

Carboxamide analog ITR-284 evokes apoptosis and inhibits migration ability in human lung adenocarcinoma A549 cells

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Abstract. Lung adenocarcinoma is the most common type of lung cancer and found in both smokers and non-smokers, but the treatment of lung cancer is limited. ITR-284 has been shown to be a potent carboxamide-derived anticancer agent and to induce apoptosis in leukemia and colon cancer cells. However, little is known whether ITR-284 has anticancer activity in human lung adenocarcinoma cells through induction of apoptosis and suppression of migration *in vitro*. We showed that ITR-284 inhibited human lung cancer A549 cells using the thiazolyl blue tetrazolium bromide (MTT) assay and evoked apoptosis via the cell cycle distribution at S phase arrest. After treatment with 20 nM ITR-284 for 24 h, apoptotic cells were induced and detected by Annexin V-FITC/PI staining. The production of reactive oxygen species (ROS) was dose-dependently increased in A549 cells caused by ITR-284. The results from immunoblotting analysis showed an elevation of protein levels of p53 and phosphorylation of p53 in A549 cells prior to ITR-284 exposure. Additionally, apoptosis-associated proteins such as Bax, cleaved caspase-3 and cleaved PARP were upregulated after ITR-284 treatment. By wound healing assay, low concentrations (1-5 nM) of ITR-284 exerted a greater effect on inhibition of A549 cell migration. The protein levels of E-cadherin and vimentin, which are the epithelial-mesenchymal transition (EMT) markers, were modulated in ITR-284-treated cells assessed by western blot analysis. Taken

together, our data suggest that ITR-284 may be an effective anticancer agent for treating lung adenocarcinoma.

Introduction

Lung cancer has the highest mortality rate of all cancers worldwide (1,2), and its incidence rate is still increasing in Taiwan (3,4). Lung adenocarcinoma accounts for ~30% of primary lung tumors in male smokers and 40% in female smokers (5). However, males and females suffer from adenocarcinoma approaching 60 and 80%, respectively among non-smokers (6). Although there is a rapid development in the treatment by targeted therapies, the 5-year survival rate is still low and <16% all over the world (7). In addition, 75-80% of lung cancer is non-small cell lung cancer (NSCLC) (8). Radical resection is the treatment of choice for NSCLC (9). Over 75% of NSCLC patients are potential candidates for chemotherapy, but chemotherapy usually produces rather poor response rates in NSCLC patients with rare complete remissions (10,11). Thus, it is imperative to develop new treatment to improve the overall disease-free survival of NSCLC patients.

Our previous study showed that a series of carboxamide derivatives were designed and synthesized as novel anticancer agents (12), and these novel compounds exhibited potent cytotoxic effect on multiple cancer cell lines (12-14). Among them, ITR-284 [N-(2-dimethylaminoethyl)-4,8-dihydrobenzo (1,2-b;4,5-b') dithio-phen-2-carboxamide phosphoric acid salt] (Fig. 1) is one of the most potent agents (12,13). It has been demonstrated that ITR-284 significantly inhibited leukemia cell proliferation (HL-60 and WEHI-3) and possessed low cytotoxicity to normal cells. In addition, ITR-284 had a greater growth inhibition than that of other compounds in human hepatocellular carcinoma cell lines (HepG2, Hep3B, SK-HEP-1 and J5) and colorectal cancer cell lines (HT 29, COLO 205, HCT 116 and SW 620 (14). Therefore, ITR-284 has the potential for treating multiple cancers, but it might be needed for further investigations.

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Tumor cells possess metastasis and invasion ability to become malignant tumor cells (15). Cancer cell migration and invasion are a complex cell spreading process from primary locations to secondary site in the body, in which cell adhesion molecules and signal transduction factors are involved (16,17). During migration and invasion, several phenotypic changes of tumor cells, including cell-cell adhesion and remodeling of cell-matrix adhesion sites have been characterized. This process is known as epithelial-mesenchymal transition (EMT) (18,19). Loss of E-cadherin expression to lead to reduction of cell-cell adhesion is a symbol of occurrence of EMT, while increase of vimentin expression, close relationship with differentiation and metastasis of cancer cell is another distinctive event in EMT (20,21). In this study, we focused on exploring the anti-proliferative effects, cell cycle arrest, apoptosis and anti-migration ability of ITR-284 on human lung adenocarcinoma A549 cells *in vitro*.

