Phenylhexyl isothiocyanate suppresses cell proliferation and promotes apoptosis via repairing mutant *P53* in human myeloid leukemia M2 cells

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Abstract. The aim of the present study was to investigate the specific effect and possible mechanisms of phenylhexyl isothiocyanate (PHI) on cell proliferation and apoptosis in acute myeloid leukemia M2 cell lines. Cell proliferation in several hematological tumor cells lines following PHI treatment was evaluated *in vitro* using a Cell Counting Kit-8. The apoptosis and cell cycle of Kasumi-1 and SKNO-1 cells (human M2 cell lines) following exposure to PHI were examined using flow cytometry. A colony-formation assay was used to identify the inhibitory effect of PHI on Kasumi-1 cells *in vitro*. Furthermore, Kasumi-1 xenograft tumor models were established. The antitumor effect of PHI was observed *in vivo* by measuring the size of the resulting xenograft tumors. The apoptosis of the xenograft tumor cells was measured using a TUNEL assay. Finally, protein expression levels were assessed by western blotting. PHI inhibited cell growth in 16 hematological tumor cell lines, with Kasumi-1 and SKNO-1 cells being the most sensitive. *In vitro* treatment induced apoptosis and inhibited cell cycle arrest at the G_0/G_1 phase in a dose- and time-dependent manner. PHI also inhibited growth and induced apoptosis *in vivo*. The compound increased the expression of caspases 3, 9 and 8, Fas and poly (ADP-ribose) polymerase. Furthermore, PHI enhanced the protein expression of p53, Bax and p21 in a dose-dependent manner. In conclusion, PHI had a specific and notable inhibitory effect on Kasumi-1 and SKNO-1 M2 cell lines *in vivo* and *in vitro*. Treatment inhibited cell cycle arrest at the G_0/G_1 phase, and induced apoptosis through the mitochondrial and Fas death receptor pathways. PHI restored the activity of mutated *P53* and reactivated the *P53* pathway, highlighting it as a potential target drug for mutated *P53*.

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Introduction

Acute myeloid leukemia (AML) is a myeloid stem cell tumor characterized by abnormal myeloid cell proliferation, which inhibits the viability of normal cells. M2 is a subtype of AML, as classified by the French-American-British classification (1), constituting 10-15% of AML cases. One of the hallmarks of M2 is the formation of a fusion protein, AML1-ETO, due to a translocation of chromosome 8 to chromosome 21. Chemotherapy and hematopoietic stem cell transplantation are the main treatments of AML. However, to date, the therapeutic effects are insufficient. For patients <60 years old, the 5-year overall survival (OS) rate is ~40%. However, for the majority of patients with AML (>60 years old), the 5-year OS rate is only ~10-20% (2,3). Furthermore, for patients with M2 AML, clinical studies have shown that the median survival time was <2 years, with a 5-year OS rate of <40% (4). Therefore, the development of novel and effective therapies for AML is urgently required, particularly for patients with type M2.

The human *P53* gene is located at 17P13.1. It is considered to be a classical tumor suppressor gene and is closely associated with human tumorigenesis (5,6). The p53 protein (a product of *P53* gene expression) acts as a transcription factor that mediates DNA repair and induces cell cycle arrest and apoptosis, thereby inhibiting the formation of tumor cells (7,8). However, the *P53* gene can be mutated, rearranged or deleted. This can cause it to lose its anticancer function and become an oncogene, promoting the formation of tumors (9,10). Animal experiments have demonstrated that *P53*-deficient mice spontaneously develop cancer. It was also revealed that >50% of patients with cancer carry mutations in the *P53* gene (11). Similarly, in hematological malignancies, the *P53* mutant occurs in 11.1% of cases, according to version R15 of the International Agency for Research on Cancer

database (12). The overexpression of cellular mutant *P53* in therapy-related myelodysplastic syndrome and AML (13) was found to be present in ~63 and 75% of patients, respectively, and was associated with poorer OS (14). In a mouse model of *P53*-deficiency, re-inducing the expression of *P53* caused a decrease in tumor volume, indicating that *P53* remains effective against established tumors (15). A number of studies have also demonstrated that restoring the function of mutant *P53* can reactive its antitumor effect (16,17). Restoring mutant *P53* may be a hopeful treatment for hematological tumors.

Natural isothiocyanates exist in all types of cruciferae, including broccoli, cabbage and watercress. PHI is an isothiocyanate derivative, which is known to be an apoptotic inducer and cell proliferation inhibitor (18). Our previous study demonstrated that this compound suppressed proliferation of the leukemia HL-60 cell line by inducing apoptosis *in vitro* and *in vivo*. However, it spared normal cells and tissues (19,20). PHI exerted an effect on PC3 prostate cancer cells by inhibiting the Akt pathway (21). Furthermore, it inhibited cell proliferation and induced apoptosis in hepatocellular carcinoma cells by inducing the accumulation of acetylated histones H3 and H4, possibly resulting in the loss of function of *P15* (22,23). However, the effect of PHI on other hematological tumors is unknown, and whether the *P53* pathway is involved in the PHI mechanism is also unclear.

In the present study, the effect of PHI was evaluated in several hematological tumor cell lines. Cell proliferation, cycle and apoptosis were investigated. The mechanism of PHI in inhibiting the growth of Kasumi-1 M2 cells was examined, with a focus on the *P53* pathway. The results revealed that PHI inhibited M2 cell growth by inducing cell apoptosis and cell cycle arrest, involving the restoration of mutant *P53* and reactivation of the *P53* signaling pathway.

