Lung cancer is one of the leading causes of cancer deaths worldwide [1]. It is also one of the most difficult cancers to treat because the current treatment options for advanced lung cancer are quite limited [2]. Furthermore, for most patients with lung cancer, chemotherapies have not dramatically changed clinical outcome, because resistance has emerged as a clinical problem [3]. Thus, there is an urgent need to identify novel anticancer agents that are nontoxic and highly effective in inducing apoptosis preferentially in lung cancer cells.

Cancer cells exhibit enhanced levels of ROS compared with normal cells. Emerging evidence suggests that cellular adaptation to ROS stress plays an important role in maintaining cancer phenotype and drug resistance because of their stimulating effects on cell growth and proliferation [4,5]. However, high levels of ROS also can trigger cell cycle arrest and apoptosis when they outstrip cellular antioxidant defenses [6]. Surprisingly, cancer cells are more sensitive to an acute increase in ROS levels than normal cells. This is because oncogenic transformation elevates ROS to such high levels that a further acute increase triggers reactivation of the apoptotic program in cancer cells [7]. Conversely, normal cells are less sensitive because of their relatively low basal ROS levels. Therefore, it is reasonable that exogenous agents enhance the intracellular ROS and then induce cell death.

FOXO3a is a member of the Forkhead box class O (FOXO) transcription factors family. In response to various stresses, such as oxidative stress, FOXO3a can activate or repress multiple target genes, such as Bim [8] and TRAIL [9] for inducing apoptosis and p27 [10], p21 [11], and cyclin D [12] for cell cycle arrest. Inhibition of FOXO3a expression and activity is critical to promote cell transformation, tumor progression, and angiogenesis. This indicates that FOXO3a has tumor-suppressive roles [13–15]. Furthermore, inactivation of FOXO3a is also correlated with poor prognosis of breast...
cancer [16], leukemia [17], and lung cancer [18]. Therefore, FOXO3a has been proposed to be an important factor influencing the efficacy of a variety of chemotherapeutic drugs. Reactivation of FOXO3a activity based on its tumor suppressor properties is considered an attractive therapeutic strategy for human cancer treatment [19, 20].

Curcumin (diferuloylmethane), a natural compound isolated from the plant Curcuma longa, has been shown to inhibit cell proliferation, induce apoptosis, suppress inflammation, and sensitize tumor cells to cancer therapies [21]. However, the clinical potential of curcumin remains limited because of its relatively low activity and poor bioavailability [22]. Consequently, analogues of curcumin with similar safety profiles but increased anticancer activity have been developed in recent years [23, 24]. New 4-arylidene curcumin analogues, which can effectively inhibit proliferation of a panel of lung cancer cells at very low concentrations, were synthesized in our previous study [25], in which T63 (compound 17 in Ref. [25]) showed high antitumor activity. It exhibited significantly improved potency in blocking the nuclear factor κB pathway by both in vivo and in vitro kinase assays and pathway analysis. However, the detailed molecular mechanisms and downstream target genes responsible for its tumor suppression activity need further investigation. This study revealed that T63 could induce cell cycle arrest and apoptosis through a specific signaling cascade initiated by ROS and transduced by p38MAPK/AKT, FOXO3a, and p27/p21/Bim and then activate caspase-3 and inhibit cyclin D1. Furthermore, in vivo studies showed that T63 significantly suppressed the growth of A549 lung cancer xenograft tumors, associated with proliferation suppression and apoptosis induction in tumor tissues, without inducing any notable major organ-related toxicity. Our in vitro and in vivo results suggested that T63 could effectively be used for the prevention and treatment of human lung cancer.

Materials and methods

Materials

T63 and curcumin were synthesized at our laboratory, dissolved in dimethyl sulfoxide (DMSO) to prepare a 10 mM stock solution, and stored at −20 °C. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyldi-trazolium bromide (MTT), propidium iodide, 3,3′-diaminobenzidine, SB203580, LY294002, diphenylene iodine (DPI), rotenone, allopurinol, PEG–catalase (PEG–CAT), and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyldi-trazolium bromide (MTT) were obtained from Sigma (Poole, UK). 4,6-Diamidino-2-phenylindole (DAPI), the secondary anti-rabbit antibody conjugated to FITC (fluorescein isothiocya-nate), Lipofectamine 2000, RPMI 1640 medium, and fetal bovine serum were obtained from Invitrogen (Carlsbad, CA, USA). Monoclonal antibodies against FOXO3a, Bim, AKT, p38MAPK, p21, ERK2, and N-acetylcysteine (NAC) were obtained from Sigma (Poole, UK). 4,6-Diamidino-2-phenylindole (DAPI), the secondary anti-rabbit antibody conjugated to FITC (fluorescein isothiocyanate). Lipofectamine 2000, RPMI 1640 medium, and fetal bovine serum were obtained from Invitrogen (Carlsbad, CA, USA). Monoclonal antibodies against FOXO3a, Bim, AKT, p38MAPK, p21, ERK2, and N-acetylcysteine (NAC) were obtained from Sigma (Poole, UK). 4,6-Diamidino-2-phenylindole (DAPI), the secondary anti-rabbit antibody conjugated to FITC (fluorescein isothiocyanate). Lipofectamine 2000, RPMI 1640 medium, and fetal bovine serum were obtained from Invitrogen (Carlsbad, CA, USA). Monoclonal antibodies against FOXO3a, Bim, AKT, p38MAPK, p21, ERK2, and N-acetylcysteine (NAC) were obtained from Sigma (Poole, UK). 4,6-Diamidino-2-phenylindole (DAPI), the secondary anti-rabbit antibody conjugated to FITC (fluorescein isothiocyanate). Lipofectamine 2000, RPMI 1640 medium, and fetal bovine serum were obtained from Invitrogen (Carlsbad, CA, USA)

