissues in comparison to normal skin or benign nevi from the dataset deposited by Haqq et al through oncomine [31], which is also consistent with the data published by Wang, et al.[10]. In this study, with a Western-blot analysis we demonstrated increased AURKA and AURKB protein levels in all six melanoma cell lines tested in comparison to normal human melanocytes (Supplemental Fig.1C). All these lines of evidence indicate that Aurora kinases A&B are overexpressed in melanoma tumor tissue and cell lines, making them ideal targets for melanoma therapeutics.

Many Aurora kinase inhibitors have been discovered, and are currently under preclinical or clinical development. PHA-739358 is among the few AKIs that have entered Phase II clinical trials for patients with solid tumors (www.clinicaltrials.gov). PHA-739358) is a small ATP competitive molecule that inhibits Aurora A, B and C kinases [14]. PHA-739358 has strong activity on Aurora A, as determined by inhibition of autophosphorylation in position Thr288 [32] and also on Aurora B as determined by inhibition of its substrate histone H3 in position Ser10 [33]. In SK Mel 28 molanoma cells, inhibition for both AURKA and AURKB were seen from 0.1 μM concentrations upward (Fig. 6A&B).

In vitro studies have shown that PHA-739358 causes a failure of cell division, resulting in polyploidy and reduction in viability in hepatocellular carcinoma and pancreatic cancer cells [34–35]. In agreement with these results, our study shows that PHA-739358 induces G2/M arrest and polyploidy (Fig.2), and inhibited proliferation in melanoma cell lines (Fig.1). Increased caspase activation (Fig.3) was also observed in melanoma cell lines upon PHA-739358 exposure. Additionally, PHA-739358 enhanced TMZ-induced caspase activation (Fig.4).

Metastasis is a crucial feature of malignant melanoma [36]. We observed that PHA-739358 significantly inhibited melanoma cell migration in a dose and time dependent manner (Fig. 5A~C). Since overexpression of MMPs is a key factor for tumor invasion and metastasis [37], and MMP-2 overexpression is closely correlated to melanoma progression and survival [23], we next investigated whether PHA-739358 can affect the expression of MMP-2. The results showed that PHA-739358 downregulated expression of MMP-2 (Fig.6C&D). Overexpression of MMPs has been frequently detected in solid tumors and associated with tumor invasion and metastasis [38]. Therefore, the antimigration effect of PHA-739358 on melanoma cells might be through the downregulation of MMP-2. As NF-kB is a key transcription factor in the regulation of MMP-2 expression [39], and Aurora kinase inhibition downregulates NFκB [40], we next studied whether PHA-739358 could inhibit the activity of NFκB signaling pathway. Consistent with the literature [40], we observed decreased IκBα phosphorylation and reduced NFκB-p65 phosphorylation indicating the inhibition of NFκB signaling pathway upon PHA-739358 exposure (Fig.6C&D). In agreement with Awasthi, et al. [24], MG132, a protease inhibitor that can inhibit NFκB activity, reduced MMP-2 expression in melanoma cells (Fig.6E&F). All these data indicate that inhibition of NFκB signaling pathway may be involved in PHA-739358-induced MMP-2 downregulation, resulting in inhibition of cell migration.

In conclusion, our results provide the first evidence for the anti-proliferative and the anti-metastatic effect of the pan-aurora kinase inhibitor, PHA-739358, on melanoma cell lines. PHA-739358 induced 40% cell migration inhibition in SK Mel28 cell line at a concentration (100nM) more than 100 times lower than the IC50 (12.45 μM) (Fig.6A&C). The same concentration (100nM) induced a 66% inhibition of cell migration in Lu1205 cell line (Fig. 5A&B). Additionally, PHA-739358-induced migration-inhibition may be through inhibition of NFκB signaling pathway resulting in decreased MMP-2 expression. Importantly, PHA-739358 enhanced temozolomide-induced caspase activation. In summary, this study provides a solid foundation for further *in vivo* or even clinical evaluation of the anti-tumor especially the anti-metastatic effects of PHA-739358 on melanoma therapeutics.

Materials and Methods

Chemicals and Reagents

PHA-739358 was purchased from Selleck Chemicals (Houston, TX). Temozolomide (TMZ) and MG132 were purchased from Sigma-Aldrich (St. Louis, MO).

