Novel imidazo[1,2-a]pyridine inhibits AKT/mTOR pathway and induces cell cycle arrest and apoptosis in melanoma and cervical cancer cells

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Abstract. The present study aimed to investigate the anti-cancer activity of imidazo[1,2-a]pyridine 5-7 in the A375 and WM115 melanoma and HeLa cervical cancer cell lines. The viability of cancer cells was analyzed by the MTT assay. Apoptosis was quantified by flow cytometry following staining of the cells with AnnexinV/propidium iodide (PI). The cell cycle was evaluated by flow cytometry after staining of cells with PI. The three compounds inhibited the proliferation of all cells for half maximal inhibitory concentration ranging from 9.7 to 44.6 μM following 48-h treatment. In addition, all cancer cells were more sensitive to compound 6 compared with the other compounds. Treatment with compound 6 induced G_2/M cell cycle arrest and a significant increased level of intrinsic apoptosis in all tested cells. Furthermore, compound 6 reduced the levels of phospho (p)-protein kinase B and p-mechanistic target of rapamycin, and increased levels of the cell cycle inhibitors p53 and p21 and of the apoptosis-associated proteins BCL2 associated X protein and active caspase-9. Silencing p53 in A375 melanoma cells reduced compound 6-induced apoptosis, which suggested that compound 6 may induce p53-partially mediated apoptosis. These results demonstrated that imidazo[1,2-a]pyridines 5-7 are potential effective compounds in the treatment of melanoma and cervical cancers.

Introduction

Cervical cancer and melanoma are aggressive cancers with increasing incidence worldwide (1). Whereas cervical cancer ranks fourth cancer for both incidence and mortality, malignant melanoma is the most serious type of skin cancer and accounts for the majority of skin cancer-associated mortalities (1,2). Cervical cancer is commonly treated with a combination of radiotherapy and platinum-based chemotherapy, which may also damage normal cells (3). At present, there is no globally accepted standard treatment that offers a significant survival benefit for patients with advanced-stage melanoma (2). Until 2011, the chemotherapeutic drugs dacarbazine, temozolomide and fotemustine were most commonly used for metastatic melanoma treatment (4); however, only a low percentage of patients who received these compounds exhibited a significant response. These treatments have been mostly replaced by the immune-checkpoint inhibitors, including cytotoxic T-lymphocyte-associated protein-4 (CTLA-4), B-Raf proto-oncogene (BRAF) and mitogen-activated protein kinase kinase (MEK) inhibitors (5). However, these therapeutic alternatives display adverse events, including secondary cutaneous squamous cell carcinomas and keratoacanthomas, which occur in ~20% of patients treated with BRAF-inhibitor (6). A previous study reported that MEK inhibitors have more serious adverse effects and a lower efficacy compared with BRAF inhibitors (5). The development of novel effective agents for the treatment of these cancers is therefore crucial.

Imidazopyridines possess a wide range of biological activities. In particular, the imidazo[1,2-a]pyridine moieties of imidazopyridine have recently gained significant interest as potential anticancer agents due to their potent inhibitory role of cancer cell growth, which is commonly due to survival kinases inhibition (7,8). Various drugs containing imidazo[1,2-a]pyridine moieties are currently used to treat cardiac disorders, insomnia, antianxiety, ulcers and HIV infections (9). Although these compounds have numerous medicinal applications, none of them have been accepted as an anti-cancer drug. However, previous studies have reported that imidazo[1,2-a]pyridines have some anti-cancer abilities. For example, Goel *et al* (9) recently reported that 3-{1-[(4-fluorophenyl)sulfonyl]-1*H*-pyrazol-3-yl}-2-methylimimoredazo[1,2-a]pyridine may be a novel PIK3CA inhibitor with an half maximal inhibitory concentration (IC_{50}) of 0.67 μM. Further optimization of the substituents resulted in thiazole groups substituted by

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imidazo[1,2-a]pyridines, which are more potent inhibitor of phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PI3KCA) with an IC_{50} of 0.0028 μM (10). In addition, this compound exerts a promising anti-proliferation effect against melanoma (A375) and cervical (HeLa) cancer cells, with IC_{50} values of 0.14 and 0.21 μM, respectively. Notably, 50 mg/kg of this compound significantly inhibits HeLa human cervical tumor xenografts growth in mice. Additional series of imidazo[1,2-a]pyridine derivatives were designed and synthesized as PI3Kα inhibitors (6). One of these compounds, comprising the bioisosteric 1,2,4-oxadiazole group as a substituent, exhibits potent PI3Kα inhibition with an IC_{50} of 2 nM. In addition, this compound inhibits various types of breast cancer cell line proliferation with an IC_{50} of >10 μM. Furthermore, Annexin V results demonstrated that it significantly increases T47D breast cancer cell apoptosis. At a molecular level, these effects are associated with PI3K signaling inhibition. Notably, this compound has some anti-angiogenic effects through VEGF expression inhibition. In addition, ethyl 6-(5-(phenyl sulfonamide)pyridin-3-yl)imidazo[1,2-a] pyridine-3-carboxylate was also reported (11). This compound has significant anti-cancer activity in non-small cell lung cancer cells. Cell treatment with this compound inhibits the phospho (p)-protein kinase B (PI3K)-protein kinase B (Akt)-mechanistic target of rapamycin (mTOR) pathway-induced intrinsic apoptosis (11). A recent study reported that selenylated imidazo[1,2-a]pyridines inhibits breast cancer cell proliferation by inducing DNA damage and apoptosis (12). Additional anti-cancer properties of imidazo[1,2-a]pyridines, including inhibition of nicotinamide phosphoribosyltransferase (13), cyclin-dependent kinases (14) and insulin-like growth factor 1 receptor tyrosine kinase (8) were also reported.