Cell viability assay

Cell viability was measured using the MTT assay. Briefly, cells (1 × 10⁴) were seeded into each well of a 96-well plate and treated with T63 or curcumin at concentrations from 0.01 to 10 μM for 24 h. After treatment, the cells were washed twice with phosphate-buffered saline (PBS), and 100 μl of 0.25 mg/ml MTT in culture medium was added to each well. The plate was incubated for 4 h at 37 °C. Then, the culture medium was removed, and DMSO (100 μl) was added to each well to dissolve the dark blue crystal. The absorbance was measured at 570 nm using a microplate reader (Spectra MAX 340; Molecular Devices, Sunnyvale, CA, USA).

Cell cycle analysis

Control and treated cells were collected into flow cytometry tubes and centrifuged at 2000 rpm for 5 min to obtain cell pellets. The cells were washed with PBS and fixed with 70% ethanol (−20 °C ice-cold) for 1 h at 4 °C. Fixed cells were washed with PBS and incubated with RNase A (0.1 mg/ml) for 30 min following incubation with propidium iodide (50 μg/ml) for 30 min at room temperature. Cell cycle analysis was performed with a Coulter Epics XL flow cytometry system (Beckman–Coulter, Miami, FL, USA). In each analysis, 10,000 events were recorded. The percentages of cells at sub-G1, G0/G1, S, and G2/M were calculated using EXPO32 ADC analysis software (Beckman–Coulter).

Determination of ROS production

ROS production was determined using the Amplex red hydrogen peroxide assay kit (Molecular Probes, Eugene, OR, USA). Amplex red is a widely used probe for detecting ROS generation; it is oxidized by horseradish peroxidase (HRP) and H₂O₂ to a fluorescent product, resorufin [26]. Briefly, control and treated cells were incubated in 50 mM potassium phosphate buffer (pH 7.4) containing 0.2 μM HRP and 50 mM Amplex red. Fluorescence was monitored at the excitation and emission wavelengths of 530 and 590 nm, in a FLEXstation 3 spectrofluorimeter (Molecular Devices). Calibration was performed using hydrogen peroxide as standard. Data were expressed as fold increase in H₂O₂ produced relative to control.

Determination of total (reduced and oxidized) glutathione

Total GSH level was measured by the enzymatic recycling method using glutathione reductase and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), in which GSH is oxidized by DTNB and reduced by NADPH in the presence of glutathione reductase. Control and treated cells were lysed in 500 μl of 5% sulfosalicylic acid for 15 min on ice. Then the cell lysates were centrifuged at 10,000 rpm for 5 min, and the supernatant was mixed with a 10-μl aliquot containing 150 μl of potassium phosphate buffer (pH 7.0) containing 5 mM EDTA, 6 mM DTNB, and 6 μM glutathione reductase. Finally, 50 μl of 96 μM NADPH was added. The absorbance was measured at 1-min intervals for 10 min at 405 nm in a microplate reader (Spectra MAX 340; Molecular Devices).

Determination of intercellular superoxide dismutase and catalase activity

The intercellular enzyme activities of SOD and CAT were determined using commercial assay kits ( Jiancheng Biochemical, Nanjing, China) and following the manufacturer’s protocol. The specific enzyme activities of SOD and CAT were expressed as U/mg protein.