Materials and methods

Materials and reagents. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), thiazolyl blue tetrazolium bromide (MTT), trypsin-EDTA, propidium iodide (PI), RNase A, sodium citrate and anti-actin antibody were purchased from Sigma-Aldrich (St. Louis, MO, USA). Primary antibodies against p53, p-p53 (Ser15), Bax, Bcl-2, caspase-3, PARP, E-cadherin and vimentin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Secondary antibodies conjugated with infrared fluorescently-labeled dye were purchased from LI-COR Biosciences (Lincoln, NE, USA).

Cell culture. Human adenocarcinoma cell line A549 was obtained from the Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan). Cells were grown in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin at a humidified environment with 95% air and 5% CO₂ at 37°C. Cells in the logarithmic phase of growth were used for the following experiments.

Cell viability assay. A549 cells (7x10³ cells/well) were seeded into 96-well plates (Costar, Corning Inc., Corning, NY, USA), allowed to attach overnight, and then exposed to various concentrations (1, 5, 10, 15 and 20 nM) of ITR-284 for 24 h. Cell viability was determined by the MTT assay, as previously described (22). The absorbance was recorded by using an enzyme-linked immunosorbent assay (ELISA) reader and control absorbance was normalized to 100%. Six replicated wells were included in each group. At least three independent experiments were done.

DNA content analysis. Cell cycle phase and sub-G1 population (apoptosis) of A549 cells were detected by flow cytometry as previously described (23,24). Cells were treated with or without 1, 5, 10, 15 and 20 nM of ITR-284 for 24 h, harvested and washed with phosphate-buffered saline (PBS). Cells were fixed in 70% ice-cold ethanol/PBS for 20 min on ice and then incubated in PI solution containing 69 mM PI, 388 mM sodium citrate, 100 µg/ml RNase A for 15 min. Cells were immediately detected with FACScan flow cytometry (BD Biosciences, San Jose, CA, USA).

Annexin V/PI double staining. A549 cells (2.5x10⁵ cells/ml) were seeded in 6-well plates and cultured overnight. Culture medium was removed and replaced with 1, 5, 10, 15 and 20 nM of ITR-284. After incubation for 24 h, cells were washed with PBS and collected in a tube before centrifugation at 1,200 rpm for 5 min. Cell pellet was washed once with PBS, re-suspended with the 0.1 ml binding buffer (1X) and mixed with 5 µl fluorescein isothiocyanate (FITC)-Annexin V and 10 µl PI staining reagent as recommended in the manufacturer's protocol (FITC Annexin V Apoptosis Detection kit I, BD Pharmingen, San Diego, CA, USA). Following staining and mixing carefully, apoptotic cell populations were detected using a flow cytometer.

Measurement of intracellular reactive oxygen species (ROS) production. ROS generation inside living cells was measured by Muse Oxidative Stress kit (Millipore, Billerica, MA, USA), and the assays were performed according to the manufacturer's instructions. Dihydroethidium (DHE), an oxidation-sensitive probe, displays blue fluorescence in the cell cytoplasm and forms a red fluorescent product, 2-hydroxyethidium upon oxidation by ROS. Briefly, untreated or treated A549 cells were stained with 0.5 µM DHE for 30 min at 37°C in the dark and subsequently assayed by flow cytometry.