Materials and methods

Chemicals and reagents. All reagents and chemicals were purchased from Sigma-Aldrich (Merck KGaA), unless stated otherwise. PHI was from Abcam, fetal bovine serum from Invitrogen (Thermo Fisher Scientific, Inc.) and RPMI-1640 medium from Gibco (Thermo Fisher Scientific, Inc.). Primary antibodies against cleaved caspase 3 (cat. no. #9664), cleaved caspase 8 (cat. no. #9748), cleaved caspase 9 (cat. no. #7237), cleaved poly (ADP-ribose) polymerase (PARP; cat. no. #5625), p21 (cat. no. #2947), p53 (cat. no. #2527), Bcl-2 (cat. no. #4223), Bax (cat. no. #5023), Fas (cat. no. #8023), α-tubulin (cat. no. #3873) and β-actin (cat. no. #3700) were purchased from Cell Signaling Technology, Inc. The horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit IgG and goat anti-mouse IgG) and pifithrin-α were purchased from Beyotime Institute of Biotechnology. The methylcellulose medium was purchased from Stemcell Technologies, Inc.

Cell culture. The human Kasumi-1 and SKNO-1 AML M2 cell lines, HL-60, NB4 and HT93 AML M3 cell lines, MV4-11 and THP-1 AML M5 cell lines, HEL AML M6 cells, KG-1 AML cells, K-562 and MEG-01 chronic myeloid leukemia cell lines, U266 myeloma cells, Raji Burkitt lymphoma cells, Jurkat acute lymphoblastic leukemia cells, and MOLT-4 and U-937 histiocytic lymphoma cell lines were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI-1640 medium with 10-20% fetal bovine serum at 37°C with 5% CO_2.

Cell proliferation assay. Cell proliferation was assessed using a Cell Counting Kit-8 (CCK-8) assay (Dojindo Molecular Technologies, Inc.). The 16 types of hematological tumor cell were seeded at $2x10^4$ cells/well in 96-well plates and incubated with increasing concentrations of PHI (5, 10, 20 and 40 μmol/l) at 37°C for 24 h in an incubator containing 5% CO_2; the Kasumi-1 and SKNO-1 cells were further incubated with 10 μmol/l PHI for 12, 24, 48 and 72 h. The cells in the control group were incubated with medium containing the same volume of dimethyl sulfoxide (DMSO). The CCK-8 solution (10 μl) was then added to each well, and the plates were incubated for 1 h at 37°C. The optical density of cells at 450 nm was measured in a microplate reader (Thermo Fisher Scientific, Inc.). The half maximal inhibitory concentration (IC_{50}) of PHI was calculated using SPSS version 13.0 (SPSS, Inc.).

Cell apoptosis detection. The Kasumi-1 and SKNO-1 cells were seeded at $2x10^6$ cells/well in 6-well plates and treated with PHI at concentrations of 5, 10, 20 and 40 μmol/l for 24 h. The same volume of DMSO was used as a control. The cells were incubated at 37°C in an incubator containing 5% CO_2. After being harvested, the cells were washed in PBS and resuspended in 200 μl binding buffer. The cells were then incubated with 5 μl Annexin V-FITC solution and 5 μl propidium iodide (PI), both from BD Biosciences, at room temperature for 15 min in the dark. The apoptosis of cells was analyzed by flow cytometry. Data were analyzed using FlowJo 10.4.1 (FlowJo LLC). All experiments were performed in triplicate.

Cell cycle analysis. The Kasumi-1 cells were seeded at $2x10^6$ cells/well in 6-well plates and incubated with increasing concentrations of PHI (1.25, 2.5 and 5 μmol/l) at 37°C for 24 h in an incubator containing 5% CO_2. The same volume of DMSO was used as a control. After being harvested and washed in PBS, the cells were fixed using precooled 70% methanol at 4°C for 2 h. The cells were then washed in PBS and stained with PI (50 mg/ml) at 37°C for 30 min. Cell cycle analysis was performed using flow cytometry. Data were analyzed using FlowJo 10.4.1 (FlowJo LLC). All experiments were performed in triplicate.

Colony-formation assay. The Kasumi-1 cells (500 cells/well) were seeded into 6-well plates and cultured in semi-solid RPMI-1640 medium consisting of 1% methylcellulose and 20% FBS with PHI (5, 10, 20 and 40 μmol/l) at 37°C for 2 weeks. The same volume of DMSO was used as the control. The numbers of clones were counted under a light microscope. Images of the clones were captured using a camera without magnification. All experiments were performed in triplicate.

Establishment of an AML xenograft mouse model and PHI treatment. Male athymic nude mice (BALB/c) aged 4-6 weeks and weighing 18-22 g were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. and were housed under specific

pathogen-free conditions. A total of 8 mice in the study were in rooms with 12:12-h dark/light cycle (lights on at 07:00), housed with wood-chip bedding and given *ad libitum* access to food and water. There is a maximum of 5 mice per cage. And the room temperature for mouse housing is maintained to be 22°C. 8 mice were grafted with 1×10^7 Kasumi-1 cells via subcutaneous injection into the right flank. The xenograft model was established, and tumors were continually measured with digital calipers until they reached a volume of >0.5 cm^3. The mice were then randomized into two cohorts (n=4 each): Group A, injected intraperitoneally with 200 μl PHI (40 μmol/l) once a day; and Group B (control), injected intraperitoneally with 200 μl of DMSO once a day. Daily measurements of tumors and animal weight were performed. The tumor volume was calculated as ab^2/2 (a, long diameter; b, short diameter). No animals presented with multiple tumors. Following a 14-day treatment period, the mice were sacrificed by cervical dislocation. Death of the mice was confirmed by ascertaining cardiac and respiratory arrest. Necropsy was then performed and the xenograft tumor was excised.

Hematoxylin and eosin (H&E) staining. The xenograft samples were fixed in 4% formaldehyde in PBS for 6 h at 37°C and then embedded in paraffin. The paraffin sections (4-6 μm) were adhered to glass slides pretreated with 0.01% aqueous solution of poly-L-lysin. The slides were then stained with hematoxylin for 40 sec and with eosin for 30 sec. The tissue slides were examined under a light microscope following mounting with mounting medium.