Cell lines and culture conditions

Human cell lines A549, H460, and BEAS-2B were obtained from the American Type Culture Collection. The cells were cultured in RPMI 1640 with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified 5% CO₂ atmosphere.
Western blot analysis

Cells were lysed in cell lysis buffer containing 1% NP-40, 20 mM Tris–HCl (pH 7.6), 0.15 M NaCl, 3 mM EDTA, 3 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 20 mg/ml aprotinin, and 5 mg/ml leupeptin. Cell lysates were centrifuged at 12,000 rpm for 30 min at 4 °C, supernatants were saved, and protein concentrations were determined by Bio-Rad DC protein assay (Bio-Rad, Hercules, CA, USA). Equal amounts of protein (20 μg) were run on 10% SDS–polyacrylamide gels and transferred to nitrocellulose membranes. After blocking with 5% nonfat milk at room temperature for 2 h, membranes were incubated with the primary antibody at 1:1000 dilution overnight at 4 °C and then incubated with a horseradish peroxidase-conjugated secondary antibody at 1:5000 dilution for 1 h at room temperature. Specific immune complexes were detected using the Western

Fig. 1. T63 induces lung cancer cell apoptosis and cell cycle arrest. (A) Chemical structure of T63 and curcumin. (B) Effects of T63 and curcumin on cell proliferation in A549 and H460 cells. Cells were treated with various concentrations of T63 or curcumin for 24 h, and then cell viability was assessed by MTT assay. Data are presented as means ± SD of three independent experiments. (C) Effects of T63 and curcumin on cell proliferation in normal human bronchial epithelial cells (BEAS-2B). Cells were treated with 2 μM T63 or 10 μM curcumin for 24 h, and then cell viability was assessed by MTT assay. (D) Effects of T63 and curcumin on cell cycle distribution in A549 and H460 cells. Cells were treated with various concentrations (0, 0.25, 0.5, 1, 2 μM) of T63 or 10 μM curcumin for 24 h, fixed in ethanol, and stained with propidium iodide, and then DNA contents were determined by flow cytometry. The percentage of cells in each phase of the cell cycle (sub-G1, G0/G1, S, and G2/M) is indicated. (E) Effects of T63 and curcumin on the expression of caspase-3 and cyclin D1. Protein lysates were prepared from A549 cells after treatment with various concentrations (0, 0.25, 0.5, 1, 2 μM) of T63 or 10 μM curcumin for 24 h. Caspase-3, cleaved caspase-3, and cyclin D1 protein expression levels were analyzed by Western blot.
Blotting Plus chemiluminescence reagent (Life Science, Boston, MA, USA).

Nuclear/cytosol fractionations

Nuclear and cytosolic fractions from A549 cells were obtained by using a nuclear/cytosol fractionation kit (BioVision), and Western blot analysis was done as described above.

Real-time reverse transcription–PCR analysis

Total RNA was isolated from cells using the EZNA HP Total RNA kit (Omega Bio-Tek, Doraville, GA, USA). The reverse transcription was performed with the PrimeScript RT reagent kit (Takara, Shiga, Japan). After the resulting complementary DNA template was mixed with FOXO3α or GAPDH primers and Takara SYBR Premix Ex Taq, quantitative real-time PCR was performed on a Bio-Rad q.iQ real-time PCR detection system (Bio-Rad). Crossing threshold values for individual genes were normalized to GAPDH. Changes in mRNA expression were expressed as fold change relative to control. Gene-specific primer pairs used in this study were as follows: FOXO3α sense 5′-GGTGCTCTTCTCAAGGATTAA-3′ and FOXO3α antisense 5′-GACCCGCATGAATCGACTATG-3′; GAPDH sense 5′-CACCCAGAAGACTGTGGATGG-3′ and GAPDH antisense 5′-GTCTACATCGCAACTGTGAGG-3′.

Transfection with small interfering RNA (siRNA)

SignalSilence FOXO3α siRNA and SignalSilence control siRNA were purchased from Cell Signaling Technology. A549 cells were transfected with 100 nM siRNA at 60% confluence in reduced-serum RPMI 1640. Lipofectamine 2000 (Invitrogen) was used for transfection following the manufacturer’s protocols. Twenty-four hours after transfection, the cells were treated with T63 for 12 or 24 h, and then the cells were collected for Western blot and flow cytometric analysis.

Immunofluorescence analysis

A549 cells were grown on chamber slides. After 12 h of cultivation, cells were treated with T63 for 4 h. The cells were fixed with 4% paraformaldehyde for 10 min and permeabilized with PBS (0.1% Triton X-100) for 30 min at room temperature. Slides were blocked for 30 min with normal goat serum and incubated overnight at 4 °C with the anti-FOXO3α antibody (1:200 dilution). After a wash step, slides were incubated with FITC-conjugated anti-rabbit IgG antibody (1:1000 dilution) for 1 h, and then nuclei were stained with DAPI. The images were acquired using a Zeiss LSM710 confocal microscope at 60× magnification with Zen2008 software (Zeiss, Oberkochen, Germany).