The present study aimed to investigated the anticancer activities of three imidazo[1,2-a]pyridines, the compounds 5-7, in melanoma and cervical cancer cells through analyses of the Akt/mTOR pathway, the cell cycle and apoptosis.

Materials and methods

Chemicals and reagents. Chemicals and solvents, radioimmunoprecipitation assay (RIPA) lysis buffer (cat. no. R0278) were used without further purification and were supplied by Sigma-Aldrich; Merck KGaA (Darmstadt, Germany), unless otherwise stated. Media and cell culture reagents were from Biological Industries (Kibbutz Beit Haemek, Israel). Western blotting reagents were obtained from Bio-Rad Laboratories Inc. (Hercules, CA, USA). DAPI and primary antibodies against poly(ADP-ribose) polymerase 1/2 (PARP1/2; cat. no. sc-7150), p53 (cat. no. sc-126), p21 (cat. no. sc-756), BCL2 associated X protein (BAX; cat. no. sc-7480), cyclin B (cat. no. sc-sc-53236), AKT (cat. no. sc-514032) and tubulin (cat. no. sc-5286) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Primary antibodies against B-cell lymphoma 2 (BCL2; cat. no. 2876) and caspase-9 (cat. no. 9502) were provided by Cell Signaling Technology, Inc. (Danvers, MA, USA). Horseradish peroxidase-conjugated secondary antibodies were provided by Bio-Rad Laboratories, Inc. (Hercules, CA, USA) and electrochemiluminescence reaction (ECL) detection system was purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA).

Synthesis of compounds 5-7. Triethylamine (0.6 g, 0.006 mol) diluted in 10 ml dry tetrahydrofuran (THF) was dropwise added to a stirring solution of hydrazonoyl chloride 1 (0.005 mol) and substituted picolines 2-4 (0.65 g, 0.006 mol) in 25 ml THF at room temperature. Stirring was continued overnight, and the solvent was evaporated *in vacuo*. The residual solid was washed with water to remove the triethylammonium salt, and the crude products were recrystallized from the appropriate solvents to obtain the compounds 5-7. The physical data of these compounds are available in the original publication (15). The chemical formulas of the three compounds were as follows: 3-[(4-Chlorophenyl)diazenyl]-2,8-dimethylimidazo[1,2-a]pyridine (compound 5), 3-[(4-Chlorophenyl) diazenyl]-2,7-dimethylimidazo[1,2-a]pyridine (compound 6) and 3-[(4-Chlorophenyl)diazenyl]-2,5-dimethylimidazo[1,2-a]pyridine (compound 7).

Cell culture and treatments. The human melanoma cell lines A375 and WM115 and the cervical cancer cell line HeLa, sourced from the Department of Human Biology, University of Cape Town (Cape Town, South Africa), were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and placed at 37˚C in a humidified incubator containing 5% CO_2. Compounds 5-7 were dissolved in dimethyl sulfoxide (DMSO) to obtain 10 mM stock solutions that were stored at room temperature for a maximum of 10 days. Control cells were treated with equivalent concentrations of DMSO (vehicle).

Small interfering RNA (siRNA). Suppression of p53 expression was achieved by siRNA that specifically targeted p53 mRNA. Cells at 70% confluence were transfected with 50 nM anti-p53 siRNA (cat. no. sc-756) or a scrambled control RNA (cat. no. sc-37007) from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA) using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The transfection reagent and siRNA complex were added drop-wise to the cells and incubated for 30 h at 37˚C. The subsequent experiments were performed 30 h after transfection.

Cytotoxicity assays. Cells were seeded in 96-well plates at 4-5x10^3 cells per well and allowed to settle for 48 h. Cells were treated with increasing concentrations of compounds 5-7 (0-100 μM) or vehicle for 48 h. Cytotoxicity was assessed using MTT assay (Roche Diagnostics GmbH, Mannheim, Germany) (16) according to the manufacturer's instructions. Briefly, 10 μl MTT solution was added to each well and cells were incubated at 37˚C for 4 h. The solubilization buffer (100 μl, 10% SDS in 0.01 M hydrochloric acid) was added on cells for 16 h at 37˚C. Absorbance was determined at 585 nm with a microplate reader, and the mean cell viability was calculated as a percentage of the mean vehicle control.

