Novel indolyl-chalcone derivatives inhibit A549 lung cancer cell growth through activating Nrf-2/HO-1 and inducing apoptosis in vitro and in vivo

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Increasing evidence indicates that Nrf-2, named the nuclear factor-erythroid 2-related factor, may perform anticancer function. In this study, a series of novel substituted phenyl- (3-methyl-1H-indol-2-yl)-prop-2-en-1-one (indolyl-chalcone) derivatives were synthesized and their effects on Nrf-2 activity were observed. We found that compounds 3a-3d and 6c elevated Nrf-2 activity. Then we evaluated their anticancer activities in vitro and in vivo by utilizing human lung cancer cell line A549. The in vitro results showed that among the compounds, 3d performed effectively anti-growth activity by inducing A549 lung cancer cell apoptosis and activating Nrf-2/HO-1 (heme oxygenase-1) pathway. In vivo, we proved that compound 3d inhibited the tumor growth effectively through inducing cell apoptosis without affecting CAM normal angiogenesis. These data suggest that our discovery of a novel Nrf-2 activator compound 3d would provide a new point of human lung cancer treatment.

Lung cancer is a considerable worldwide public health concern. Comparing with the survival rates of other cancers, 5-years survival rate of lung cancer is lower¹. Therefore, much more attention has been paid to the discovery of new anticancer drugs².

Cytotoxicity induced by xenobiotic can cause cell death³. It has been reported that cell death plays a crucial role in the progress of cancer. As we know, apoptosis and programmed necrosis are two major types of cell death which show different cell morphologies and pathways. Furthermore, as a new alternative target in tumor treatment, autophagy needs us to pay more attention⁴. Discovering new agents in anticancer therapy by inducing different cell death types is meaningful.

Chalcones, are diffusely existing in natural plant products. It has been reported that chalcones have many pharmacological and biological activities, such as anti-oxidative, anti-cancer, anti-mutagenic, anti-inflammatory, etc. The biological activities of chalcones maybe changed through the interaction with different compounds⁵. There are a lot of chalcone derivatives that have been synthesized and identified by researchers in the laboratory through different chemical methods⁶. Many reports have displayed that minor structural transformation of chalcones could induce considerable difference in the effect of anticancer, anti-inflammatory or autoimmune diseases⁷,⁸. For example, compound II, a novel dithiocarbamate–chalcone derivative could apparently inhibit the growth of SK-N-SH cells by triggering apoptosis and blocking the cell cycle⁹. Chalcones could prevent cancer by inhibiting p53 degradation⁹. In human breast cancer, Cathepsin-K contributes to tumor spread. Chalcones agents can suppress Cathepsin-K enzyme activity and effectively inhibit tumor invasiveness in body¹⁰. As we know that

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PI3K/Akt/mTOR pathway which modulates cell proliferation, metabolism, apoptosis, autophagy and other cellular biological activities is important in tumorigenesis13,14. A novel quinazoline chalcone derivative (QC) has been reported to inhibit PI3K/Akt/mTOR signaling pathway and trigger human HCT-116 cells apoptosis15. A pyrrole derivative of chalcone, (E)-3-phenyl-1-(2-pyrrolyl)-2-propenone (PPP) performs anti-inflammatory effect through inhibiting the activity of Syk, Src, and TAK114. It has been also reported that the dysfunction or abnormal proliferation of immune cells may cause autoimmune diseases, atherosclerosis, and tuberculosis15,16. Chalcones as immunomodulatory drugs show different effects on various immune cells, including triggering apoptosis in dendritic cells17, inhibiting superoxide anion production by weakening the activity of PKC in PMA-induced rat neutrophils18, performing anti-inflammatory activity in monocytes and macrophages19, suppressing rabbit platelets aggregation caused by arachidonic acid or collagen20,21, inhibiting the generation of functional cytotoxic T cells from mouse spleen and so on22.

Indole is another important chemical group in this series of compounds which we have synthesized. It has been known that indole as its heterocyclic system, it involves in the protein synthesis in the form of tryptophan23. Indole alkaloids with biological activity are plentiful in the nature, such as strychnine and lysergic acid diethylamide. Various indole alkaloids isolated from plants have been reported for some therapeutic value, including anticancer, the treatment of Hodgkin’s diseases23 and psychiatric disorders23, anti-inflammatory, cytotoxicity, antiviral23,24, being as antimicrobial agents25 and so on. Therefore, indole derivatives have attracted many researchers’ attention and a lot of indole derivatives have been synthesized or extracted from natural resources23.