Cell culture

Normal human melanocytes were isolated from discarded new-born foreskin and cultured in MCDB153 medium (Sigma, St. Louis, MO) containing 2% fetal bovine serum (FBS), 0.3% bovine pituitary extract (Cambrex, Walkersville, MD), 10 ng/ml TPA, 2 mM $CaCl_2$, 5 μg/ml insulin, and 0.1 mM IBMX (Sigma)[41]. Melanoma cell lines, WM3211, Lu1205, c83-2C, A375, SK Mel28, or SK-Mel5, were cultured in RPMI 1640, L15/MCDB, F10, DMEM, EMEM, or AMEM media, respectively. MDA-MB-231, a breast cancer cell line, was grown in DMEM. Each medium was supplemented with 5% Fetal Bovine Serum (FBS), 5% Neonatal Bovine Serum (NBS), and 2% penicillin (100 U/ml)/streptomycin (0.1 mg/ml). All cells were kept at 37°C in 5% CO_2 incubator.

Cell cycle analysis using flow cytometry

Cells were seeded in 100 mm tissue culture dishes (6×10^5 cells per dish) (Corning, Lowell, MA) and grown overnight before drug treatment. For cell cycle analysis, WM3211, Lu1205, and SK Mel28 cells were treated with various concentrations of PHA-739358 as indicated in the figure legends for 72hrs. The drug-treated cells and untreated control samples were harvested by trypsinization and stained with propidium iodide (Sigma-Aldrich, St. Louis, MO) in a modified Krishan buffer for one hour at 4°C. The propidium iodide-stained samples were analyzed with a FACS Calibur Flow Cytometer (BD Immunocytometry Systems, San Jose, CA). Histograms were analyzed for cell cycle compartments, and the percentage of cells at each phase of the cell cycle was calculated using CellQuest Software (BD Immunocytometry Systems).

Sulforhodamine B (SRB) cell viability assay

Cells were seeded in 96-well culture plate at a density of 4000 cells/well at 24hr before drug treatment to allow attachment. Cells were then exposed to serial dilutions of PHA-739358 for 24, 48 or 72hrs followed by SRB assay as described previously [35]. Briefly, after drug treatment, cells were fixed with 10% TCA solution, and then were stained with 0.04% SRB.

After washing with 1% acetic acid, SRB dye was dissolved with 50mM Tris-base. Plates were finally read at OD 564 nm using a BIO-RAD Model 680 Microplate Reader (Hercules, CA). Cell viability was calculated by dividing the average of the reading number for vehicle treated control wells. The dose-response curves were plotted and the IC50 values (concentration required to achieve 50% growth inhibition) were determined using the Prism 4 software (GraphPad Software).

Caspase 3/7 activity-based apoptosis assay

Cells were seeded in 6-well plates (2×10^5 cells per well) and incubated for 24 hr at 37°C to allow attachment. Then cells were treated with various concentrations of drugs as indicated in the figure legends for different cell lines. After washing with PBS solution, the cells were detached by trypsinization and combined with the culture media for each sample. The cell suspension was pelleted by centrifugation at 1000 rpm for 5 min. 150 μl of cell lysis buffer (Cell Signaling Technology, Danvers, MA) was then added into the cell pellet and mixed by pipetting and incubated on ice for at least 30 min. The lysed cell mixture was spun down at 13,200 g for 15 min to remove cell debris. Protein concentrations were determined using the BCA protein assay kit (Thermo Scientific, Rockford, MI). Caspase 3/7 activity was measured using the Caspase-Glo® 3/7 Assay kit (Promega) according to the manufacture instructions. Briefly, an equal volume (50μl) of Caspase-Glo® 3/7 reagent was added to each cell lysate sample (50μl) in a Half Area 96-well assay plate (Corning, NY) with a final assay volume of 100μl. Samples were incubated at room temperature for 1hr (protected from light) with shaking, and the luminescence of each sample is measured using a Combination-Modulus (Turner Biosystems, Sunnyvale, CA). The Caspase 3/7 activity was normalized to the amount of total protein contained in the cell lysate as determined by the BCA protein assay (Thermo Scientific, Rockford, MI).

In vitro migration assay

The migration ability of the cells with or without treatment was tested using BD BioCoat Tumor Invasion Assay System (BD Biosciences, Bedford, MA) according to manufacture's protocol. Briefly, cells were treated with various concentrations of PHA-739358 as indicated in the figure legends. After drug treatment, viable cells from each treatment group (75,000 cells/500 μl of SFM, confirmed by trypan blue staining) were seeded onto the upper chamber, while medium containing 0.5~1% FBS (determined by cell type) was added into the lower compartment as a chemo-attractant. After overnight incubation in a 5% CO_2 humidified incubator at 37°C for 24 hrs, the cells on the upper chamber were carefully removed with a cotton swab and the cells that had traversed to reverse face of the membrane were fixed with methanol for 20 min, and stained with Hematoxylin for 10min. Photographs were taken and the number of cells on the reverse side of the filters were quantified by a Celestron 1600X LCD Deluxe Digital Microscope (Celestron, LLC., Torrance, CA).