Western blotting. A549 cells (1x10⁷ cells/dish) were incubated in a 10-cm dish for 24 h. Culture medium was removed and replaced with the indicated concentrations of ITR-284. After incubation for 24 h, cells were collected, treated with cell lysis buffer, disrupted by sonication, and then centrifuged at 12,000 rpm for 30 min. The protein-containing supernatant was collected and stored at -80°C before use. Quantitation of protein was carried out by using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. A total of 50 µg sample was prepared and mixed with sample buffer, boiled in a 95°C water bath for 5 min, and cooled on ice. Samples were separated on 10% SDS-polyacrylamide gel under 100 V and transferred onto a polyvinylidene fluoride (PVDF) membrane (Immobilon-P Transfer Membrane, Millipore), which was presoaked in methanol for 30 sec for activation and then immersed in transfer buffer. After transferring at 4°C under 100 V for 2 h, the PVDF membrane was soaked in blocking buffer containing 5% skim milk for 1 h. The primary antibodies dissolved in the Tris-buffered saline with Tween-20 (TBST) buffer solution containing 0.1% Tween-20, 150 mM NaCl, and 10 mM Tris-HCl (pH 8.0) were diluted with an appropriate ratio, including p53 (1:1,000), p-p53 (1:1,000), Bax (1:1,000), Bcl-2 (1:1,000), caspase-3 (1:1,000), PARP (1:1,000), E-cadherin (1:1,000), vimentin (1:1,000) and actin (1:1,000). After probed at 4°C overnight, PVDF membranes were washed with the TBST buffer three times and incubated with the infrared fluorescently-labeled dye-conjugated secondary antibody (IgG) at room temperature for 1 h. PVDF membranes were washed with the TBST buffer three times and scanned by the Odyssey infrared imager (LI-COR Biosciences) for measurement of protein expression intensity.

Wound healing assay. Cells were seeded in 6-well plates and incubated for 24 h to reach ~80-90% confluency. A linear

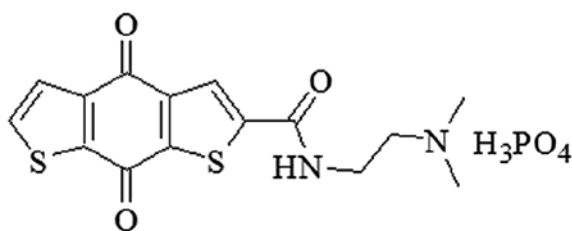


Figure 1. ITR-284 chemical structure.

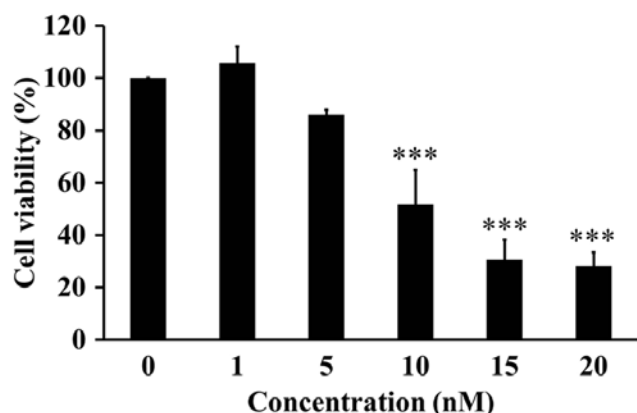


Figure 2. Effect of ITR-284 on cell viability of the lung cancer A549 cells. Cells were treated with 0, 1, 5, 10, 15 or 20 nM of ITR-284 for 24 h, and cell viability was measured by the MTT assay. Each histogram represented cell viability from three independent experiments. Data are summarized as mean \pm SD. *** $p < 0.005$ indicates a significant difference compared with untreated control.

wound was created by dragging a 200- μ l pipette tip through the monolayer. Cellular debris was removed by gentle washes with culture medium. Cells were treated with 1, 2, 3, 4 and 5 nM of ITR-284 for 24 h. The healing process was photographed after the wounding using a microscope (Olympus 600 auto-biochemical analyzer, Tokyo, Japan). Migration distance of A549 cells was measured from 5 field images, and the gap size was determined using Image-Pro Plus 6.0 software (Media Cybernetics, Silver Spring, MD, USA).

Statistical analysis. All data are presented as the mean \pm standard deviation (SD). One way analysis of variance (ANOVA) with Student's t-test was used for multiple comparisons among groups. A value of $p < 0.05$ was considered statistically significant.

Results

ITR-284 inhibits the proliferation of A549 cells. MTT assay was performed to investigate the cytotoxic effect of ITR-284 on the cell proliferation and viability in A549 cells. We found that ITR-284 obviously inhibited the cell viability of A549 cells in a dose-dependent manner (Fig. 2). The IC_{50} (50% inhibitory concentration) of ITR-284 was 10 nM after a 24-h treatment in A549 cells.