It has been reported that chalcones or indoles have a lot of medicinal value in the treatment of diseases, and various chalcones or indole derivatives have been synthesized. However, to date, few indolyl-chalcone compounds have been reported. Novel indoly-chalcone derivatives (CITs) have been synthesized and identified to play a role in anti-cancer treatment through inducing cell death and inhibiting proliferation in PC3, A549, CLR2119 and PAN02 cells26,27. 1,α-Cyano bis (indolyl) chalcone inhibits the growth of A549 lung cancer cells effectively by promoting tubulin polymerization30.

As we know, Nrf-2 (nuclear factor-erythroid 2-related factor) encoded by NFE2L2 (nuclear factor, erythroid 2 like 2) gene belongs to the basic-leucine zipper (bZIP) family of transcription factors and is expressed in various tissues28. Nrf-2 maintains cellular redox balance by Kelch-like ECH-associated protein 1 (Keap1)-Nrf-2-antioxidant response element (ARE) pathway to make response to endogenous and exogenous stresses29. It has been reported that oxidative stress involves in the initiation of cancer, Nrf-2 might exert anticancer function and is implicated in chemoprevention. For example, as an Nrf-2 activator, the natural product sulforaphane (SFN) that presents in cruciferous vegetables has been researched in clinical trials of different cancers30. Moreover, a recent study shows that cisplatin as a well-known anticancer drug has been proved to induce oxidative damages and cell death in Hep-2 cells through improving the expression of Nrf-2 and HO-1. According to the current studies, intensifying Nrf-2 activity seems to be an attractive strategy in the process of cancer treatment.

Here, we synthesized a novel series of indolyl-chalcone derivatives and identified a new Nrf-2 activator named indolyl-chalcone derivative 3d, which dramatically inhibited tumor growth in vitro and in vivo by inducing A549 lung cancer cell apoptosis and activating Nrf-2/HO-1 pathway. Additionally, compound 3d did not show any significantly autophagy or necrosis in A549 lung cancer cells. Consequently, the discovery of the novel Nrf-2 activator would provide a new point of human lung cancer therapy.

Chemistry: synthesis of compounds
A series of indolyl-chalcone derivatives were designed and synthesized (Fig. 1A). Compound 2 was synthesized according to the reported method34. Compound 3 was synthesized from compound 2. Firstly, the mixture of compound 2 (1 mmol) and NaOH (2 mmol) in ethanol (10 ml) were stirred at room temperature, and then substituted aldehyde (1.2 mmol) in ethanol (5 ml) was added dropwise. The mixture was stirred for 2–4 h. The end of reaction was detected by TLC. Then the mixture was poured into cold water and filtered. The crude product was recrystallized from ethanol to obtain compound 3 in 60–90% yield. Compounds 4 and 5 were synthesized according to previous reported method35,36. Compound 6 was procured by the following reactions. The mixture of substituted acetophenone (1 mmol) and NaOH (2 mmol) in ethanol (10 ml) were stirred at room temperature, and then compound 5 (1.1 mmol) in ethanol (5 ml) was added dropwise. The mixture was stirred for 2–4 h. The end of reaction was detected by TLC. Then the mixture was poured into cold water and filtered. The crude product was recrystallized from ethanol to obtain compound 6 in 60–90% yield.

Results
Indolyl-chalcone derivatives (3a-3d, 6c) activate Nrf-2 significantly. As Nrf-2 activators have shown much incredible potential in disease prevention35, especially in cancer treatment, we firstly analyzed endogenous Nrf-2 activity in HeLa cells which were transfected with luciferase-based Nrf-2 reporter plasmid after treatment with a series of novel substituted phenyl-(3-methyl-1H-indol-2-yl)-prop-2-en-1-one, indolyl-chalcone derivatives (3a-3d, 6a-6c). The luciferase assay suggested that compounds 3a, 3b, 3c, 3d and 6c (10μM) elevated Nrf-2 activity significantly compared with the control after treatment for 12 h or 24 h (Fig. 1B).