A549 cell xenograft in mice

Six-week-old male BALB/c nude mice purchased from the Experimental Animal Center at Sun Yat-sen University were used for in vivo experiments. The procedures involving mice and their care were approved by the Animal Experimentation Ethics Committee of Sun Yat-sen University. Mice were injected with 5 × 10⁶ A549 cells in the right shoulder and allowed to form xenografts. When tumors became visible (approximately 3 × 3 mm in size), the mice were randomly divided into three groups of eight animals and treated intraperitoneally with T63 at a dose of 0.5 or 2 mg/kg body wt every other day for 4 weeks, whereas the control group was treated with an equivalent volume of normal saline. Tumor size and body weight were measured every 2 days. The tumor volume was calculated using the formula V = 1/2 × larger diameter × (smaller diameter)², and growth curves were plotted using average tumor volume within each experimental group at the set time points. At the end of treatment, the animals were sacrificed, and the tumors were removed and weighed for use in histology (hematoxylin and eosin (H&E), cleaved caspase-3, cyclin D1) and Western blot studies.

Tumor histology and immunohistochemistry

Tumor tissues were fixed in formalin and embedded in paraffin. Sections (5 μm) were cut and stained with H&E. For immunohistochemical staining, sections were deparaffinized and hydrated, and endogenous peroxidase activity was blocked with 3% H₂O₂ in water for 10 min. Antigen retrieval was done with 10 mM citrate buffer (pH 6.0) for 10 min. Slides were incubated with Biocare blocking reagent for 10 min to block nonspecific binding. Then, they were incubated with anti-cleaved caspase-3 or cyclin D1 overnight at 4 °C. Slides were washed in PBS twice and then incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antibodies for 30 min at room temperature and then washed. Finally, slides were incubated with 3,3′-diaminobenzidine and counterstained with hematoxylin.

Statistical analysis

All values are reported as means ± SD of three independent experiments unless otherwise specified. Data were analyzed by two-tailed unpaired Student’s t test between two groups and by one-way ANOVA followed by Bonferroni’s test for multiple comparison involved. These analyses were performed using GraphPad Prism software version 5.0 (GraphPad Software, La Jolla, CA, USA). A p < 0.05 was considered statistically significant.

Results

Effects of T63 on cell viability

We first determined the effects of T63 (Fig. 1A) on cell proliferation of lung cancer cells. A549 and H460 cells were treated with T63 or curcumin (0.01 to 10 μM) for 24 h, and then the viability was determined by MTT assay. As shown in Fig. 1B, T63 significantly suppresses proliferation of A549 and H460 cells within a 24-h treatment period. More important, the inhibitory effects were observed at a dose of 1 μM, whereas curcumin showed no significant inhibitory effect upon A549 or H460 cell proliferation even at concentrations up to 10 μM. Furthermore, T63 has lower toxicity on normal human bronchial epithelial cells (BEAS-2B), with the cell viability percentage remaining above 75% when treated with up to 5 μM T63 for 24 h (Fig. 1C).

Effect of T63 on cell cycle arrest and apoptosis

To further examine the effects of T63 on cell proliferation and survival, cell cycle phase distribution of cells treated with 0, 0.25, 0.5, 1, and 2 μM T63 for 24 h was analyzed by flow cytometry after propidium iodide staining. Treatment with T63 on A549 and H460 cells resulted in a significant increase in the proportion of cells at G0/G1 phase and reduction in the proportion of cells at S and G2/M phases at 0.25 and 0.5 μM, compared with the untreated control or 10 μM curcumin-treated cells (Fig. 1D). Consistently, cyclin D1, a key regulator of G1 to S phase progression, showed a significantly decreased expression in a dose-dependent manner (Fig. 1E). Moreover, the proportion of cells at apoptotic sub-G1 phase was obviously increased at 1 and 2 μM (Fig. 1D). The apoptotic outcome
was further corroborated by showing a significant increase in cleaved caspase-3 protein levels at 1 and 2 μM by Western blot (Fig. 1E). However, cleaved caspase-3 expression was significantly lower in 10 μM curcumin-treated cells than in T63-treated cells. These results indicated that T63 induces a cell cycle arrest at the G0/G1 phase and apoptosis at very low doses, whereas no such effect is observed with curcumin.

**Effect of T63 on ROS generation**

The generation of ROS has an important role in the effects of various anticancer agents on tumor cell cycle transition and apoptosis [7]. Recently, it has been reported that curcumin treatment results in the production of ROS [27,28]. Therefore, we investigated the possibility that T63 induces cell cycle arrest and apoptosis by allowing for the accumulation of ROS. ROS generation was detected by Amplex red assay. As shown in Fig. 2A, treatment with T63 significantly increased Amplex red oxidation (H2O2 generation) in a dose-dependent manner. Furthermore, the ROS scavenger NAC or cell membrane-permeative analogue PEG-CAT significantly blocked T63-induced Amplex red oxidation (Fig. 2B). Altogether, these results suggested that treatment of A549 cells with T63 induced a significant increase in ROS production, which may be involved in T63-induced cell cycle and apoptosis.