Detection of xenograft apoptosis using a TUNEL assay. Apoptosis of the xenograft cells was detected using a TUNEL assay with an *in situ* cell death detection kit (Wuhan Boster Biological Technology, Ltd.). Following treatment with protease K, the sections of xenograft tissue were treated with dUTP tagged with digoxin for 2 h under a reaction of terminal deoxynucleotidyl transferase, in order to promote transfer to the 3'-OH end of the DNA fragment. The streptavidin-biotin complex was added for 30 min. Diaminobenzidine (DAB) was then used for staining. Cells exhibiting yellow nuclei were positive, as examined under a light microscope. The apoptotic cells were counted at x400 magnification, and the apoptotic index (AI) was calculated using the following formula: AI = number of apoptotic cells/numbers of total cells x100%. All experiments were performed in triplicate.

Treatment with pifithrin-α. The Kasumi-1 cells were divided into four groups: PHI group, cells treated with 10 μmol/l PHI for 24 h; pifithrin-α + PHI group, cells treated with 10 μmol/l pifithrin-α for 30 min, and then 10 μmol/l PHI for 24 h; pifithrin-α group, cells treated with 10 μmol/l pifithrin-α for 24 h; and control group, cells treated with an equal volume of DMSO for 24 h. The grouped Kasumi-1 cell were seeded at 5×10^6 cells/well in 10 cm culture dishes and incubated at 37°C with 5% CO$_2$.

Western blot analysis. The cells were lysed in RIPA buffer containing 150 mM NaCl, 50 mM Tris-base, 5 mM EDTA, 1% NP-40, 25% deoxycholate (pH 7.4), according to methods previously described (24). The protein concentrations of the lysates were detected using a Bradford protein assay. The proteins (20 μg) were separated on a 10 or 12% gel by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto PVDF membranes (Merck KGaA). Following blocking with 5% skimmed milk at 37°C for 1 h, the membrane was incubated with primary antibodies (1:1,000 dilution) overnight at 4°C. The membrane was then incubated with horseradish peroxidase-conjugated secondary antibodies (1:500 dilution) for 2 h at 37°C. The signals were detected using an enhanced chemiluminescence kit (Thermo Fisher Scientific, Inc.). The intensity of the bands was quantified with ImageJ software (version 1.520; National Institutes of Health). β-actin and α-tubulin served as internal controls.

Statistical analysis. Student's t-test was used for the statistical analysis between the control and treated groups. The statistical differences between multiple groups were detected using one-way analysis of variance followed by Tukey's post hoc test. The comparative data are expressed as the mean ± SD of at least three independent experiments. P<0.05 was considered to indicate a statistically significant difference.

Results

PHI inhibits the proliferation of hematological tumor cell lines. A total of 16 hematological tumor cell lines were treated with 10, 20 and 40 μmol/l PHI for 24 h. Compared with the control, cell proliferation was inhibited in a dose-dependent manner in all hematological tumor cells. (Fig. 1A and B). The results demonstrated that Kasumi-1 and SKNO-1 cells were more sensitive to PHI than the other cell lines, providing the lowest IC$_{50}$ values (7.45±0.89 and 7.86±0.77 μmol/l, respectively; both P<0.05, compared with the other cell line). No significant difference was observed between the IC$_{50}$ values of the two cells types (P>0.05) (Fig. 1C).

Subsequently, the Kasumi-1 and SKNO-1 cells were treated with 10 μmol/l PHI for 12, 24, 48 and 72 h. The viability rates of the Kasumi-1 cells were 68.3±1.1, 35.6±1.1, 21.3±2.2 and 12.1±2.1%, respectively, at these time points. For the SKNO-1 cells, the viability rates were 71.2±1.6, 36.3±1.6, 22.6±2.1 and 12.7±2.5%, respectively, indicating a time-dependent effect (P<0.05). No significant difference was noted between the Kasumi-1 and SKNO-1 groups (P>0.05) (Fig. 1D).

PHI induces Kasumi-1 and SKNO-1 cell apoptosis. Following treatment of the Kasumi-1 and SKNO-1 cells with 0, 5, 10, 20 and 40 μmol/l PHI for 24 h, cell apoptosis was observed to increase in a dose-dependent manner. In the Kasumi-1 group, the early apoptotic rates were 1.3±0.03, 3.1±0.13, 34.2±0.98, 41.2±1.12 and 60.5±1.7%, respectively, following exposure to the aforementioned concentrations of PHI (Fig. 2A and C). In the SKNO-1 cells, the early apoptotic rates were 2.5±0.05, 4.5±0.12, 10.7±0.82, 59.8±2.3 and 69.7±2.15%, respectively (P<0.05; Fig. 2B and D).