Compounds 3c, 3d, 6a-6c inhibit the growth of A549 lung cancer cells at low IC50 values. In order to find out how these compounds influenced tumor cells growth as Nrf-2 activators, we selected A549 lung cancer cells for the following research. We firstly observed the morphological changes of A549 lung cancer cells after treatment with the compounds 3a-3d, 6a-6c for 24 h or 48 h by using a phase contrast microscope to investigate the anti-cancer activity of the compounds (Fig. 2A). There was no remarkable morphological change of A549 lung cancer cells treated with the compounds at the dose of 2.5 μM except for compounds 3d and 6c. Comparing with control group, the cell density reduced in response to the treatment of these compounds. Additionally, we observed that morphology of A549 lung cancer cells significantly shrunk, bleb protrusions formed in the cell...
membrane and apoptosis body released after treatment with compound 3d and 6c. Sulforhodamine B (SRB) assay suggested that compound 3d inhibited the growth of A549 lung cancer cells most efficiently (Fig. 2B, Table 1).

Apoptosis assay of compounds 3c, 3d, 6c in A549 lung cancer cells. To detect whether compounds 3c, 3d, 6c (2.5 μM) induced apoptosis of A549 lung cancer cells, we performed Hoechst 33258 staining assay. The data suggested that compound 3d (2.5 μM) could notably trigger apoptosis in A549 lung cancer cells through inducing chromatin condensation and nuclear fragmentation formation (Fig. 3A). We further detected the protein level of cleaved-PARP for researching the effect of compound 3d (2.5 μM, 5 μM) in A549 lung cancer cells. As a major member of Poly (ADP-ribose) polymerases (Parps) family, PARP plays a crucial role in modulating DNA repair and death of cells. Moreover, up-regulation of cleaved-PARP is one of the main characteristics of cell apoptosis. The results showed that the level of cleaved-PARP (89 KDa) increased after treatment with compound 3d (2.5 μM, 5 μM) (Fig. 3B). It indicated that compound 3d induced A549 cell apoptosis obviously. In the other hand, we investigated if these compounds could induce autophagy or necrosis in A549 lung cancer cells by performing acridine orange (AO) staining assay, western blot analysis (microtubule-associated protein1

Figure 1. Effects of compounds 3a-3d, 6a-6e on Nrf-2 activity. Chemical structures of compounds 3a-3d, 6a-6e (A). HeLa cells which contain Nrf-2-responsive/pGL4-3×ARE-basic luciferase reporter vector were treated with compounds 3a-3d, 6a-6e at 10 μM for 12 h or 24 h. The control group (Ctrl) was treated with 0.1% DMSO (V/V). Luciferase activity was determined by luciferase assay, and normalized to cell viability measured by SRB assay. Results are mean ± SEM (*p < 0.05, **p < 0.01 vs control. N = 3).
light chain 3 II, LC3-II) and LDH assay. The results demonstrated that compound 3d did not cause autophagy or necrosis in A549 lung cancer cells (data not shown).

Compound 3d increases oxidative stress to induce A549 lung cancer cell apoptosis. It has been reported that oxidative stress can alter the redox balance in tumor microenvironments and impact metabolic pathways in cancer cells39. Cancer cells are vulnerable to high levels of reactive oxygen species (ROS)40. Evidence indicates that ROS influences proliferation and apoptosis in various cancers41. The overproduction of ROS results in oxidative stress and induces cell apoptosis42. Therefore, we detected the level of ROS after treatment with compound 3d (2.5 μM) for 12 h and 24 h. The data demonstrated that 3d time-dependently increased the level of ROS (Fig. 4).

Compound 3d induces Nrf-2 nuclear translocation in A549 lung cancer cells. Based on our previous research results, we combined Nrf-2 activity with immunofluorescence analysis to investigate whether compound 3d could induce Nrf-2 nuclear translocation. The luciferase assay suggested that compound 3d elevated Nrf-2 activity significantly in dose dependent manner after treatment for 12 h or 24 h (Fig. 5A). In addition, immunofluorescence analysis suggested that compound 3d promoted Nrf-2 nuclear translocation conspicuously (Fig. 5B).

Compound 3d enhances the expression of HO-1 in A549 lung cancer cells. It has been reported that the oxidative stress could induce HO-1 gene transcription in tumor cells through activation of Nrf-243.