Western blotting analysis

Cell lysates containing equal amount of protein (40 μg) were loaded on 10% SDS-PAGE gels. The separated proteins were transferred to PVDF membranes. Membranes were then probed with primary antibodies against Aurora kinase A (AURKA) (GeneTex, Irvine, CA), Phospho-AURKA (Thr^{288}), Aurora kinase B (AURKB), Phospho-Histone H3 (Ser^{10}),

MMP-2, Phospho-IκBα, Phospho-NFκB-p65, and β-actin (All from Cell Signaling Technology Inc., Danvers, MA). β-Actin was included to serve as an internal control. The bound primary antibodies were detected using peroxidase-conjugated secondary antibodies (Cell Signaling) and chemiluminescence by the Immobilon™ Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA) according to manufacturer's instructions. The luminescent signal of the membrane was then detected by photographic film.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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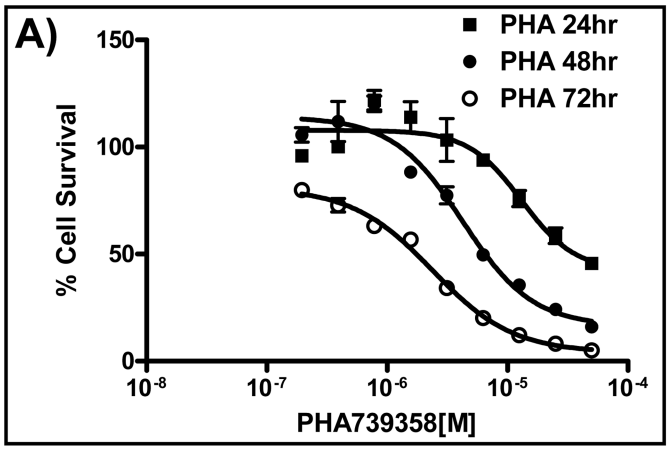
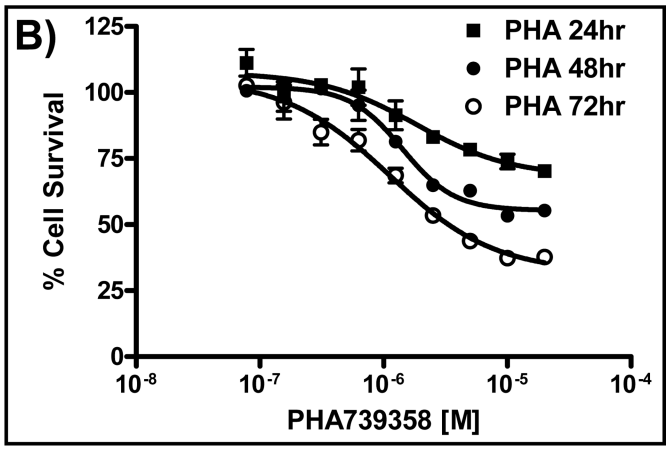
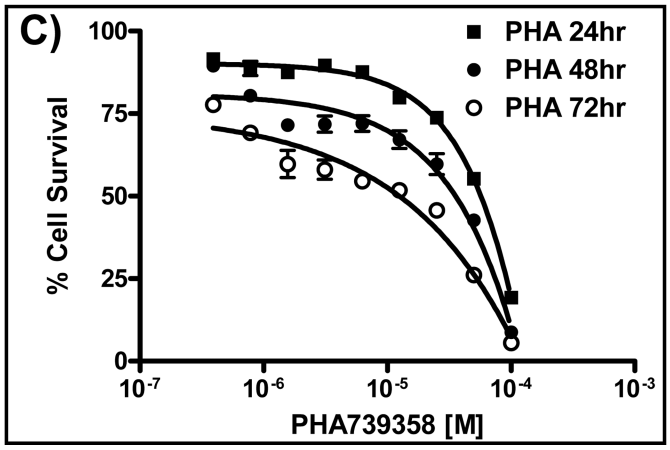