Cell cycle analysis. The effect of compound 6 on the cell cycle profile of cancer cells was determined according to a previous protocol (17). Briefly, cells were seeded at 3-4x10^5 cells per

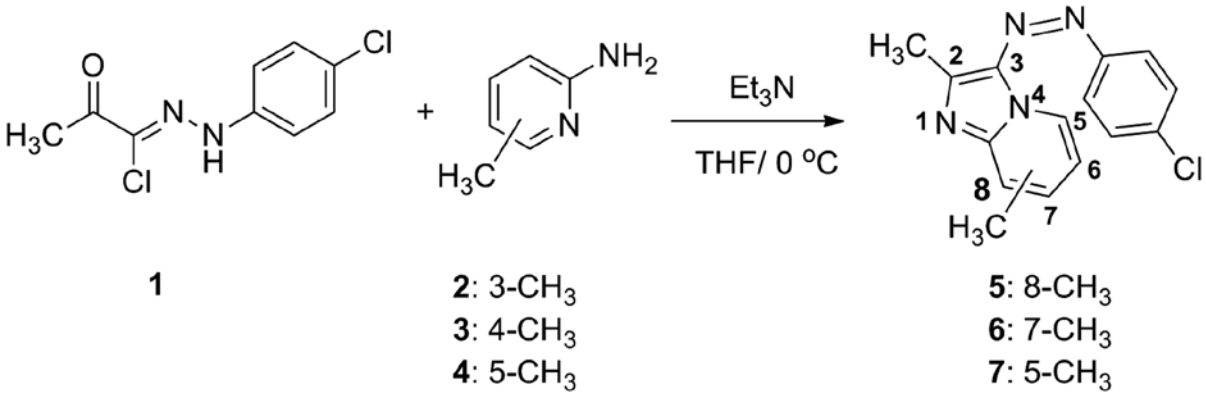

Figure 1. Schematic representation of the imidazo[1,2-a]pyridines 5-7 compounds synthesis and structures (15). Et_3N, triethylamine; THF, tetrahydrofuran.

6-cm dish and allowed to settle for 24 h. Log-phase cultures were exposed to compounds 5-7 (0 to 100 μM) or vehicle for 48 h. Cells were trypsinized, washed with PBS and fixed overnight in 95% ethanol at 4˚C, and subjected to RNase treatment and stained for 30 min at room temperature with propidium iodide (PI; 500 μg/ml; Sigma-Aldrich; Merck KGaA). Cellular DNA content was determined by flow cytometry with a FACSCalibur flow cytometer with a 488 nm Coherent laser (BD Biosciences, Franklin Lakes, NJ, USA). The 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).

Detection of apoptosis. Log-phase cultures (60-70% confluence) were treated with compound 6 or vehicle for 48 h. Adherent and floating cells were collected and double-labelled with Annexin V-Fluorescein isothiocyanate (FITC) and PI (Sigma-Aldrich; Merck KGaA) for 10 min at room temperature. Annexin V-FITC was used to determine apoptotic cells percentage whereas PI stained all dead cells. Cells were analyzed by flow cytometry with a 488 nm Coherent laser equipped with FACStation running CellQuest software (BD Biosciences).

Nuclear fragmentation. Melanoma and cervical cancer cells were treated with compound 6 (10 and 35 μM, respectively) for 24 h at 37˚C and stained with DAPI (10 μg/ml) nuclear stain for 10 min at room temperature. Cells were observed by fluorescence microscopy (Zeiss GmbH, Jena, Germany).

Western blotting. Cells were harvested with RIPA lysis buffer for 30 min on ice. The lysates were collected, and the protein concentrations were determined using Bradford's reagent (Bio-Rad Laboratories, Inc.), according to the manufacturer's instructions and using albumin as a standard. A total of 20 μg protein was separated by 8-15% SDS-PAGE, and transferred to a Hybond ECL nitrocellulose membrane (GE Healthcare, Chicago, IL, USA) (17). Following blocking for 1 h at room temperature with PBS containing 0.05% Tween-20 and 5% powdered skim milk, membranes were incubated overnight with primary antibodies (1:1,000) at 4˚C. After primary antibody incubation, membranes were incubated with appropriate HRP-conjugated secondary antibodies (1:5,000) for 1 h at room temperature. Bands were detected using enhanced chemiluminescence detection system (Thermo Fisher Scientific, Inc., Waltham, MA, USA) (18). Relative expression level of the proteins was analyzed by UN-SCAN-IT gel 6.1 software by Silk Scientific Corporation (Orem, UT, USA) and normalized to the loading controls.