To further assess the source of ROS production, we investigated the effects of various inhibitors on ROS-generating systems: rotenone (mitochondrial respiratory chain inhibitor), allopurinol (xanthine oxidase inhibitor), and DPI (NADPH oxidase (NOX) inhibitor) on T63-induced ROS levels. As shown in Fig. 2C, rotenone significantly and DPI partially, but not allopurinol, inhibited Amplex red oxidation in response to T63. These findings suggested that mitochondria and NADPH oxidase are important sources of ROS by T63 treatment. However, the detailed mechanisms of ROS production by T63 are not clear, and further studies to clarify this mechanism are needed.

**Effects of T63 on intracellular GSH, SOD, and CAT levels**

GSH is a major antioxidant and protects cells from oxidative stress. Several studies demonstrated that depletion of GSH was necessary for an initial increase in ROS production [29–31]. Indeed, in our study, T63 induced a rapid depletion of intracellular GSH (Fig. 2D). Exposure of cells to 0.25, 0.5, 1, or 2 μM T63 for 1 h led to intracellular GSH depletion of about 14, 21, 45, and 68%, respectively.

The intercellular levels of SOD and CAT, two important antioxidant enzymes, were also measured. As shown in Fig. 2E, treatment with T63 did not show any significant effect on the CAT levels. The SOD activity of low-dose T63-treated cells was also similar to that of control cells but a moderate increase was observed at 2 μM T63-treated cells (Fig. 2F).

**Role of ROS in T63-induced cell cycle arrest and apoptosis**

To verify the role of ROS in T63-induced cell cycle arrest and apoptosis, A549 cells were treated with 2 μM T63 for 24 h in the presence or absence NAC (5 mM), and then DNA content was determined by flow cytometry. Our results showed that NAC could almost completely reverse the cell cycle arrest and apoptosis induced by T63 (Fig. 3A). Consistently, blocking the production of ROS by NAC resulted in a decrease in cleaved caspase-3 expression and increase in cyclin D1 expression (Fig. 3B). Furthermore, we tested the effects of the cell membrane-permeative analogues PEG-CAT and PEG-SOD on T63-induced cell cycle arrest and apoptosis. Cell cycle analysis showed that PEG-CAT
significantly reduced T63-induced cell apoptosis (sub-G1 group) (Fig. 3C). In contrast, PEG-SOD was unable to reverse T63-induced cytotoxicity and increased cell apoptosis when used alone or in combination with T63 (Fig. 3C). PEG-CAT, but not PEG-SOD, reversed the expression of cleaved caspase-3 and cyclin D1. Together, these data suggested that ROS (mainly H₂O₂) accumulation was critical in mediating T63-induced cell cycle arrest and apoptosis.

T63 enhances FOXO3a expression and transcriptional activity

As a stress-activated transcription factor, FOXO3a plays a very important role in cell cycle arrest and apoptosis in response to ROS [32,33]. Thus, we examined whether FOXO3a was functionally activated in A549 cells in response to T63. We first determined the expression characteristics of FOXO3a in T63-treated A549 cells. As shown in Fig. 4A, treatment with T63 for 12 h markedly increased the FOXO3a protein levels in a concentration-dependent manner. However, curcumin did not induce a significant change in FOXO3a protein expression at higher concentrations (10 µM). Real-time quantitative PCR also showed an increase in FOXO3a mRNA expression within 12 h of treatment with T63. The increase reached to fourfold at 2 µM (Fig. 4B). These data suggested that the expression regulation of FOXO3a by T63 occurred at the transcriptional and protein levels.

Then, we investigated whether the upregulation of FOXO3a was associated with an increased transcriptional activity. Indeed, we found that, together with the upregulation of FOXO3a, transcriptional targets p27, p21, and Bim were also upregulated by T63 treatment, but not by curcumin treatment (Fig. 4C). Treatment of A549 cells with T63 showed a dramatic increase in FOXO3a level as early as 8 h, and this expression level reached a maximum by 16 h, whereas the expression levels of p27, p21, and Bim were increased somewhat later (Fig. 4D). The upregulation of p27, p21, and Bim is indicative of the fact that FOXO3a activity is activated by T63. Furthermore, cotreatment of A549 cells with NAC blocked T63-induced FOXO3a, p27, p21, and Bim accumulation. It suggested that both FOXO3a expression and transcriptional activity induced by T63 were due to ROS accumulation.