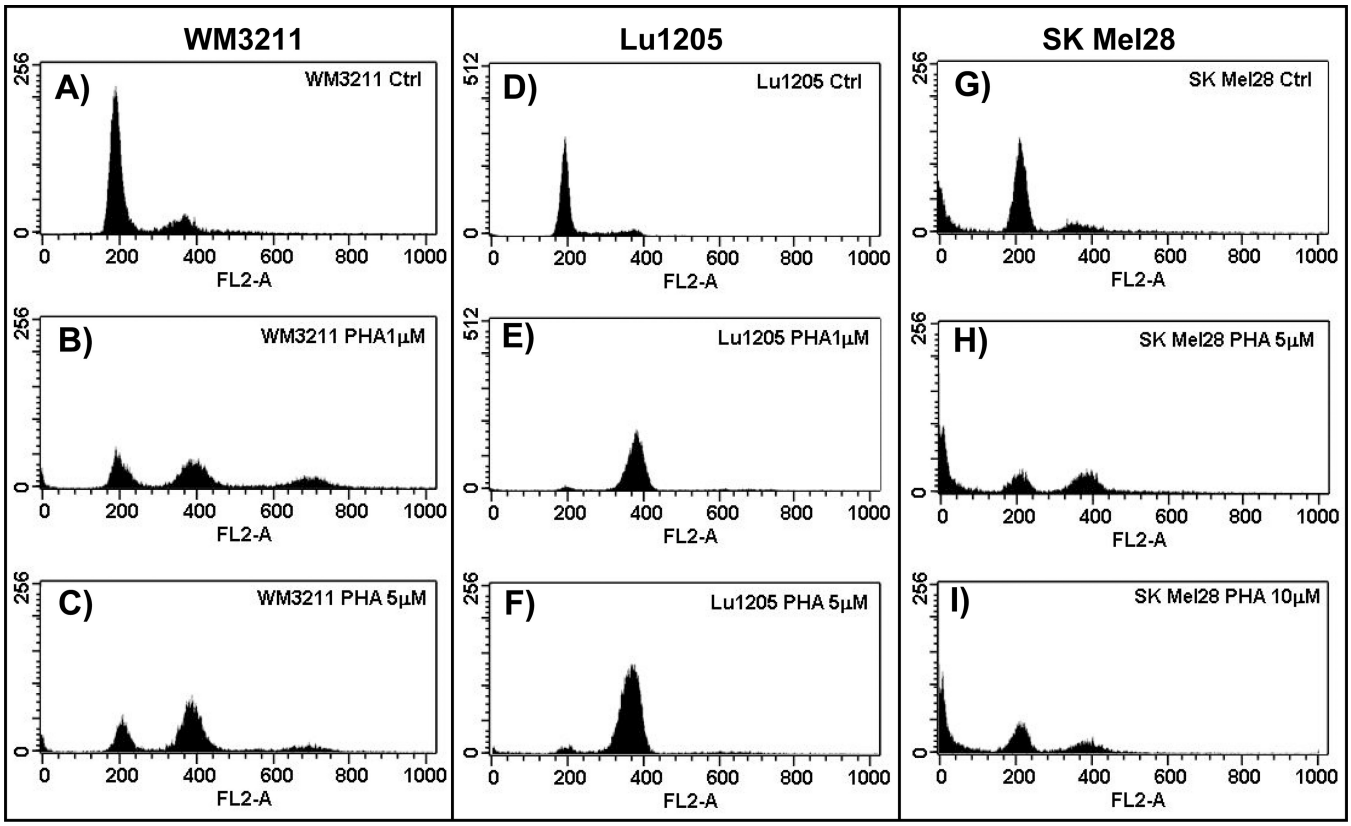


D)

Cell Lines	IC_{50}s (μM)
WM3211	1.76±0.04
Lu1205	3.34±0.05
SK Mel28	12.45±0.27

Figure 1. Dose and time effects of PHA-739358 on cell viability in three melanoma cell lines

WM3211, Lu1205, and SK Mel28 cells were treated with serial dilutions of PHA-739358. Cell viability was measured by SRB assay at 24hr, 48hr, or 72hr after drug treatment, respectively. The dose-response curves (A: WM3211; B: Lu1205; and C: SK Mel28) were plotted and the IC50 values (concentration required to achieve 50% growth inhibition) after 72 hr drug treatment (D) were determined using the Prism 4 software.