PHI induces cell cycle arrest at the G_0/G_1 phase. Following treatment of the Kasumi-1 cells with PHI, cell cycle was arrested at the G_0/G_1 phase. The percentage of cells in the G_0/G_1 phase was 44.8±1.2, 50.5±1.8, 64.3±3.1 and 71.9±1.6% following exposure to 0, 1.25, 2.5 and 5 μmol/l PHI, respectively
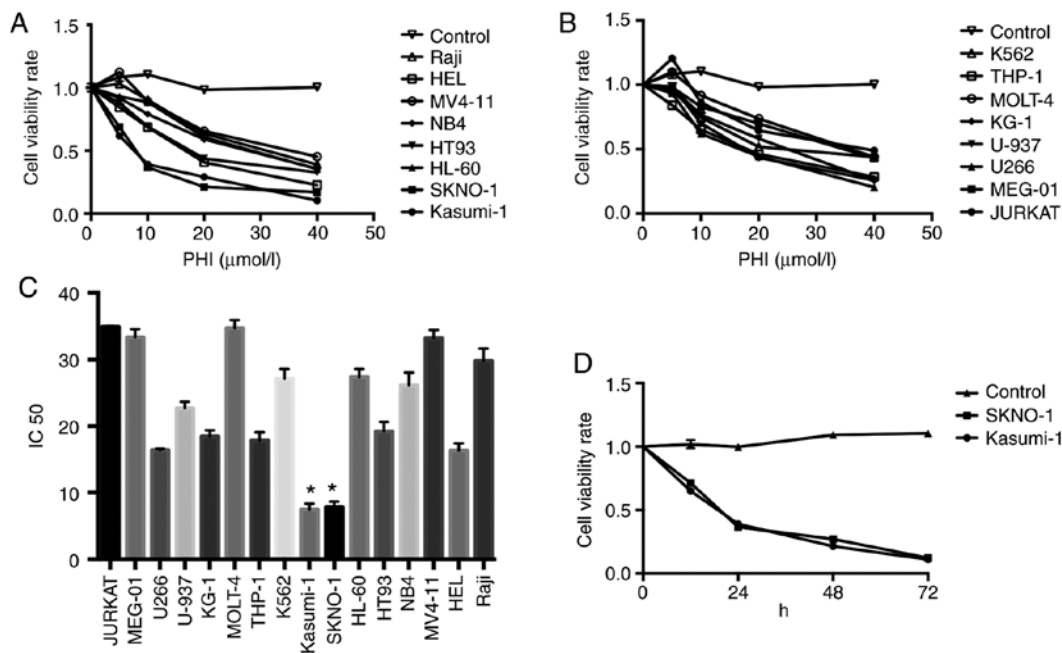
Figure 1. PHI inhibits the proliferation of hematological tumor cell lines. Following treatment with 10, 20 and 40 μmol/l PHI for 24 h, the cell viability rate of (A) Raji, HEL, MV4-11, NB4, HT93, HL-60, SKNO-1, Kasumi and (B) K562, THP-1, MOLT-4, KG-1, U-937, U266, MEG-01 and Jurkat hematological tumor cell lines were measured. (C) The IC_{50} of the 16 cell lines is presented in the histogram. Compared with the other cell lines, Kasumi-1 and SKNO-1 cells exhibited the lowest IC_{50}. *$P<0.05$. (D) Cell viability rates of Kasumi-1 and SKNO-1 cells following treatment with 10 μmol/l PHI for 12, 24, 48 and 72 h. PHI inhibited the viability of Kasumi-1 and SKNO-1 cells in a time-dependent manner. $P<0.05$. PHI, phenylhexyl isothiocyanate; IC_{50}, half maximal inhibitory concentration.

(Fig. 3A). Compared with the control, the increases in cell numbers in this cell cycle phase were statistically significant (all $P<0.01$; Fig. 3B). The lowest percentage of cells in the S phase and the peak in apoptosis were observed at the concentration of 5 μmol/l PHI.

PHI inhibits colony formation in Kasumi-1 cells. The colony-formation ability of Kasumi-1 cells was inhibited following treatment with PHI. The numbers of cell colonies were 92±3, 52±4, 38±4 and 21±2 following treatment with 5, 10, 20 and 40 μmol/l PHI (Fig. 4A and B). Compared with the control (130±6 colonies), the decrease in colony-formation ability was statistically significant ($P<0.01$).

PHI inhibits the growth of Kasumi-1 xenografts. During treatment with PHI, no obvious differences were observed in the feeding behavior, mental state or weight of the mice between the control and PHI groups. The tumor volumes were 0.77±0.12, 1.02±0.14, 1.35±0.11, 1.65±0.21 and 1.91±0.18 cm^3 on days 3, 6, 9, 12 and 14, respectively, following administration of 40 μmol/l PHI. The tumor volumes in the control group were 1.05±0.15, 1.55±0.12, 2.28±0.18, 3.20±0.12 and 3.82±0.12 cm^3, respectively, at these time points. From day 3 onward, a smaller size of xenograft tumor was observed in the PHI group compared with that in the control (all $P<0.05$; Fig. 5A-C).

PHI induces apoptosis of Kasumi-1 cells in vivo. Following 14 days of treatment, the xenograft tumor was removed and sliced for staining. Using microscopy and H&E staining, the tumors of the control group were observed to have grown markedly in a solid trabecular or nested arrangement; the tumor cells exhibited a large, polygonal or oval shape, and hyperchromatic nuclei were visible (Fig. 6A). Following exposure to 40 μmol/l PHI, the tumor cells shrunk and were loosely arranged. Irregular liquefactive necrosis, karyopyknosis or even loss of nuclei, and connective tissue proliferation were observed (Fig. 6B). A TUNEL assay revealed that the AI was 28.5±0.5 in the PHI group, but apoptosis was absent in the controls. The difference between the two groups was significant ($P<0.01$; Fig. 6C-E).

PHI induces apoptosis by activating mitochondrial and death receptor pathways in Kasumi-1 cells. Following treatment of the Kasumi-1 cells with 5, 10, 20 and 40 μmol/l PHI for 24 h, the expression levels of apoptosis-associated proteins cleaved caspases 3, 8 and 9, and cleaved PARP, were increased in a dose-dependent manner. The expression of cleaved caspase 9 began to rise at the PHI concentration of 10 μmol/l. However, the expression of cleaved caspases 3 and 8 and PARP began to rise at 5 μmol/l PHI (Fig. 7A and C). Treatment with this compound downregulated Bcl-2, and upregulated Bax, increasing the Bax/Bcl-2 ratio (Fig. S1). The expression levels of p53, Fas and p21 were also increased following exposure to PHI. All of these changes occurred in a dose-dependent manner (Fig. 7B and D).