Statistical analysis. Results are presented as the mean ± 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 (version 5; GraphPad Software, Inc., La Jolla, CA, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Imidazo[1,2-a]pyridines induces cytotoxicity and cell cycle arrest. Imidazo[1,2-a]pyridines 5-7 were synthesized according to our published method (15). As presented in Fig. 1, the reaction of hydrazonoyl chloride 1 with the appropriate substituted methyl-2-aminopicolines 2-4 in the presence of triethylamine as a base at 0˚C gave the compounds 5-7 (Fig. 1).

The cytotoxic effects of compounds 5-7 on melanoma and cervical cancer cell lines were investigated. A375, WM115 and HeLa cells were treated with increasing concentration of compounds 5-7 (0 to 100 μM) for 48 h prior to assessing cell viability with MTT assay. The results demonstrated that all compounds inhibited cell proliferation of the three cell lines with different IC_{50} ranging from 9.7 to 44.6 μM (Fig. 2A). Notably, compound 6 was the most potent compound to induce melanoma and cervical cancer cell toxicity. In addition, compound 6 was more toxic to melanoma cells than cervical cancer cells. The effect of compound 6 on cell cycle profile was then explored. To do so, melanoma and cervical cancer cells were treated with 10 and 35.0 μM compound 6, respectively, for 48 h prior to analyzing cell cycle. The results in Fig. 2B and C demonstrated that compound 6 induced a significant G_2/M cell cycle arrest in all cell lines, which was mainly at the expense of G_1 phase cell populations. In addition, A375 cell treatment with compound 6 caused an increase in the cell population

A

Compound	IC_{50}		
	HeLa	A375	WM115
Compound 5	44.697±4.732	33.370±4.380	33.900±5.793
Compound 6	37.841±3.324	9.7346±2.435	11.346±2.459
Compound 7	42.036±2.603	19.941±4.267	22.458±1.293

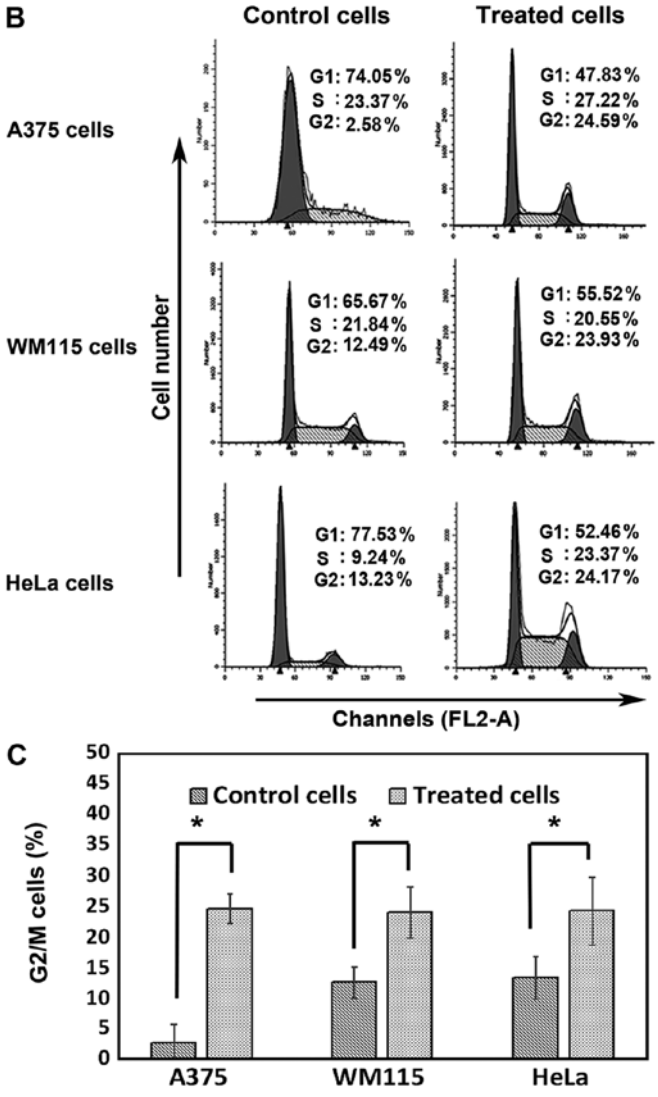

Figure 2. Compounds 5-7 inhibit cancer cell proliferation. (A) IC_{50} values for the treatment of A375, WM115 and HeLa cell lines with compound 6 for 48 h. Cell survival rate was measured by MTT assay and IC_{50} values were calculated from sigmoidal plots with GraphPad Prism version 5. Results represented the means ± standard error of the mean of at least three experiments performed in quadruplicate. (B) A375 and WM115 cells were treated with vehicle or compound 6 (10 μM), and HeLa cells were treated with vehicle or compound 6 (35 μM) for 48 h. Cell cycle profile was determined by cell staining with propidium iodide and DNA content was assessed by flow cytometry. (C) Proportion of cells in the G_2/M phase was expressed as a percentage of the total number of cells analyzed and represented as the means ± standard error of the mean of at least three experiments. $^*P<0.01$, one way analysis of variance with Tukey post-test comparing treated samples to the relevant untreated control. IC_{50}, half maximal inhibitory concentration.