J)

Flow cytometry analysis of the effect of PHA on cell cycle progression

Cell line	Group	G0-G1(%)	S(%)	G2/M(%)	Polyploidy	Debris
	Ctrl	68.79	3.65	15.97	8.45	3.35
	PHA 1μM	23.56	3.05	31.42	26	16.18
WM3211	PHA 5μM	19.69	2.75	48.39	16.34	13.15
	Ctrl	70.12	7.93	11.69	3.52	6.86
	PHA 1μM	3.75	2.2	81.89	6.81	5.45
Lu1205	PHA 5μM	4.1	2.23	79.9	5.44	8.43
	Ctrl	45.05	10.3	2.02	6.62	36.2
	PHA 5μM	17.02	2.03	24.63	8.15	48.37
SK Mel28	PHA 10μM	24.33	3.09	14.84	2.95	55.08

Figure 2. Effect of PHA-739358 on Cell Cycle Progression in Melanoma Cell Lines

WM3211 (A~C), Lu1205 (D~F), and SK Mel28 (G~I) cells were treated with various concentrations of PHA-739358 as indicated in the figures. After 72 hr of drug treatment, flow cytometry analysis of cell cycle was performed using propidium iodide staining (A~I). Cell cycle distribution of control and treated cells for all three cell lines are shown in J.