PHI restores mutant P53 and leads to cell apoptosis. To further confirm whether *P53* is key in the PHI-induced apoptosis in Kasumi-1 cells, pifithrin-α (a p53 inhibitor) was used to investigate PHI-induced changes in p21, Bax (two downstream factors of *P53*) and cleaved caspase 3. Compared with the control group, the pifithrin-α group had no effect on the activation of cleaved caspase 3, but had marginally decreased
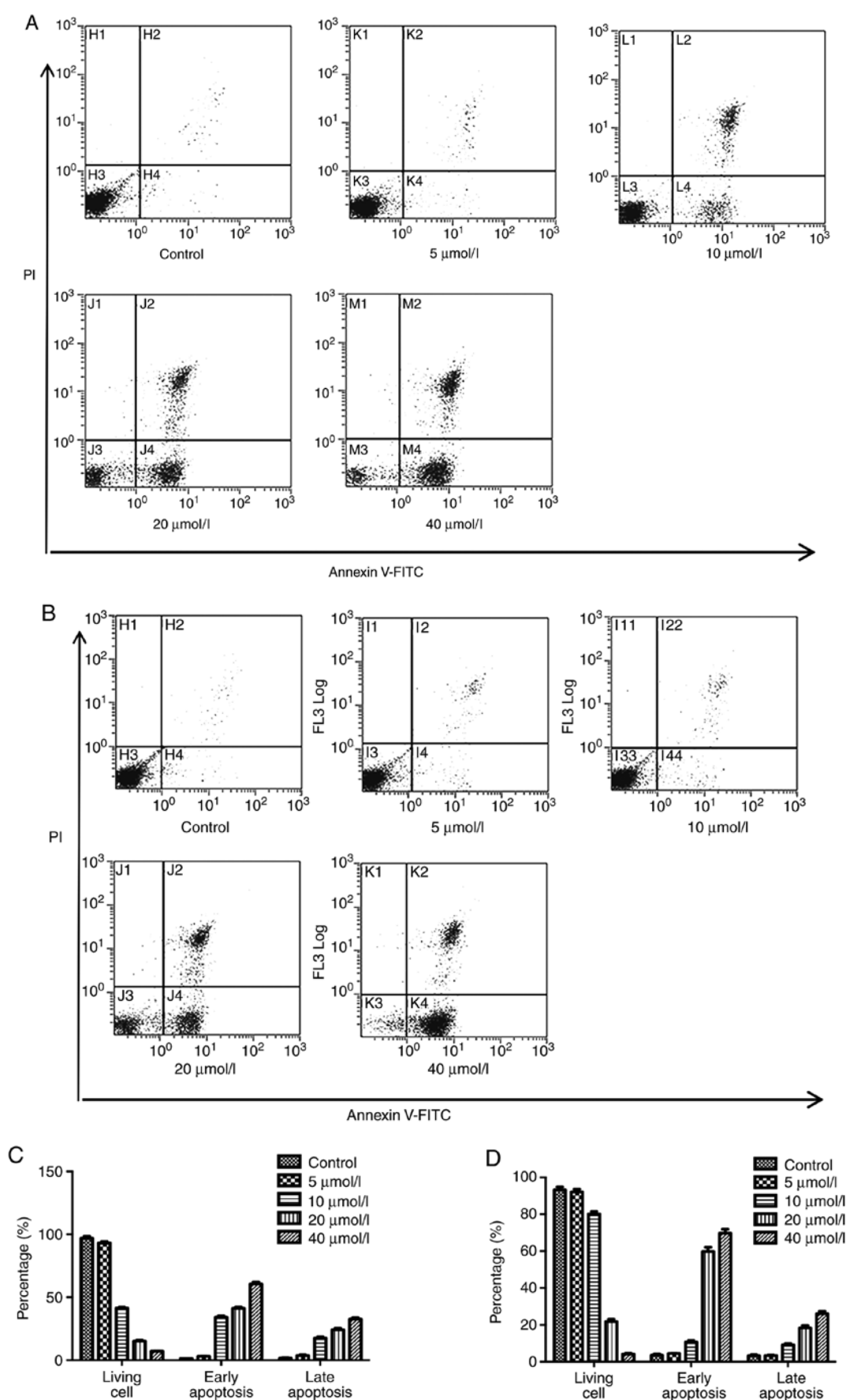
Figure 2. PHI induces apoptosis in Kasumi-1 and SKNO-1 cells. Kasumi-1 and SKNO-1 cells were treated with 5, 10, 20 and 40 μmol/l PHI for 24 h. Subsequently, the early (Annexin V-FITC-positive and PI-negative) and late (Annexin V-FITC-positive and PI-positive) apoptosis of (A) Kasumi-1 and (B) SKNO-1 cells were observed. The percentages of apoptotic cells in the (C) Kasumi-1 and (D) SKNO-1 groups were measured. Apoptosis was induced by 10 μmol/l PHI in the Kasumi-1 cells and 20 μmol/l in the SKNO-1 cells. The numbers of apoptotic cells in the two groups increased in a dose-dependent manner. P<0.05. PHI, phenylhexyl isothiocyanate; FITC, fluorescein isothiocyanate; PI, propidium iodide.

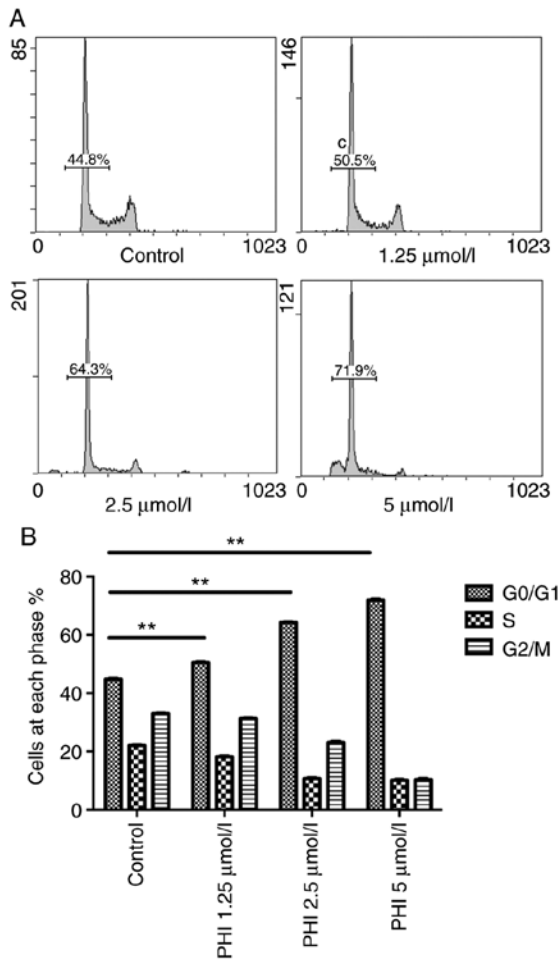Figure 3. PHI induces cell cycle arrest at the G_0/G_1 phase in Kasumi-1 cells. Kasumi-1 cells were treated with 1.25, 2.5 and 5 μmol/l PHI for 24 h. (A) Cell cycle phases. (B) Percentages of cells in the G_0/G_1, S, and G_2/M phases. The percentage of Kasumi-1 cells in the G_0/G_1 phase increased in a dose-dependent manner. The cell percentage in the S phase was significantly decreased. The differences between the control and the treated group were statistically significant. **P<0.01. PHI, phenylhexyl isothiocyanate.
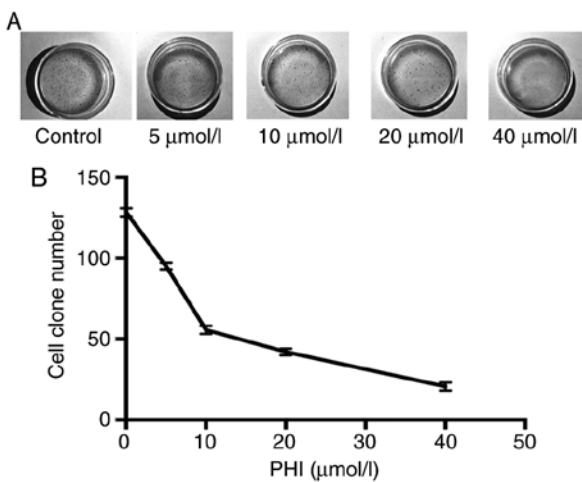