To activate the downstream targets, FOXO3a has to reside in the nucleus. This suggests that T63 may also regulate the subcellular localization of FOXO3a. To test this, we used confocal microscopy to investigate the subcellular localization of FOXO3a in A549 cells in response to T63 treatment at various concentrations (0, 0.25, 0.5, 1, and 2 µM) at a time point (8 h) simultaneous with the induction of FOXO3a expression. As shown in Fig. 4E, FOXO3a is mainly located in the cytoplasm of untreated cells. However, there is a significant increase in FOXO3a nuclear location after treatment with T63. This is particularly true in the cases of high T63 concentrations (1, 2 µM); almost all FOXO3a...
was translocated into the nucleus. The change in FOXO3a localization was further confirmed by Western blot analysis of nuclear/cytoplasmic lysates. Consistent with the confocal microscopy findings, treatment with T63 resulted in a decrease in FOXO3a level in the cytoplasm and a parallel increase in FOXO3a in the nucleus (Fig. 4F).

Silencing of FOXO3a by siRNA results in a reduction in T63-induced cell cycle arrest and apoptosis

To show that FOXO3a has an essential role in response to T63 treatment, RNAi studies were carried out to knock down endogenous FOXO3a expression using FOXO3a-specific siRNA. Western blot analysis showed that FOXO3a-specific siRNA significantly inhibited the induction of p27, Bim, and cleaved caspase-3 by T63 treatment and also increased the expression of cyclin D1 (Fig. 5A). Consistent with the above results, cell cycle analysis showed that silencing of FOXO3a by siRNA can partially rescue cells from T63-induced cell cycle arrest and apoptosis. This is evident from the increase in proportion of cells in S and G2/M phase and decrease in proportion of cells in sub-G1 phase in siFOXO3a-transfected A549 cells compared with control siRNA-transfected cells (Fig. 5B). These results suggested that activation of FOXO3a by T63 was essential for T63-induced cell cycle arrest and apoptosis.

Inactivation of AKT is required for FOXO3a activation in response to T63

We next sought to identify the signal transduction pathways involved in activating FOXO3a in response to T63 treatment.
To this end, we used total and phospho-specific antibodies to monitor the expression levels and activation statuses, respectively, of key members of several signal transduction pathways. As shown in Fig. 6A, FOXO3a phosphorylation at the Thr 32 residue, a known AKT phosphorylation site [13], showed a peak after 1 h of treatment of T63. This effect was transitory, and then the phospho-FOXO3a levels declined, reaching levels lower than those of the untreated cells by 4 h. Consistently, the phosphorylation of AKT at Ser473, which reflects its activation status, was also transiently increased at 1 h, followed by a marked and persistent
reduction. These data indicated that inhibition of AKT activation by T63 could prevent FOXO3a phosphorylation (Thr32) and result in FOXO3a nuclear translocation and subsequent transcriptional activation of its target genes. ERK, another important oncogenic kinase, has been recently reported to regulate FOXO3a activity [15]. After treatment with T63, the expression of phospho-ERK1/2 was increased at 1 h, but rapidly decreased and stayed at a basal expression level throughout the time course (Fig. 6A). This indicated that ERK might not be essential for T63-induced FOXO3a activation.

$p38MAPK$ is involved in FOXO3a activation in response to T63

Previously, some studies showed that oxidative stress can activate FOXO via a JNK-dependent pathway [34,35]. Therefore, we monitored JNK activity in A549 cell treatment with T63. As shown in Fig. 6A, T63 did not affect phosphorylation levels of JNK at the concentration of 2 μM. However, the phosphorylation level of another stress-activated protein kinase, p38MAPK, was significantly increased at 2 h after T63 treatment and remained elevated throughout the time course (Fig. 6A). Thus, we further tested the role of constitutive activation of p38MAPK in the regulation of FOXO3a transcriptional activity. Inhibition of the p38MAPK pathway by SB203580 partially inhibited FOXO3a nuclear localization (Fig. 6B) and decreased the expression of p21, p27, and Bim induced by T63 (Fig. 6C). Similarly, inhibition of the AKT pathway by a specific inhibitor, LY294002, induced FOXO3a nuclear translocation, whereas SB203580 also partially abrogated this effect and decreased FOXO3a transcriptional activity (Figs. 6B and 6C). Activation of FOXO3a transcriptional activity by p38MAPK was not due to dephosphorylation of FOXO3a (Thr32), because the level of phospho-FOXO3a (Thr32) remained constant after treatment with T63 or LY294002 in the presence or absence of SB203580 (Fig. 6C). These data suggested that the effect of p38MAPK on FOXO3a transcriptional activity is independent of AKT signaling. Taken together, upon treatment of A549 cells with T63, both inhibition of AKT and activation of p38MAPK were involved in the regulation of FOXO3a activity.

ROS generation is upstream of p38MAPK and AKT

To further determine the relationship between ROS generation and p38MAPK activation or AKT inhibition in T63-induced cell cycle arrest and apoptosis, the effects of NAC or PEG-CAT were investigated. As shown in Fig. 7A, NAC could almost completely reverse the T63-induced p38MAPK phosphorylation or AKT dephosphorylation. Similarly, treatment with PEG-CAT could prevent p38MAPK phosphorylation and AKT dephosphorylation caused by T63 (Fig. 7B). However, the AKT inhibitor LY294002 induced only a very slight increase in H2O2 production when used alone or in combination with T63. Furthermore, the p38MAPK inhibitor SB203580 also showed no effect on T63-induced H2O2 generation (Fig. 7C). These results indicated that ROS production induced by T63 contributed to the activation of p38MAPK and the inhibition of AKT.