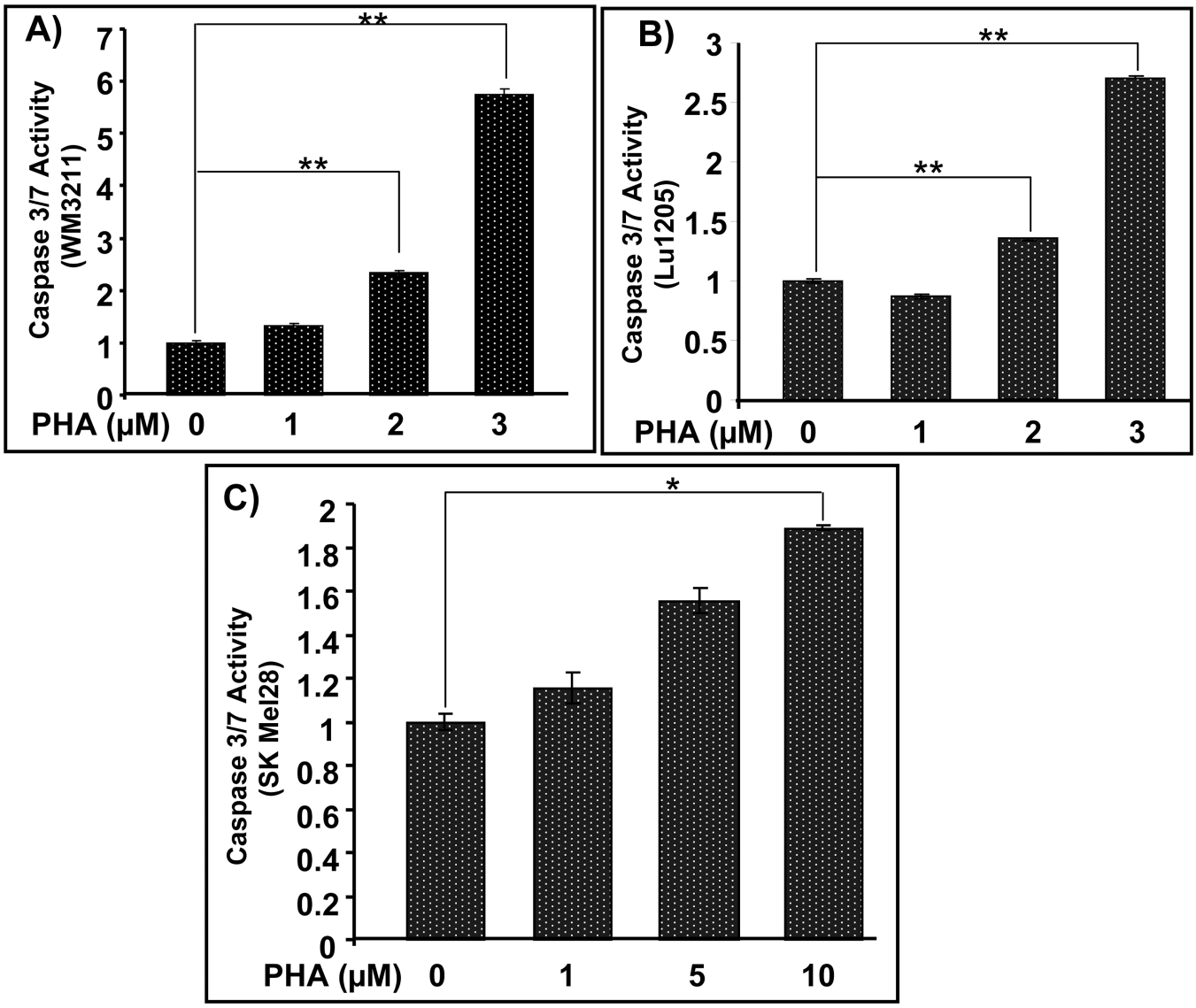


Figure 3. PHA-739358-induces caspase activation in melanoma cell lines

WM3211 (A), Lu1205 (B), SK Mel28 (C) cells were treated with various concentrations of PHA-739358 as indicated in the figures for 72 hrs and then subjected to evaluation of caspase activity levels by the Caspase 3/7 Glo Assay. An asterisk (*) indicates $P<0.005$ while double asterisks (**) indicate $P<0.001$ when means from PHA-739358 treated samples are compared with control samples.

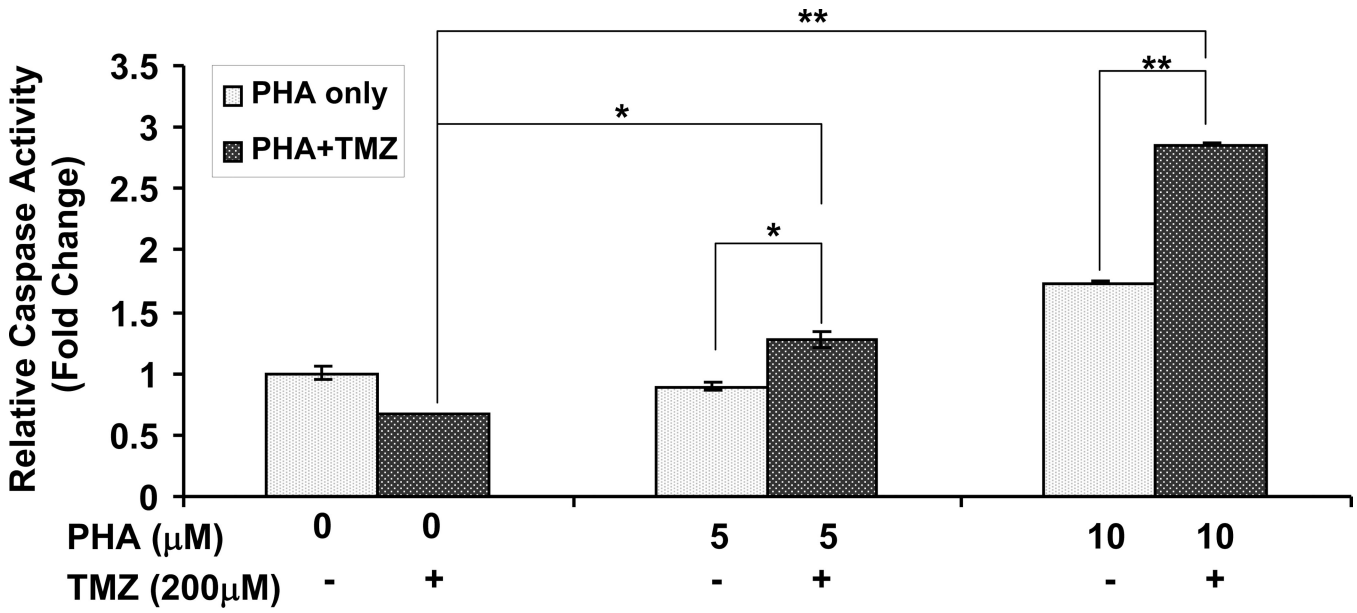


Figure 4. Increased caspase activation induced by the combination treatment of PHA-739358 and TMZ

SK Mel28 cells were treated with PHA-739358 (0, 5 or 10μM) with/or without TMZ (200μM) for 72 hrs and then subjected to caspase activity evaluation using Caspase 3/7 Glow Assay. An asterisk (*) indicates $P<0.05$ while double asterisks (**) indicate $P<0.0001$ when means from PHA-739358 plus TMZ - treated samples are compared with either TMZ or PHA-739358 alone.