ITR-284 induces S phase arrest in A549 cells. It is well known that cell cycle is a reflection of cell growth and division, which

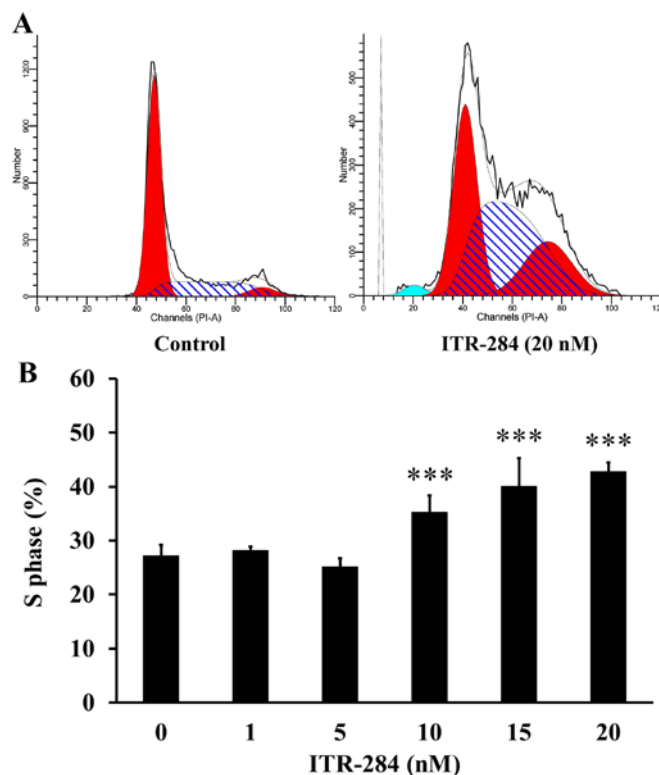


Figure 3. Effect of ITR-284-treated A549 cells on cell cycle progression. (A) Cells were treated with or without ITR-284 cells at the indicated concentrations for 24 h. Treated cells were harvested, stained with propidium iodide (PI) and analyzed by flow cytometry. Cell cycle distribution for 0, 1, 5, 10, 15 or 20 nM of ITR-284 at 24 h was performed, and the percentage of S phase was increased in a dose-dependent manner. (B) Each histogram represents cell cycle distribution from three independent experiments. Data are summarized as mean \pm SD. *** $p < 0.005$ indicates a significant difference compared with untreated control.

is easily disturbed by drug treatment (25). We examined the cell cycle distribution in ITR-284-treated A549 cells. The cells were treated with different concentrations of ITR-284 for 24 h and analyzed by flow cytometry after PI staining. The data demonstrated that the percentage of A549 cells in S phase was increased in a dose-dependent manner after exposure to 10, 15 and 20 nM of ITR-284 (Fig. 3A). The proportion of cells at S phase was $42.4 \pm 1.78\%$ ($p < 0.05$) in 20 nM ITR-284-treated A549 cells for 24 h (Fig. 3B).

ITR-284 triggers apoptosis in A549 cells. ITR-284 possesses inhibitory ability via inducing apoptosis when leukemia and colon cancer cells were treated with ITR-284, as previous studies reported (13,14). To investigate the effect of ITR-284 on apoptosis in A549 cells, Annexin V/PI double staining assay was conducted. After treatment with 0, 1, 5, 10, 15 and 20 nM of ITR-284 for 24 h in A549 cells, the profiles from flow cytometric analysis were performed (Fig. 4A) and the percentage of A549 apoptotic cells (Annexin V⁺/PI⁺) significantly increased (Fig. 4B) prior to 10-20 nM ITR-284 exposure for 24 h. Thus, ITR-284 showed apoptotic evidence in A549 cells after challenge.

ITR-284 promotes ROS production in A549 cells. Reactive oxygen species (ROS) generation plays an important role in