T63 inhibits tumor growth in vivo

To evaluate the role of T63 in tumor proliferation in vivo, we examined the ability of T63 to suppress the growth of A549 tumor xenografts in nude mice. Mice were treated with T63 every other day for 4 weeks. T63 significantly inhibited the growth of tumor xenografts (Fig. 8A). The excised tumors from the control group weighed between 1.5 and 1.9 g, whereas these from the 0.5 and 2 mg/kg body wt; 296.9 ± 74.6) and control group (692.5 ± 61.2; Fig. 8C).

T63 treatment significantly enhanced the expression of FOXO3a, p27, p21, and Bim in A549 cell tumors (Fig. 8D). In addition, we observed that p38MAPK was constitutively activated, whereas phosphorylation of AKT at Ser473 was drastically suppressed by T63 treatment. Immunohistochemistry staining of excised tumor sections also revealed a higher expression of cleaved caspase-3, but decreased expression of cyclin D1 in T63-treated tumors (Fig. 9A). Consistently, high levels of condensed apoptotic nuclei, an important apoptotic feature, were observed in tumors from T63-treated animals after H&E staining (Fig. 9B). Taken together, these data show that T63 has the ability to inhibit lung cancer growth in nude mice bearing A549 xenografts via proliferation suppression and apoptosis induction in tumor tissues.

To examine potential cytotoxic side effects of T63 on normal tissues, non-tumor-bearing mice were intraperitoneally treated with T63 (2 mg/ kg) every 2 days for 3 weeks. There was no significant loss in body weight in the animals (data not shown). H&E staining of the major organs collected at the end of the study also suggested no major organ-related toxicities (Fig. 10). Together, these results indicate that treatment with T63 potently suppresses tumor growth without affecting normal tissues in mice.
The ability of cancer cells to avoid apoptosis has been identified as one of the major mechanisms for development of cancers [36]. Induction of cell cycle arrest and cell apoptosis in cancer cells is a critical feature of chemotherapeutic agents. Curcumin has been shown to inhibit proliferation of various human tumor cells, including lung, colon, breast, myeloma, leukemia, and prostate carcinoma [21,37]. The chemotherapeutic action of curcumin might be due to its ability to induce apoptosis through several pathways [38]. However, curcumin has poor bioavailability [23]. It is therefore critical to develop structural analogues of curcumin that are effective at lower in vivo and in vitro concentration. We have previously revealed that T63, a new synthesized curcumin analogue, can inhibit cell proliferation [25]. In the present study, we have confirmed that the novel curcumin analogue T63 could significantly inhibit both in vitro and in vivo growth of lung cancer cells. In particular, at low doses, T63 was highly effective at inducing cell cycle arrest and apoptosis. However, consistent with previous reports [39], at a dose of 10 μM curcumin did not affect lung cancer cell growth.

Elevated levels of ROS in cancer cells play an important role in the initiation and progression of cancer. However, excessive ROS can also cause toxicity to cancer cells [7,40]. Recently, some studies showed that excessive ROS induced by exogenous agents cause oxidative mitochondrial damage and inactivation of redox-sensitive molecules and selectively kill cancer cells [41–44]. Using the Amplex red probe, we found that T63 induced a significant increase in ROS production, with evidence of a rapid and significant Amplex red oxidation upon T63 treatment. It is
thought that ROS are produced via multiple processes, such as the mitochondrial electron transport chain, NADPH oxidase, and xanthine oxidase [45]. By observing the effects of various inhibitors of ROS-generating systems, we demonstrate that the mitochondrial electron transport chain and NADPH oxidase are involved in ROS production by T63. Furthermore, our results showed, first, that NAC or PEG-CAT abrogates T63-induced production of ROS and significantly inhibits T63-induced cell cycle arrest and apoptosis; second, that NAC abolished the T63-induced expression of FOXO3a and its targets; and third, that NAC prevents AKT dephosphorylation and p38MAPK phosphorylation resulting from T63 treatment. Taken together, all these results suggest that induction of ROS is a critical upstream event for T63-induced cell cycle arrest and apoptosis.