in the G_2/M phase from 2.58±3.1% (control) to 24.59±2.4%. Similarly, WM115 cell treatment with compound 6 increased cell population in the G_2/M from 12.49±2.6% (control) to 23.93±4.2%. In cervical cancer cells, compound 6 treatment increased the G_2/M phase cell population from 13.23±3.5% (control) to 24.17±5.6%. Notably, whereas no significant effects of compound 6 were observed on the S phase of melanoma cells, HeLa cells treated with compound 6 exhibited a significant increase in the S phase cell population which raised from 9.24±2.3% (control) to 23.37±4.8%. These results suggested that compounds (5-7), particularly compound 6, may inhibit cancer cells proliferation and induce G_2/M cell cycle arrest in cancer cells.

Compound 6 induces intrinsic apoptosis. To determine whether compound 6 induced apoptotic cell death, melanoma and cervical cancer cells were treated with 10 and 35.0 μM compound 6, respectively, for 48 h and stained with PI and Annexin V-FITC. The results from flow cytometry demonstrated that compound 6 significantly increased apoptosis in all cancer cell lines (Fig. 3A). In addition, the levels of apoptotic melanoma cells were higher than the levels of apoptotic HeLa cells following compound 6 treatment. A375 cells treated with 10 μM of compound 6 for 48 h increased significantly from 4.58±2.34% (control) to 27.38±3.4%. Similar results were obtained for WM115 cells that exhibited an increase in apoptosis from 3.48±1.49% (control) to 22.49±3.23% following 48-h treatment with 10 μM compound 6. However, the level of apoptosis induced by 35 μM compound 6 was only 16.38±3.23% in HeLa cells. In addition, following nuclear staining and fluorescence microscopy analysis, cancer cells treated with compound 6 exhibited fragmented chromatin, which was characteristic of apoptotic cells (Fig. 3B). To further confirm that compound 6 induced apoptosis at a molecular level, and to investigate the mechanisms underlying compound 6-induced cell death, western blotting of key apoptotic proteins, including cleaved PARP, BAX, BCL2 and caspase-9 were performed (Fig. 3C). The results demonstrated that PARP cleavage was increased in all cancer cell lines following 24 and 48 h of treatment with compound 6. Furthermore, the markers of intrinsic apoptosis, including BAX cleaved caspase-9, were increased in the three cancer cell lines following 48 h of treatment with compound 6. In addition, the level of the anti-apoptotic protein BCL2 decreased in all treated cells. These results indicated that compound 6 induced intrinsic apoptotic pathways in melanoma and cervical cancer cell lines.

Compound 6 inhibits AKT/mTOR pathway. Previous studies have reported the ability of different imidazo[1,2-a]pyridines to inhibit AKT/mTOR pathway in cancer cells (12,13). These studies demonstrated that these compounds bind to the ATP-binding site of PI3K with a high affinity, which induces the PI3K/AKT/mTOR pathway inhibition. To investigate whether compound 6 exerts its effect through the same mechanism, western blotting of key proteins from this pathway, including p-AKT (using the specific antibody for phosphorylated Ser 473 of Akt1), AKT and p-mTOR were performed. The results demonstrated that both p-AKT and p-mTOR levels were reduced in all cancer cells following 48-h treatment with compound 6 (Fig. 4A). These results indicated that compound 6 inhibited AKT, which may regulate other important proteins, including as p53.