Figure 4. PHI inhibits the colony-formation ability of Kasumi-1 cells. (A) Following treatment with 5, 10, 20 and 40 μmol/l PHI for 14 days, the colony formation of Kasumi-1 cells was observed. (B) Numbers of cell colonies (y-axis) of the Kasumi-1 cell were counted and are presented in the scatter diagram. PHI inhibited the colony formation in a dose-dependent manner (P<0.05). The lowest colony number (21±2) was observed under the treatment with 40 μmol/l PHI. PHI, phenylhexyl isothiocyanate.
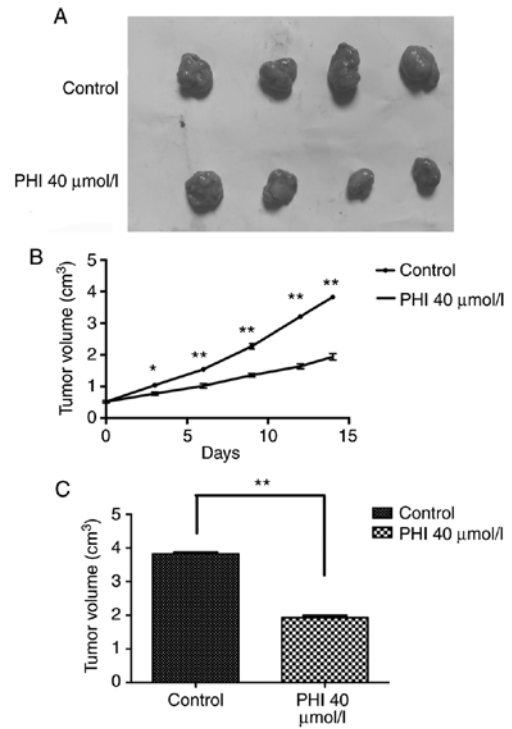

Figure 5. PHI inhibits Kasumi-1 cell proliferation *in vivo*. The nude mice with Kasumi-1 cell xenograft tumors were treated with 40 μmol/l PHI or DMSO (control) for 14 days. (A) On day 14, the xenograft tumors were removed and images were captured. (B) Tumor volumes on days 0, 3, 6, 9, 12 and 14 were recorded. Significant differences were observed between the control and PHI groups on all days following treatment. (C) Histogram demonstrating that the volume of a xenograft tumor in the PHI group was markedly smaller than that in the DMSO group on day 14. *P<0.05; **P<0.01. PHI, phenylhexyl isothiocyanate; DMSO, dimethyl sulfoxide.
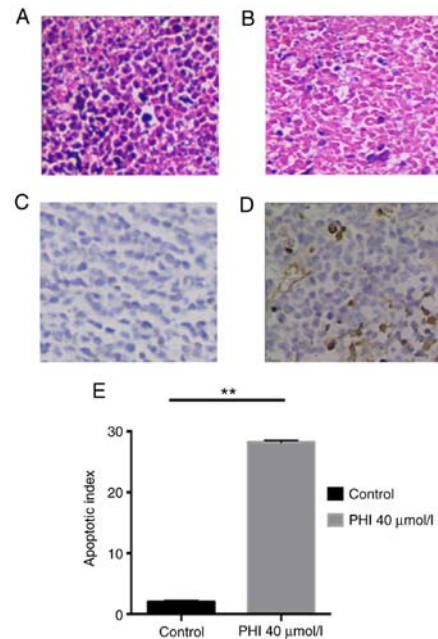