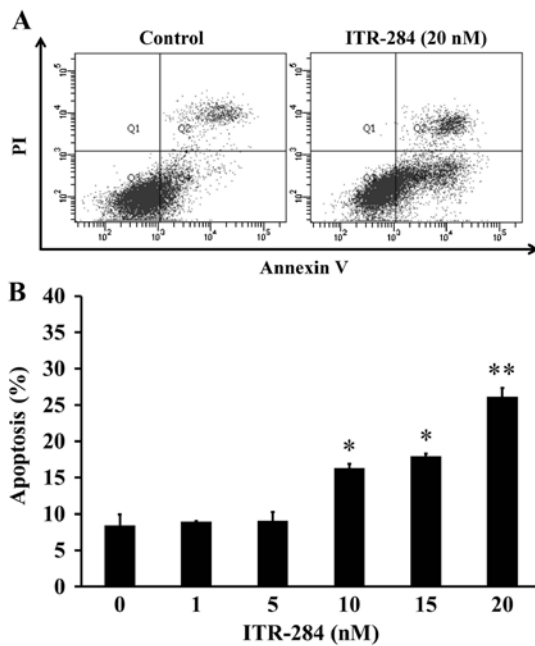


Figure 4. Effect of ITR-284 on induction of apoptosis in A549 cells. Cells were treated with or without ITR-284 cells at the indicated concentrations for 24 h, and then stained with Annexin V and PI to assay for apoptotic cells. (A) Fluorescence intensities were measured by flow cytometry using FITC-A (Annexin V) and PI-A (PI) channels. The value (Q3) shown in the lower left quadrants of each panel represent the percentage of viable cells. The values (Q2 and Q4) shown in the lower right and upper right quadrants of each panel represent the percentage of apoptotic cells. (B) Representative histograms are the mean \pm SD of percentage of apoptotic cells. Data were summarized from three independent experiments and each experiment was done in triplicate. * $p < 0.05$ and ** $p < 0.01$, respectively, indicates a significant difference compared with untreated control.

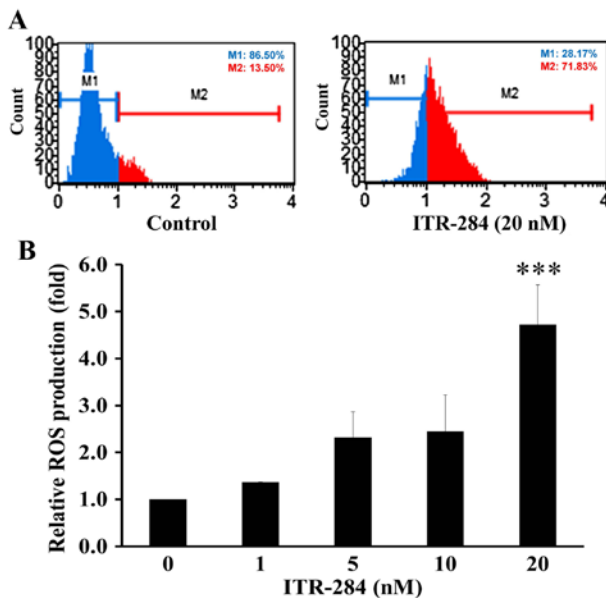


Figure 5. Effect of ITR-284 on cellular oxidative stress of A549 cells. (A) ROS generation was assessed using dihydroethidium (DHE) dye in ITR-284-treated A549 cells. DHE, a small-molecule fluorescent probe specific for superoxide radical, reacted with superoxide radical and generated a red fluorescent product analyzed by flow cytometry. (A) Cells showing high fluorescence intensity were positive. Positive cells were elevated in a dose-dependent manner. (B) Representative histograms are the mean \pm SD of the percentage of positive cells. Data are summarized from three independent experiments and each experiment was done in triplicate. *** $p < 0.005$ indicates a significant difference compared with untreated control.