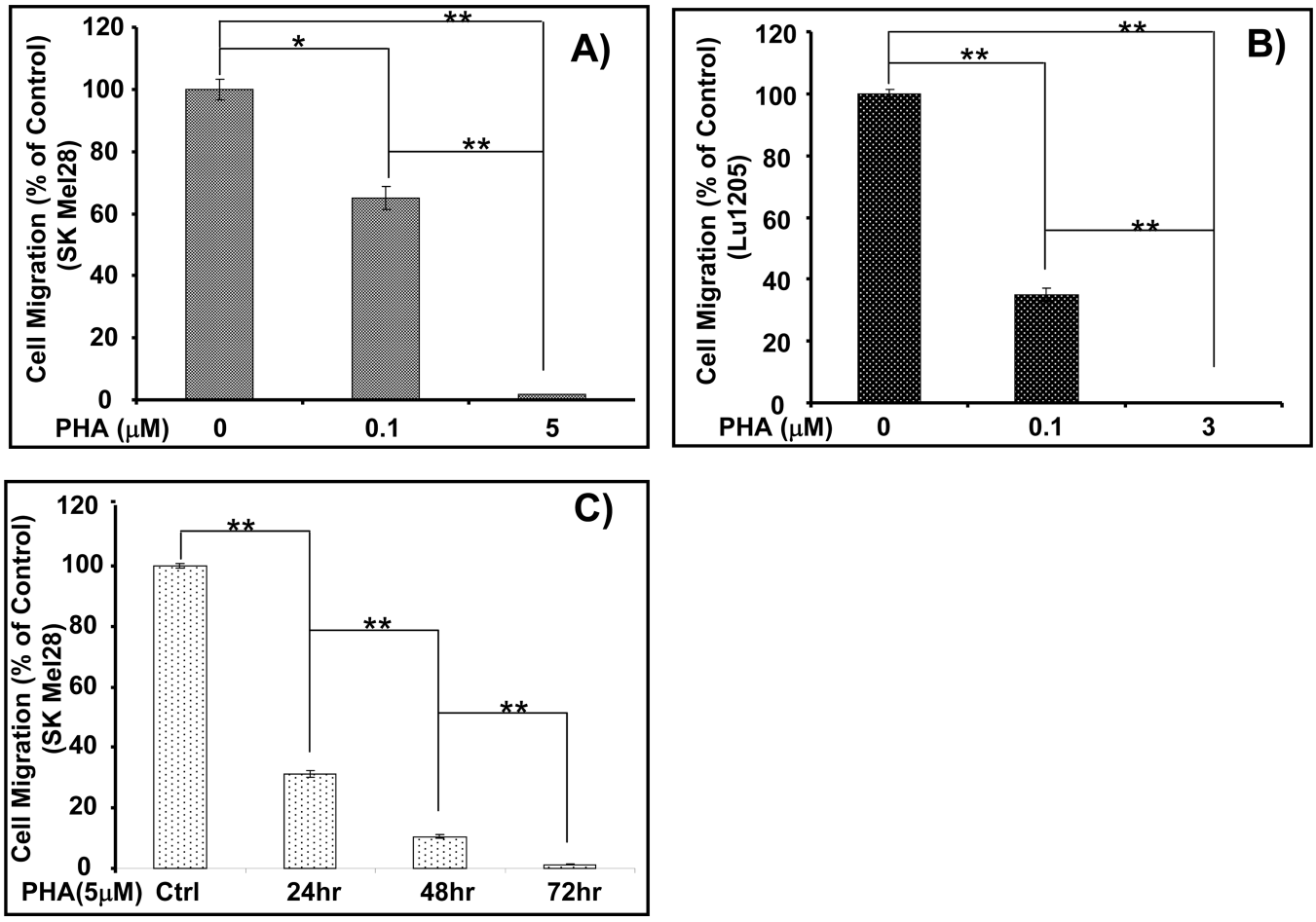


Figure 5. Time and dose dependent cell migration inhibition of PHA-739358 in melanoma cell lines

SK Mel28 and Lu1205 cells were treated with various concentrations of PHA-739358 as indicated in the figures for 72 hrs. Surviving cells were harvested and equally seeded onto the BD BioCoat chamber (BD Biosciences, Bedford, MA) (75,000 cells/500μl of SFM/ chamber), while medium containing 0.5~1% FBS was added into the lower compartment, and then incubated for 24 hrs at 37°C. A) Effect of PHA-739358 at 0.1 and 5 μM on migration ability of SK Mel28 cells in comparison to untreated control cells; B) Effect of PHA-739358 at 0.1 and 3 μM on migration ability of Lu1205 cells in comparison to untreated control cells. For the time course experiment, SK Mel28 cells were treated with 5μM of PHA-739358. Surviving cells were harvested at 24, 48, or 72 hrs, respectively, and were equally seeded onto the BD BioCoat chamber and then incubated for 24 hrs at 37°C for the measurement of cell migration activity. The effect of 5μM PHA-739358 exposure for 24, 48, or 72hr on migration ability of SK Mel28 cells in comparison to untreated control cells was shown in C. The values represented the means ± SE. * indicates $p<0.005$ while ** indicates $p<0.0001$.