### Table 1. The IC_{50} values (48 h) of compounds 3a-3d, 6a-6e and 5-FU in A549 lung cancer cells.

| Compounds | 3a     | 3b     | 3c     | 3d     | 6a |
|-----------|--------|--------|--------|--------|----|
| IC_{50} (μM) | 35.78  | 49.35  | 14.03  | 2.46   | 13.56 |
| Compounds  | 6b     | 6c     | 6d     | 6e     | 5-FU |
| IC_{50} (μM) | 15.12  | 6.02   | 26.44  | 8.09   | 8.21 |

Figure 2. Effects of compounds 3a-3d, 6a-6e on morphology and viability of A549 lung cancer cells. A549 lung cancer cells were treated with compounds 3a-3d, 6a-6e (2.5μM) or 0.1% DMSO (control) for 24 h or 48 h (A). Microscopic photographs (200×) were taken by using the inverted phase contrast microscope (Nikon). Scale bar: 20 μm. A549 cells were treated with compounds 3a-3d, 6a-6e at 0.1, 1, 2.5, 5, 10 (μM) for 24 h or 48 h (B). The control group (Ctrl) was treated with 0.1% DMSO (V/V). Cell viability was analyzed by SRB assay. 5-FU was utilized as a positive drug control. Results are mean ± SEM (*p < 0.05, **p < 0.01 vs control. N = 3).
Therefore, we detected the mRNA level of HO-1 after treatment with compound 3d (10 μM) for 0.5, 1, 3, 6, 12, 24, 48 h. The data revealed that compound 3d elevated the expression of HO-1 remarkably in A549 lung cancer cells (Fig. 6).

**Compound 3d inhibits the tumor xenograft growth in the chick embryo chorioallantoic membrane (CAM) model.** We further investigated whether compound 3d could inhibit tumor growth effectively in vivo. The chick embryo chorioallantoic membrane (CAM) has been widely used in studying tissue grafts, tumor growth, angiogenic or toxicological analysis as the chick's immunocompetent system and the immune rejection are not fully developed. Therefore, we investigated the effect of 3d on tumor growth and normal angiogenesis by CAM model. 5-FU was used for positive control drug. After seeding A549 lung cancer cells on the CAM surface for 2 days, the tumor tissue masses formed locally. Then from the day 3 to 8, we treated the tumor tissue masses with PBS, PBS/3d or PBS/5-FU every 2 days. The results demonstrated that 3d significantly...
suppressed tumor growth (Fig. 7A). TUNEL assay was used to detect whether 3d could induce cell apoptosis in solid tumor. After the TUNEL staining and confocal microscopy analysis of frozen sections of tumors, we discovered that 3d could promote tumor apoptosis significantly in vivo (Fig. 7B). We further determined the effect of 3d on normal CAM angiogenesis. The data revealed that 3d had no negative effect on CAM normal angiogenesis (Fig. 7C). Therefore, in vivo, 3d could inhibit tumor growth effectively by inducing apoptosis without affecting CAM normal angiogenesis.

**Compound 3d does not induce cell cycle arrest of A549 lung cancer cells.** We also explored if compound 3d had any effects on cell cycle of A549 lung cancer cells. The flow cytometry analysis showed that after being treated with 3d (2.5 μM) for 48 h, the cell cycle of A549 lung cancer cells has not been arrested (Supplementary Figure 1).

### Discussion

Apoptosis or programmed cell death is a natural way of removing cells which are under the circumstance of pathology or aging from the body. There are a lot of anti-cancer therapies triggering apoptosis induction to kill malignant cells. However, long-term treatment with certain drugs might induce a decline of drug sensitivity in cancers which is caused by resistance45. In order to settle with therapy resistance in cancers, exploring new drugs to resist tumors is urgent. More importantly, for the purpose of promoting the development of new therapies for cancer or other human diseases, targeting apoptosis regulators is an attractive strategy46. Our data indicated that compound 3d inhibited the growth of A549 cells effectively through the way of causing apoptosis in vitro (Fig. 3) and in vivo (Fig. 7B).

In addition, compound 3d increased the level of ROS (Fig. 4) which is accompanied by Nrf-2 activation (Fig. 5A). As a redox sensitive transcription factor, Nrf-2 plays an important role in antioxidant defense and protects cells from oxidative stress injury47. It has been reported that HO-1 is one of the stress response genes which is regulated by Nrf-2 through consensus cis-elements called ARE48. Therefore, we next detected the mRNA level of HO-1 and the data suggested that 3d increased the expression of HO-1 (Fig. 6) in a time-depended manner.