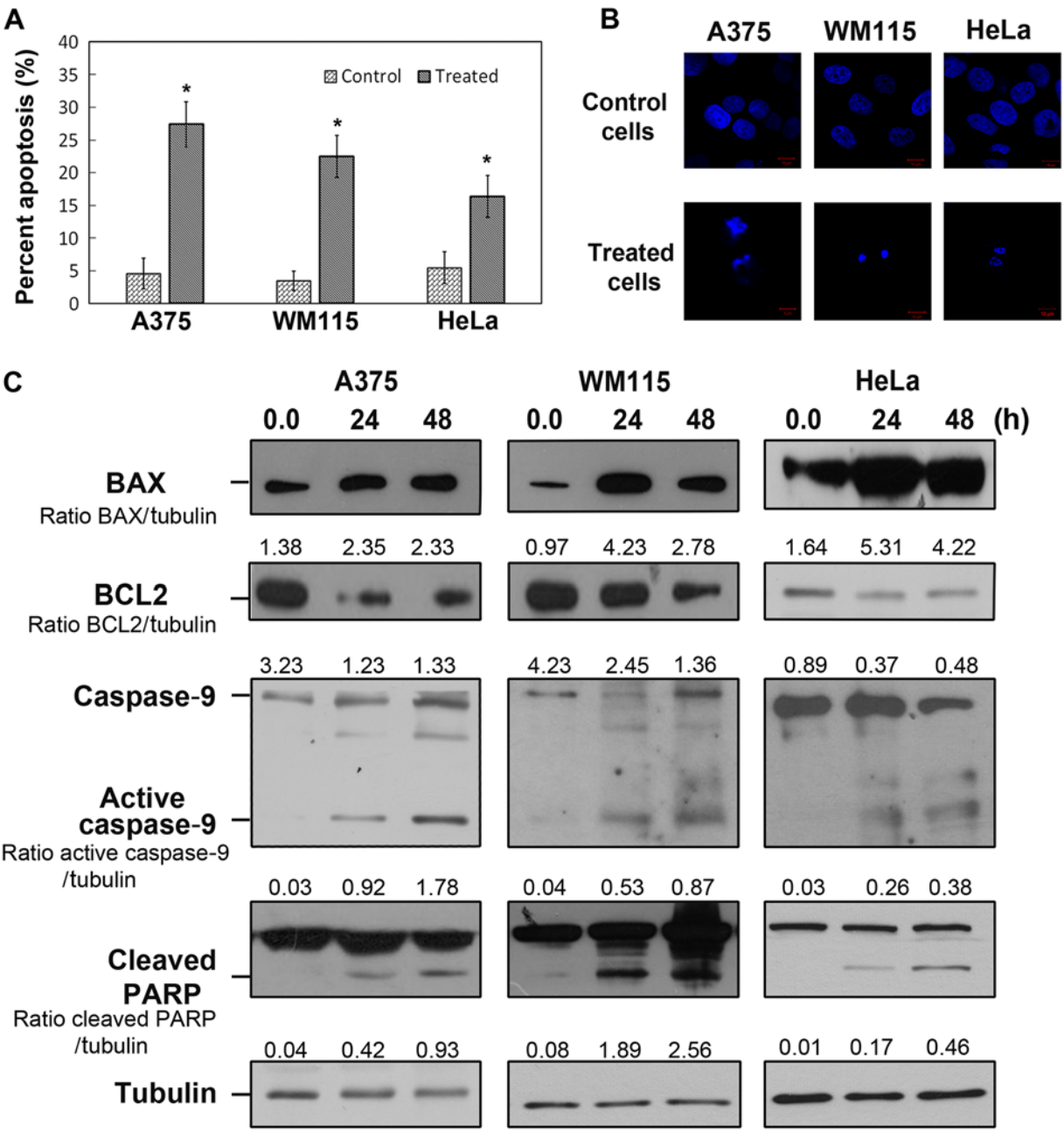

Figure 3. Compound 6 activates intrinsic apoptosis. (A) Flow cytometry analyses of Annexin V-fluorescein isothiocyanate/propidium iodide staining presented the apoptosis induction in melanoma and cervical cells treated with compound 6 at 10 and 35 μM, respectively, for 48 h. The graph represented the percentage of cells undergoing apoptosis (early and late) and the results are presented as the mean of three independent experiments ± standard error of the mean. *P<0.05. (B) Fluorescence microscopy demonstrated that cancer cells treated as in (A) and stained with DAPI displayed fragmented chromatin characteristic of apoptotic cells. (C) Western blotting of proteins from cancer cells treated as in (A) presented BAX, BCL2, caspase-9 and cleaved PARP levels. BCL2, B-cell lymphoma 2; BAX, BCL2 associated X protein; FITC, fluorescein isothiocyanate, PARP, poly(ADP-ribose) polymerase.

p53 partially mediates compound 6 cytotoxicity. A previous study revealed that AKT facilitates p53 degradation by increasing MDM2 proto-oncogene expression (19). The effect of compound 6 on p53 response was therefore investigated. To do so, western blotting of p53 and its downstream target p21was performed. As presented in Fig. 4A, p53 and p21 protein levels were increased in all cancer cells following treatment with compound 6. In addition, treatment with compound 6 reduced cyclin B level in all cancer cells which supports the G_2/M cell cycle arrest detected as shown in Fig. 2B and C. The potential roles of p53-mediated signaling in compound 6-induced cell death were investigated by inhibiting p53 expression via si-RNA transfection. A375 cells transfected with sip53 presented attenuated p21 expressions in control and compound 6-treated cells (Fig. 4B). This was further evidenced by the decreased level of cleaved PARP observed following compound 6 treatment in A375 sip53 cells (Fig. 4B). These observations were reinforced by Annexin V assay, which demonstrated that compound 6 treatment induced a moderate increase in apoptosis (15.65±3.23%) in A375 sip53 cells, compared with un-transfected cells (34.23±3.45%) (Fig. 4C). These observations suggested that compound 6 may induce apoptosis partly through p53 regulation.