Excessive levels of ROS induce cell cycle arrest and apoptosis by triggering the activation of multiple signaling pathways, including p38MAPK and JNK [46,47], or some other signaling proteins such as Nrf2 [48], p53 [49], and FOXO3a [50]. In this study, we showed that activation of FOXO3a by T63-induced ROS accumulation was essential for T63-induced cell cycle arrest and apoptosis. FOXO3a plays an important role in regulation of cell cycle and apoptosis [8–13]. FOXO3a can serve as a therapeutic target in various cancers by mediating the cytostatic and cytotoxic effects of various chemotherapeutic drugs. For example, the chemotherapeutic drugs paclitaxel [51], gefitinib [52], and triciribine [53], which are clinically used for the treatment of breast carcinoma and acute myeloid leukemia, can activate FOXO3a by reducing AKT activity. In this study, we found that T63 can activate FOXO3a in vitro and in vivo and subsequently increase the expression of p21, p27, and Bim. Inhibition of FOXO3a by siRNA blocked T63-induced upregulation of p21, p27, and Bim, and then increased cyclin D1 protein levels and decreased cleaved caspase–3 protein levels. Our data suggested that activation of FOXO3a by T63 is essential for T63-induced cell cycle arrest and apoptosis.

**Fig. 9.** T63 induces tumor cell cycle arrest and apoptosis in vivo. Tumor tissues derived from control and T63-treated mice were resected, fixed, sectioned, and placed on slides. (A) Tumor specimens were subjected to immunohistochemical staining with antibodies specific to cleaved caspase-3 and cyclin D1. (B) Tissue slides were used for H&E staining assays. More apoptotic condensed nuclei (Apo) were found in tumor tissue from mice treated with T63. Only a few apoptotic cells and many more nonapoptotic cells (Non-apo) were found in tumor tissue from mice treated with solvent.
apoptosis in lung cancer cells. Most notably, T63-induced expression of FOXO3a and its targets was fully blocked by the antioxidant NAC, suggesting that a ROS–FOXO3a pathway is a critical mediator in T63-induced cell cycle arrest and apoptosis.

The ability of intercellular ROS to trigger activation of FOXO3a has been reported in many systems [32,50,54]. Several signaling pathways have been implicated in regulating the activity of FOXO3a in response to oxidative stress [55]. For example, activation of JNK by ROS increased FOXO activity through several mechanisms, including direct phosphorylation of FOXO [35] or disruption of 14-3-3 binding [34] and, indirectly, by repressing PI3K activity [51]. In this study, we showed that treatment with T63 significantly increased p38MAPK phosphorylation levels. NAC or PEG-CAT could prevent p38MAPK phosphorylation. Moreover, inhibition of p38MAPK by SB203580 could antagonize T63-mediated FOXO3a nuclear translocation and transcriptional activity. The results suggested that p38MAPK was an important positive regulator of FOXO3a in response to T63-induced ROS accumulation. According to previous reports [56], it is likely that p38 phosphorylation of FOXO3a on Ser7 is essential for its nuclear localization and activation in response to elevated ROS levels. In addition, we found that activation of p38MAPK caused FOXO3a nuclear translocation and transcriptional activity, irrespective of FOXO3a phosphorylation (Thr32) status. Despite the fact that AKT phosphorylation is regarded as a marker for cytoplasmic-localized FOXO3a [57], some results showed a significant level of AKT-phosphorylated FOXO3a resides (Thr32) in the nucleus [58]. Moreover, our results also showed that T63-induced ROS accumulation (mainly H2O2) reduced FOXO3a phosphorylation (Thr32) levels by inhibition of AKT. This finding suggested that the relationship between T63-induced ROS and the effects on AKT activity differ from those of a previous report showing that ROS activate AKT [59,60]. Recently, some studies showed that a ROS inducer (or pretreatment with H2O2) could inhibit AKT phosphorylation, and quenching of ROS by NAC blocked the inhibitory effects of AKT signaling [61–64]. Furthermore, LY compounds (PI3K–AKT inhibitor) induced an increase in H2O2 production at doses that effectively blocked AKT phosphorylation, providing strong evidence linking inhibition of AKT with intracellular H2O2 production [65]. However, the mechanisms underlying the inhibition of AKT by ROS (or H2O2) are less clear, although recent studies have provided important insights into potential candidates, such as PTEN phosphorylated on Ser380/Thr382/383 by oxidative stress, leading to decreased phosphorylation of AKT through increased PIP3 production [66]. Further investigation is required to clarify the effects of these candidates on T63-induced ROS (mainly H2O2) inhibition of AKT activation.

In conclusion, our studies showed that T63 induced significant ROS generation, activated proapoptotic p38MAPK signaling, and inhibited prosurvival AKT signaling; subsequently elevated the expression of FOXO3a and its target genes; and then induced lung cancer cell cycle arrest and apoptosis. Moreover, our in vivo studies showed that treatment with T63 significantly suppressed the growth of A549 lung cancer xenograft tumors, associated with proliferation suppression and apoptosis induction in tumor tissues, without inducing any notable major organ-related toxicity. All these data provide evidence for the development of T63 as a potential chemotherapeutic agent for lung cancer and for further research and development.

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