Figure 6. PHI induces apoptosis in Kasumi-1 cells *in vivo*. Sections of xenograft tumors from mice treated with (A) DMSO or (B) 40 μmol/l PHI were observed under a microscope, following hematoxylin and eosin staining. A TUNEL assay was used to detect the apoptosis in xenograft tumors treated with (C) DMSO or (D) 40 μmol/l PHI. (E) A significantly larger apoptotic index (y-axis) was measured in the PHI-treated group than in the control group. Magnification, x400. **P<0.01. PHI, phenylhexyl isothiocyanate; DMSO, dimethyl sulfoxide.
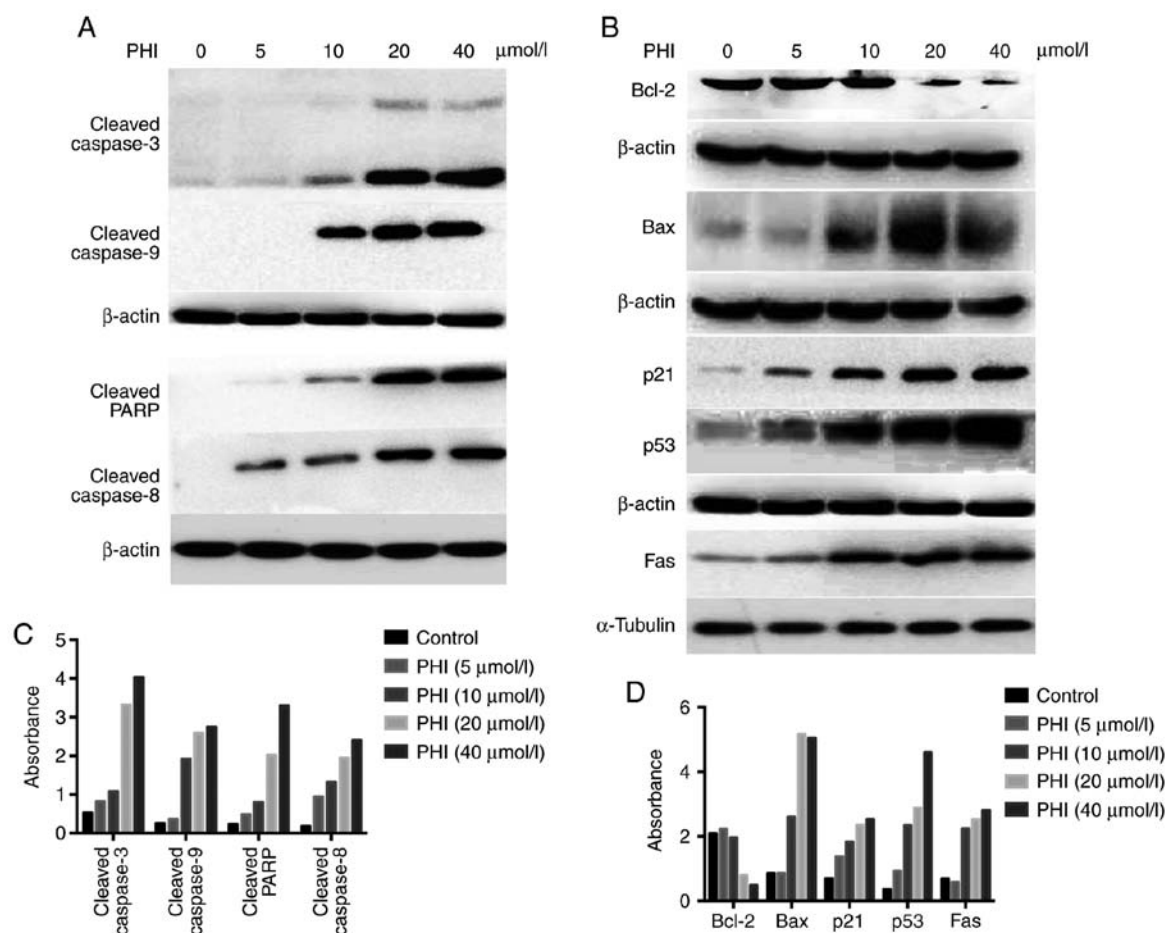
Figure 7. PHI regulates the expression of apoptosis-associated proteins. Following treatment with 5, 10, 20 or 40 μmol/l PHI for 24 h, apoptosis-associated proteins in Kasumi-1 cells were detected by western blotting. The absorbance values of the protein bands for (A) cleaved caspases 3, 6, and 9 and PARP and for (B) Bcl-2, Bax, p21, p53 and Fas are shown in the histograms. (C) Expression of cleaved caspase 9 began to rise at the concentration of 10 μmol/l, and the expression of cleaved caspases 3 and 8, and PARP began to rise at 5 μmol/l. (D) PHI downregulated Bcl-2 and upregulated Bax, resulting in an increased ratio of Bax/Bcl-2. The expression of p53, Fas and p21 were also increased following exposure to PHI. All changes occurred in a dose-dependent manner. PHI, phenylhexyl isothiocyanate; PARP, poly (ADP-ribose) polymerase.

expression levels of p21, and no obvious difference in the expression of Bax. Treatment in the PHI group significantly improved the expression levels of cleaved caspase 3, p21 and Bax. Compared with the PHI group, the pifithrin-α + PHI group exhibited notable increases in the expression of cleaved caspase 3, p21 and Bax (Fig. 8A and B).

Discussion

Previously, PHI has been demonstrated to affect HL-60 leukemia, PC3 prostate cancer and hepatocellular carcinoma cell lines. In the present study, PHI was demonstrated to exhibit a more marked inhibitory effect on M2 cell lines compared with other types of hematological tumor cell. This compound induced cell cycle arrest at the G_0/G_1 phase and inhibited colony formation in the M2 cells. Further evidence revealed that PHI induced the apoptosis of M2 cells *in vitro* and *in vivo*.

The pathways of apoptosis mainly include the mitochondrial and the receptor signaling pathways. In the mitochondrial pathway, numerous proteins, including Bcl-2, Bax, induced myeloid leukemia cell differentiation protein Mcl-1 and BH3-interacting domain death agonist, are activated. Subsequently, mitochondria release cytochrome *c*,

which activates caspases 9 and 3 (25). Reactive oxygen species (ROS) have been tightly linked to activation of the mitochondrial pathway. ROS, including H_2O_2 and superoxide, can cause the release of cytochrome *c* from mitochondria and the induction of apoptosis through the mitochondrial pathway (26). The death receptor pathway involves Fas and tumor necrosis factor receptor superfamily member 10B, and activates caspases, including caspases 8 and 10 (27). Caspase-3 is further activated; this is a protein-cutting enzyme that cleaves a series of important enzymes, including PARP, eventually leading to cell apoptosis (28). The present data showed that PHI led to the cleavage of caspases 8 and 9, resulting in the cleavage of caspase 3 and PARP. This indicates that PHI induced apoptosis through the mitochondrial and death receptor pathways. Furthermore, treatment enhanced the expression of Fas, indicating that the Fas/Fas ligand apoptotic pathway may also be involved in PHI-induced apoptosis. To determine whether ROS is involved in the mitochondrial pathway, further investigations will be performed in the future.