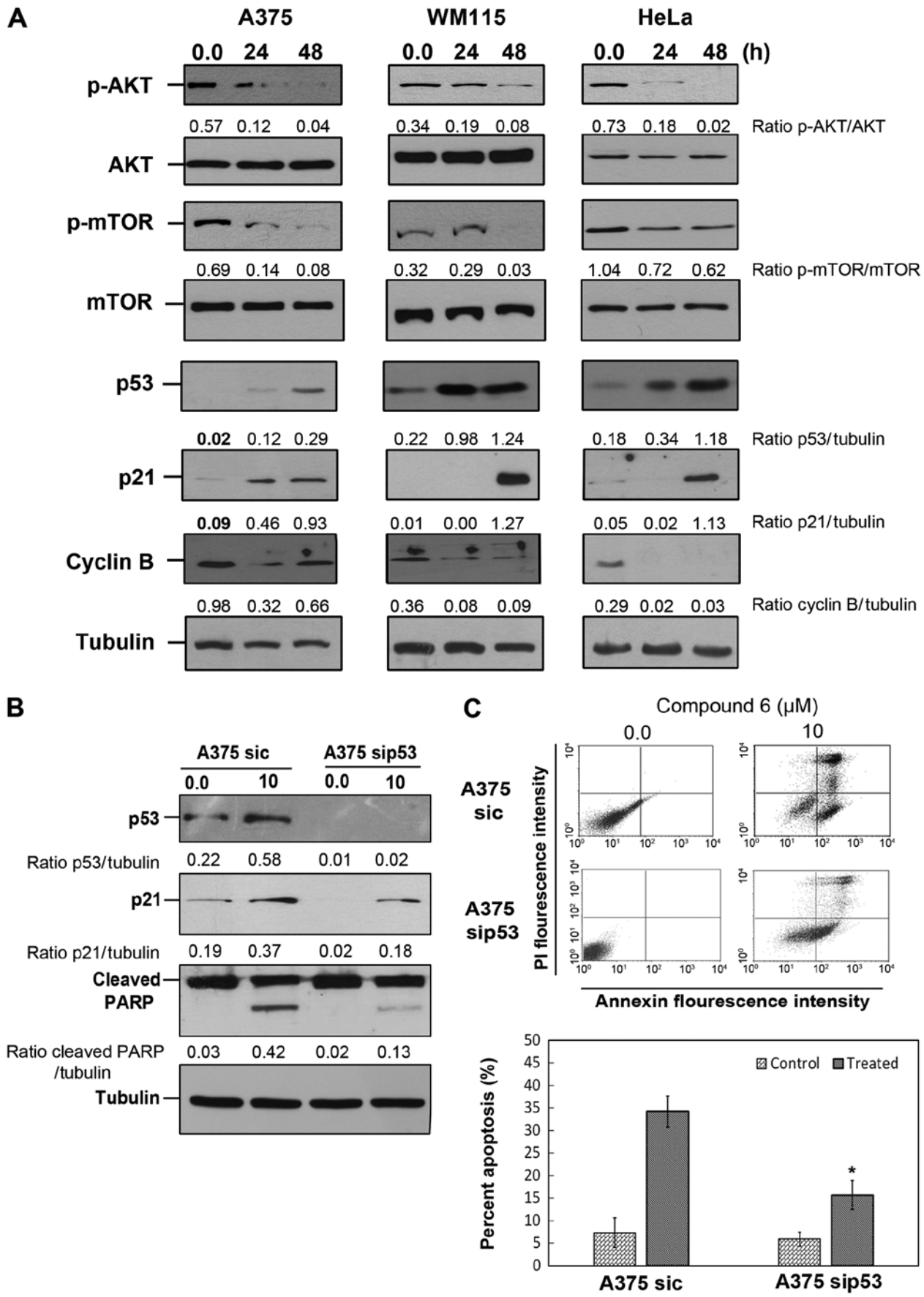

Figure 4. Compound 6 inhibits AKT/mTOR pathway and induces p53 dependent apoptosis. (A) Melanoma cells and HeLa cells were treated with vehicle or compound 6 at 10 and 35 μM, respectively, for 48 h. p-AKT, AKT, p-mTOR, p53, p21, cyclin B and tubulin proteins were analyzed by western blotting. (B) A375 cells were transfected with sip53 and treated with compound 6 (10 μM) for 48 h. Protein extracts were harvested and p53, p21 and PARP were analyzed by western blotting. (C) Annexin V/propidium iodide double staining exhibited a percentage of cell death induced by compound 6 after sip53 transfection lower than in the un-transfected group (B). *P<0.05. AKT, protein kinase B; c, control; mTOR, mechanistic target of rapamycin; p, phospho; si, small interfering.