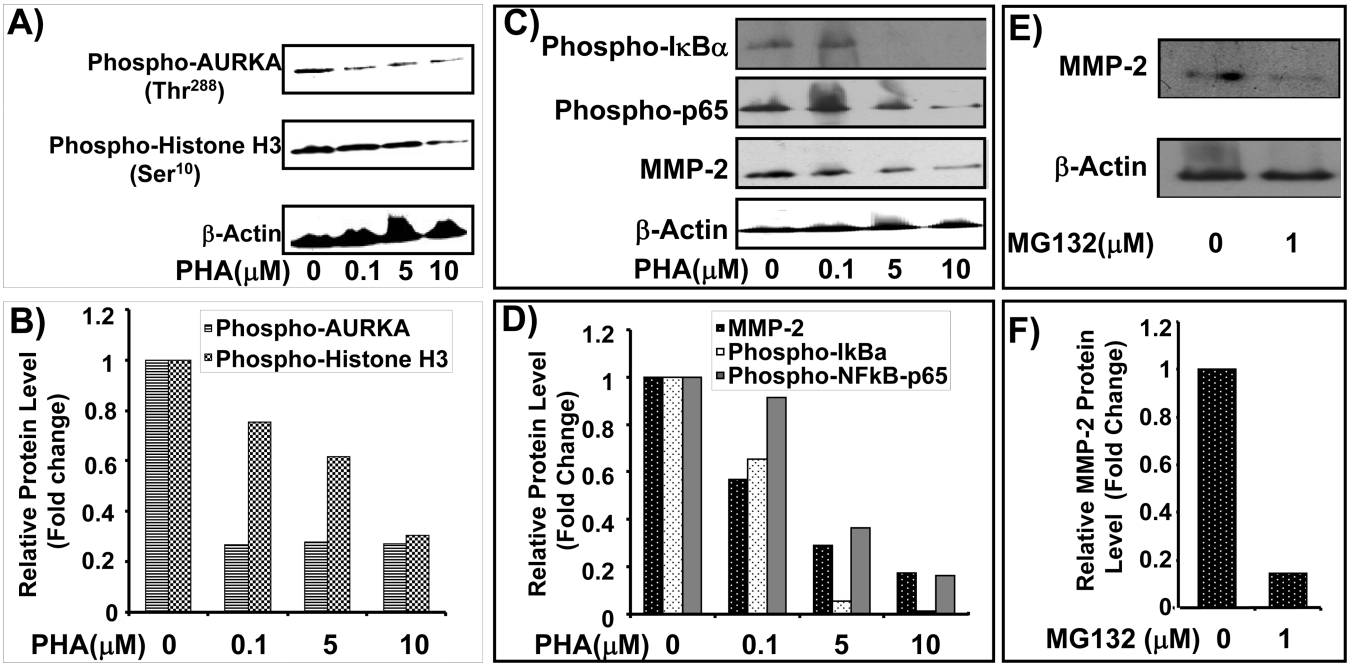


Figure 6. Inhibition of NFκB signaling may be involved in PHA-739358-induced MMP-2 downregulation

SK Mel28 cells were treated with PHA-739358 (0, 0.1, 5, 10 μM) or MG132 (1μM). 72 hrs after drug treatment, cell lysates were collected and equal amounts of total protein (40μg) were loaded in all lanes. Immunoblots for PHA-739358 treated samples were probed with anti-phospho-AURKA (Thr^{288}) and anti-phospho-Histone H3 (Ser^{10}) antibodies to determine the effects of PHA-739358 on AURKA autophosphorylation and AURKB-mediated Histone H3-phosphorylation (A). Samples were also probed with anti-MMP-2, anti-phospho-NFκB-p65, and anti-phospho-IκB antibodies (C). Anti-MMP-2 was also probed for MG132 treated samples (E). β-actin was used as a loading control. The intensities of the bands were quantified using NIH imaging J software. The quantified results for Figs A), C), and E) are shown in Figs B), D), and F), respectively.