The *P53* gene is a classic tumor-suppressor gene, and the p53 protein is the product of its translation. Its main function is to detect the integrity of the cell genome and repair DNA damage (29). If cells cannot be repaired, *P53* can be activated
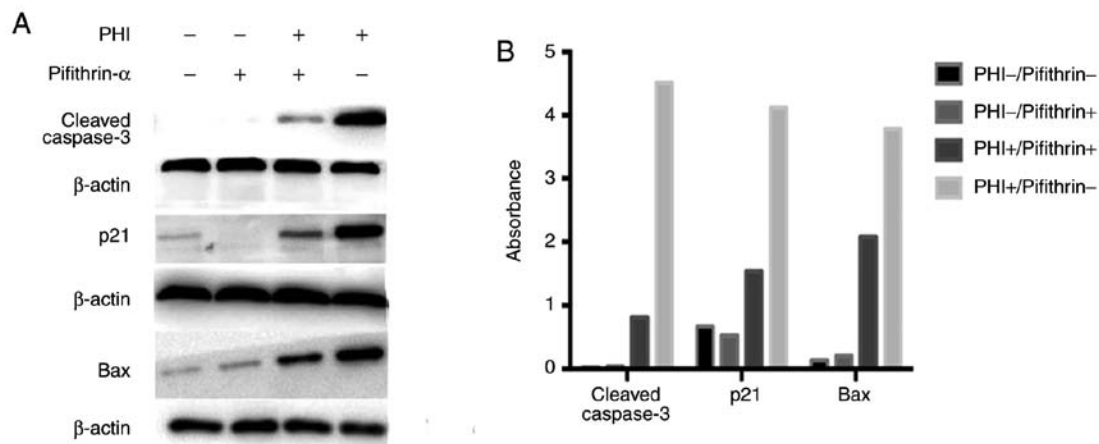
Figure 8. PHI induces cell apoptosis by restoring mutant *P53* and reactivating the *P53* pathway. Kasumi-1 cells were treated separately in four groups: PHI, pifithrin-α + PHI, Pifithrin-α, and DMSO (control) for 24 h. (A) Protein expression levels were then detected by western blot analysis. (B) Quantification of protein expression. Compared with the control, the PHI group exhibited significantly higher expression levels of cleaved caspase 3, p21 and Bax. Compared with the PHI group, the pifithrin-α + PHI group exhibited reversal of the changes in protein expression induced by PHI. PHI, phenylhexyl isothiocyanate; DMSO, dimethyl sulfoxide.

by various signaling pathways and helps the cell to remain stable without degradation. Furthermore, it can enhance the transcription of downstream genes, including cyclin-dependent kinase inhibitor *P21* and apoptotic protein *BAX*. This further induces cell cycle arrest, cell apoptosis and aging, and inhibits angiogenesis (30).

P53 mutations are common in hematological malignancies (12-14). The p53 protein is composed of an N-terminal transcriptional activation region, an intermediate DNA binding region and a C-terminal polymer region. Mutations mainly occur in the DNA-binding domain of the gene, and the majority of these are missense mutations. Hot-spot mutations include R175, G245, R248, R249, R273 and R282. These missense mutations usually result in three-dimensional structural changes in the protein, altering its DNA-binding capacity and resulting in p53 losing its function as a transcription factor, and thus its anti-cancer effect (31). This is an important factor in tumorigenesis; therefore, reactivating wild-type p53 and restoring the function of mutant p53 are important directions in cancer therapy research. At present, certain drugs are available targeting mutant p53. These can restore the DNA-binding ability of the mutant protein, delete the mutated sequence or inhibit downstream pathways. R248Q is a hot-spot mutation that lies within the DNA-binding domain of the p53 protein. This leads to the inhibition of p53 transcriptional activity and eventually results in the loss of function of *P53*. Targeted drugs for the *P53* R248Q mutation have been developed. Recently, it has been reported that phenethyl isothiocyanate, a derivative of PHI, can reactivate and repair mutant p53 and inhibit tumor growth (32).

In the present study, the Kasumi-1 and SKNO-1 cells used carry the R248Q mutation, causing p53 to lose its function as a transcription factor. Therefore, the *P53* pathway was inactive. However, the present data showed that the expression of total p53 protein increased following exposure to PHI. Furthermore, PHI upregulated the expression of Bax and p21, which are downstream proteins of *P53*. Therefore, PHI may have the ability to repair the mutation, recover the function of *P53* and reactivate its pathway. To further demonstrate whether the apoptosis induced by PHI is associated with *P53*, a p53 inhibitor (pifithrin-α) was

used. Compared with the controls, pifithrin-α had no effect on the levels of cleaved caspase 3 or Bax, and marginally decreased the expression levels of p21. The PHI group exhibited significantly higher levels of these three proteins. Compared with the PHI group, the pifithrin-α + PHI group demonstrated near reversal of these expression levels. This demonstrated that the expression of apoptotic proteins induced by PHI was reversed when the p53 inhibitor was used, resulting in the inhibition of apoptosis. These data provide evidence that PHI induced apoptosis by reactivating the p53 signaling pathway in Kasumi-1 cells.

Taken together, the results of the present study demonstrated that PHI had a specific and notable inhibitory effect on M2 cell lines (Kasumi-1 and SKNO-1) *in vivo* and *in vitro*. PHI inhibited cell arrest at the G_0/G_1 phase of the cell cycle. Additionally, PHI inhibited cell proliferation and induced apoptosis by restoring mutant p53 in Kasumi-1 cells, reactivating the transcription of downstream genes of *P53*, including *BAX* and *P21*. Overall, this compound may lead to cell cycle inhibition, gene repair and apoptosis.

Availability of data and materials

The datasets used and/or analyzed in the present study are available from the corresponding author on reasonable request.

Authors' contributions

XM conceived and designed the experiments; YZ performed the experiments; YH and YZ analyzed the data; YZ wrote the manuscript. All authors have read and approved this manuscript.

Ethics approval and consent to participate

All animal experiments were approved by the Ethics Committee of Zhangzhou Affiliated Hospital of Fujian Medical University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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