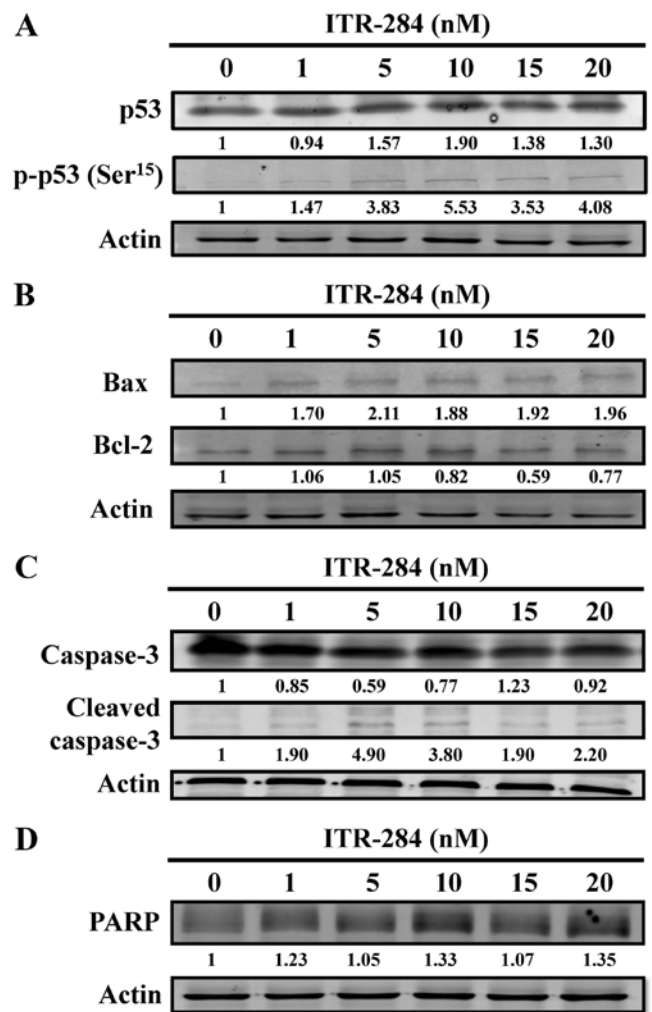


Figure 6. Effect of ITR-284 on apoptotic evidence in A549 cells. Cells were untreated or treated with the indicated concentrations of ITR-284 for 24 h before being subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by western blotting. Immunoblots show (A) a dose-dependent effect of the tumor suppressor p53 and phosphorylated p53, (B) the changes of Bax and Bcl-2, as well as a dose-dependent processing of (C) caspase-3, cleaved caspase-3 and (D) cleavage of poly-(ADP-ribose) polymerase (PARP), respectively. Blots were reprobed for actin to confirm equal loading of the samples.

mitochondria-mediated apoptotic pathway (26,27). ITR-284 has been shown to induce apoptosis through ROS generation in other cancer cells (13,14). This study indicated that ROS production was elevated in A549 cells after 20 nM ITR-284 challenge (Fig. 5A and B) consistent with previous findings (13).

ITR-284 alters protein levels of p53, Bcl-2 family and caspase-3 in A549 cells. To confirm the results of ITR-284-induced S phase arrest and apoptosis in A549, we investigated whether increased p53 phosphorylation, Bcl-2 family protein and cleaved caspase-3 were observed in A549 cells after ITR-284 treatment by western blotting. A dose-dependent increase in p53 and p-p53 protein levels was found (Fig. 6A). When A549 cells were treated with ITR-284, we observed that there was a decrease of the expression level of Bcl-2 and an increase of Bax expression; these effects were dose-dependent (Fig. 6B). The expression levels of cleaved caspase-3 (Fig. 6C)