Discussion

The PI3K-AKT signaling pathway is one of the most investigated cascades in cancer cells. Increasing evidence has confirmed the crucial role of the PI3K-AKT pathway in cancer initiation and progression (18). The phosphatase and tensin homolog (PTEN) tumor suppressor is the most common regulator of this pathway, and tumors exhibiting PTEN loss usually have a high p-AKT level (20). *In vitro* and *in vivo* investigations have demonstrated that tumors with mutant proto-oncogene B-Raf (BRAF) also present high p-AKT levels, which may contribute to the development of BRAF-inhibitor resistance (19,20). Similarly, PI3K-AKT pathway is commonly dysregulated in cervical cancer, which indicates that it may be a potential therapeutic target in the treatment of melanoma and cervical cancer (21). Numerous PI3K inhibitors have been developed and recently evaluated in clinical trials. These comprise PI3K specific inhibitors and dual targets inhibitors, including PI3K-mTOR and AKT inhibitors (11,22). Subsequently, some imidazo[1,2-a]pyridine compounds were developed and tested for their PI3K-AKT pathway inhibiting ability (23-25). Our group has therefore designed and synthesized novel imidazo[1,2-a]pyridines (compounds 5-7) (15). The current study, aimed to investigate the exact anticancer effect of these compounds in melanoma and cervical cancer cells. Notably, compound 6 exhibited the most potent cytotoxic effect in both types of cancer cell. The anticancer effect of compound 6 and its mechanism of action in melanoma and cervical cancer cell lines were therefore deeper examined. The results from this study suggested that compound 6 may be considered as a promising novel chemotherapeutic drug for melanoma and cervical cancers treatment.

Compound 6 exhibited potent cytotoxicity in A375 and WM115 cell lines with low IC_{50} values (<12 μM), which was of crucial importance, considering that metastatic melanoma cells seem to be resistant to chemotherapy. For example, the dose of dacarbazine, which is the common current treatment of metastatic melanoma, necessary to inhibit melanoma cell proliferation is ~25 μM (18). However, some imidazo[1,2-a]pyridines presented similar or more potent cytotoxicity against melanoma cancer cells (23,24). A novel series of imidazo[1,2-a]pyridine compounds inhibit A375 human melanoma cell line proliferation with a low IC_{50} <1 μM (23). A recent study that screened the cytotoxic effects of novel pyrido-imidazodiazepinones reported that seven of these compounds significantly inhibit melanoma cell growth at 1 μM (24).

In the present study, cancer cell treatment with compound 6 induced a decrease in p-AKT level. This effect was observed after 24 h in HeLa cells, and was even more important following 48-h treatment with compound 6 in melanoma cells. Notably, AKT inhibition was mirrored with the inhibition of its downstream target mTOR. Previous studies have reported that AKT and target m-TOR inhibitions increase p21 expression and activate checkpoint kinase 2, which results in G_2/M cell cycle arrest (26-28). Furthermore, these studies showed that the blockage of cell cycle arrest at the G_2/M phase is associated with a decrease in the cell cycle regulatory protein cyclin B level. Similarly, the results from the present study demonstrated that compound 6 induced a decrease in cyclin B1 level. Notably, compound 6 increased p53 and p21 levels, which could explain the G_2/M cell cycle arrest observed.

The PI3K/AKT/mTOR pathway is involved in cell survival, and its inhibition results in high p53 and BAX apoptotic proteins levels (18). Active BAX disrupts the mitochondrial membrane integrity and induces the cytochrome *c* release from mitochondria into the cytosol. Cytoplasmic cytochrome c and active caspase-9 are then involved in the apoptosome formation and caspase-3 activation (29). However, cytochrome c release does not happen when the anti-apoptotic protein BCL2 is present. The present study demonstrated that compound 6 stimulated intrinsic apoptosis by increasing BAX and active caspase-9 levels, and decreasing BCL2 level. The results suggested that the G_2/M cell cycle arrest and intrinsic apoptosis induced by compound 6 may be mediated by AKT/mTOR inhibition. Notably, p53 silencing significantly decreased the compound 6-induced apoptosis, which suggested that p53 may serve an important role in compound 6-induced cell apoptosis. Numerous imidazopyridine derivatives are currently used in the clinic for the treatment of other diseases, including alpidem (anxiolytic) and zolpidem (hypnotic), which exhibit low toxicity levels (30). This could indicate that the compounds tested in the present study may present minor adverse effects.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

SA designed the biological study, supervised different steps and was the major contributor in the writing of the manuscript. AMA synthesized and tested the chemical compounds. RYM supervised and characterized the synthesized compounds. MG contributed to the tissue culture experiments. HA contributed to the tissues culture, western blotting experiments and cell cycle analysis. AYA contributed to the western blotting and tissue culture experiments. EAA contributed to the western blotting experiments. YMA contributed to the apoptosis assay. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patients consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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