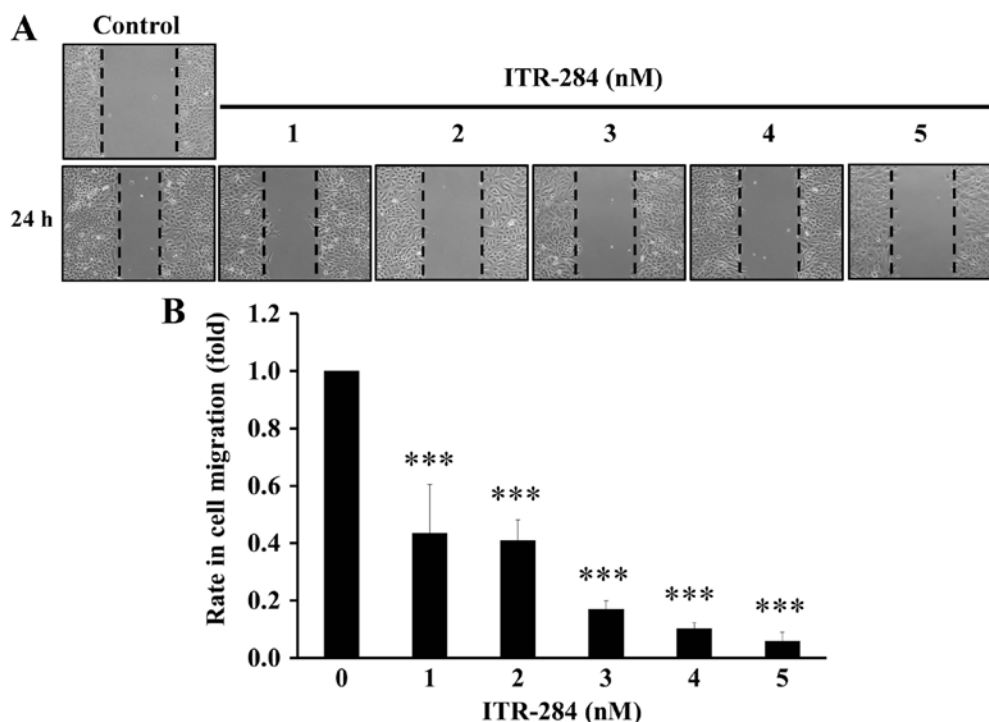


Figure 7. Effect of ITR-284 on A549 cell migration. (A) Images of wound healing assays (x100 magnification). Cells were seeded into 6-well cell culture plates, cultured in DMEM supplemented with 10% FBS, and allowed to grow to near confluence. Confluent monolayers were carefully wounded and the cellular debris was gently washed away with PBS. The wounded monolayer was re-incubated in FBS-free DMEM containing 0, 1, 2, 3, 4 or 5 nM of ITR-284 for 24 h. (B) Migration distances were qualified as described in Materials and methods. Error bars represent three independent experiments and each experiment was done in triplicate. *** $p < 0.005$ indicates a significant difference compared with untreated control.

and cleaved PARP (Fig. 6D) were increased in a dose-dependent manner in ITR-284-treated A549 cells. Thus, ITR-284 induced A549 cell apoptosis through mitochondria-dependent pathway.

ITR-284 inhibits cell migration in A549 cells. To examine the anticancer ability of ITR-284, we further investigated whether ITR-284 suppresses A549 cell migration by a 'scratch wound healing' assay. Cells were seeded for near confluency and scratched with a pipette tip. Cells were treated with low concentrations (1, 2, 3, 4 and 5 nM) of ITR-284 for 24 h. Motility of cells at different concentrations after generation of the wound was monitored under a microscope. Closure of the wound was inhibited within 24 h compared to the untreated control A549 cells. In contrast, ITR-284-treated A549 cells migrated toward the wound at a much slower rate (Fig. 7A). The quantitative data show that ITR-284 dose-dependently suppressed cell migration in A549 cells (Fig. 7B). Thus, the inhibitory effect of ITR-284 on A549 cell migration was performed *in vitro*.

ITR-284 modulates E-cadherin expression and vimentin expression in A549 cells. To evaluate the anti-cell migration ability of ITR-284, we investigated effects of ITR-284 on expression of E-cadherin and vimentin in ITR-284-treated A549 cells. Results indicated that E-cadherin protein level was dose-dependently increased (Fig. 8). We also observed that ITR-284 induced a decrease in the protein level of vimentin in A549 cells dose-dependently (Fig. 8). Therefore, ITR-284-suppressed cell migration might be mediated

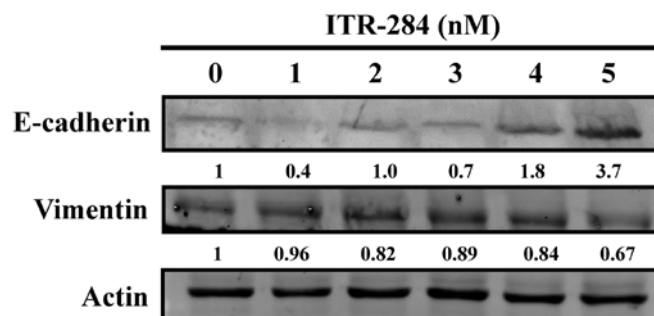


Figure 8. Effect of ITR-284 on EMT-associated protein levels in A549 cells. Western blot analysis of the E-cadherin or vimentin proteins in A549 cells after treatment with the indicated concentrations of ITR-284 for 24 h. Ratio of densitometric values of E-cadherin or vimentin to actin is shown.

through modulating E-cadherin and vimentin signaling in A549 cells.

Discussion

In this study, we examined the effects of ITR-284 on the proliferation, cell cycle, apoptosis and migration of A549 cells *in vitro*. We found that ITR-284 inhibited cell proliferation in a dose-dependent manner and changed the morphology of A549 cells *in vitro* (Fig. 2). Furthermore, ITR-284 treatment significantly reduced the levels of Bcl-2 expression and induced the levels of Bax, cleaved caspase-3 and cleaved PARP expression in A549 cells (Fig. 6B and C). In addition, ITR-284 induced

cell cycle arrest at S phase and triggered cell apoptosis in A549 cells (Fig. 3). Cell cycle arrest is associated with inhibition of tumor cell proliferation, and cell cycle progression is positively regulated by a series of cyclins and CDKs, but negatively regulated by CDK inhibitors of p21, p16 and p27 (28,29). We found that treatment with ITR-284 significantly induced the levels of p53 and phosphorylated p53 expression in A549 cells (Fig. 6A). Given that p53 is an important regulator of p21 expression (30). It is possible that ITR-284 treatment may upregulate p53 expression (Fig. 6A) and in turn elevate the expression of p21 to inhibit the cyclin1/CDK complex, leading to cell cycle arrest at S phase in A549 cells. To the best of our knowledge, this was the first study to reveal potent antitumor activity of ITR-284 in A549 cells. Our novel findings may aid in the design of new therapy for intervention of human NSCLC.

Once cell cycle distribution is disrupted, cells trigger cell cycle arrest to examine the status of cells (28). Induction of cell cycle S phase arrest is associated with triggering cell apoptosis (31). To understand the molecular mechanisms underlying the action of ITR-284 in inhibiting the proliferation of A549 cells, we examined the effect of ITR-284 on the survival of A549 cells. We found that treatment with ITR-284 triggered apoptosis of A549 cells by Annexin V/PI staining (Fig. 4). There are two distinct apoptotic signaling pathways (32,33). Intrinsic apoptotic pathway is mitochondria associated, which induces the formation of cytochrome *c* complex, which activates caspase-9 and in turn activates caspase-3, leading to cell apoptosis. Cell apoptosis can be regulated positively by the pro-apoptotic Bcl-2 family members, such as Bax, Bad and others, and negatively regulated by anti-apoptotic Bcl-2 family members, such as Bcl-2 and Bcl-xL. The balance of pro-apoptotic and anti-apoptotic factors is crucial for the development of apoptosis (32,33). In this study, we found that ITR-284 treatment significantly increased the levels of Bax, cleaved caspase-3 but decreased the levels of Bcl-2 expression in A549 cells (Fig. 6). Therefore, ITR-284 induced A549 cell apoptosis through activating the mitochondrial pathway.

Cancer cells can migrate and invade the adjacent and distant tissues, which increases the risk for poor outcome of patients with NSCLC (15,17). Tumor cell migration is regulated by many factors, such as chemokines, their receptors, the EMT process, matrix degrading enzymes and antioxidant enzymes, such as MMPs, which mediate the degradation of extracellular matrix (ECM) proteins (18,19). EMT is accompanied by profound changes in cell characteristics that enable the epithelial cells to detach from tight junctions, change the cell's shape and polarity, delaminate, and migrate (19). During tumor metastasis transition, EMT-associated molecules are changed accordingly, such as increasing the expression of mesenchymal cells specific proteins (vimentin, fibronectin, and decreasing the expression of epithelial cell adhesion protein (E-cadherin) (20,21). We found that ITR-284 treatment significantly inhibited the migration of A549 cells, accompanied by significantly attenuating the expression of vimentin and increased E-cadherin levels (Fig. 8). We observed the changes of EMT phenotype and found that vimentin and E-cadherin expression contributed to regulate EMT in A549 cells caused by ITR-284, and ITR-284 affected the invasion and metastasis of tumor cells, leading to cell functional changes.

In conclusion, our results indicate that ITR-284 treatment significantly inhibits the A549 cell proliferation by inducing their cell cycle arrest at S phase through upregulating p53 and phosphorylation of p53 expression. ITR-284 triggers apoptosis of A549 cells by activating the caspase-3-dependent pathway. Furthermore, ITR-284 significantly inhibited the migration of A549 cells by increasing E-cadherin expression and reducing vimentin expression. Therefore, our data suggest that ITR-84 may be a promising candidate for intervention of NSCLC, and our findings may provide a new insight into understanding the pharmacological mechanisms underlying antitumor activity of ITR-284.

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