We hypothesized that the apoptosis of A549 lung cancer cells induced by compound 3d may be due to its strong oxidative stress injury to cells, which lead to the activation and nucleus transportation of Nrf-2. In addition, the expression of antioxidant gene HO-1 increased prominently. However, the system of its own antioxidant stress system was difficult to resist compound 3d induced oxidative stress injury, and ultimately caused programmed cell death of A549 cells. This is the possible mechanism by which compound 3d induced A549 cells apoptosis. It has been reported that Nrf-2 is bound to Keap1 under normal conditions. While the balance of intracellular reactive oxygen species is broken and oxidative stress occurs, Nrf-2 is separated from Keap1 and transports to nucleus47. Therefore, in other situations, 3d may influence the interaction between Nrf-2 and Keap1 by suppressing Keap1. However, the exact mechanism of compound 3d inducing A549 cells apoptosis is necessary to be investigated in our next study.
Conclusion

After treatment with a series of novel substituted phenyl-(3-methyl-1H-indol-2-yl)-prop-2-en-1-one compounds, we observed a dose-dependent and time-dependent inhibition of growth in A549 lung cancer cells. Among the nine indolyl-chalcone derivatives, compound 3d inhibited the viability of A549 lung cancer cells obviously through inducing apoptosis and activating Nrf-2/HO-1 pathway. In vivo, 3d performed anti-growth activity of the tumor in an avian embryo model effectively and there was no negative effect on normal CAM.
Figure 6. Effects of compound 3d on the mRNA level of HO-1. A549 lung cancer cells were treated with compound 3d at 10μM for 0.5, 1, 3, 6, 12, 24, 48 h. The relative mRNA level of HO-1 was detected by quantitative reverse transcription–polymerase chain reaction (qRT-PCR). Results are mean ± SEM (*p < 0.05, **p < 0.01 vs control. N = 3).

Figure 7. Effects of 3d on the tumor xenograft growth and normal CAM angiogenesis in vivo. Images of control and treated tumors photographed by biomicroscopy (A). Tumor volume was quantified. The volume of 3d-treated tumor is smaller than control group. Bar, 1.5 mm, N = 5. TUNEL staining analysis of the frozen sections of tumors treated differently, and apoptotic rate was quantified (B). 3d (100μM, 200μM) induced tumors' apoptosis most obviously compared to the treatment of control (0.1% DMSO) and 5-FU (200μM). Bar, 5μm, N = 5. Angiogenesis on gelatin sponge with the treatment of 3d or DMSO (control) photographed by biomicroscopy and quantified (C). The results showed that 3d did not affect capillary formation. Bar, 1.5 mm, N = 5. Data are mean ± SEM (*p < 0.05, **p < 0.01 vs control. N = 5).
angiogenesis. It indicates that the novel indolyl-chalcone derivative 3d possesses great potential as an activator of Nrf-2 in cancer therapy.

**Materials and Methods**

**Ethics statement.** All experimental procedures and animal care in this work were performed in accordance with the ARRIVE guidelines 39 and approved by the ethics committee in Shandong University.

**Apparatus and chemicals.** $^1$H NMR (300 MHz or 400 MHz) and $^{13}$C NMR (75 MHz or 100 MHz) spectra were acquired on a Bruker Avance 300 spectrometer or Bruker Avance 400 spectrometer, with $d_6$-DMSO or $d_6$-Acetone used as a solvent and tetramethylsilane (TMS) as an internal standard. High resolution mass spectrometry (HRMS) involved a Q-TOF6510 spectograph (Agilent). Unless otherwise stated, all reagents were used without further purification from merchants. Twice-distilled water was used throughout all experiments.

**Cell culture.** Human lung cancer cell line A549 grew in RPMI-1640 medium (Gibco, 3180-022) containing with 10% (V/V) bovine calf serum. HeLa cells which were transfected with luciferase-based Nrf-2 reporter plasmid were grown in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, 12800-058) with 10% bovine calf serum. All cell lines were cultured at 37 °C in humidified air with 5% CO$_2$. Cells were seeded in 24 well plates or other appropriate dishes (30000 cells/ml).

**Cell viability assay (SRB).** A549 lung cancer cells were cultured onto 96 well plates as previously described. Next, treating cells with 0.1% DMSO or compounds 3a-3d, 6a-6c and 5-FU at 0.1, 1, 2.5, 5, 10μM for 24h or 48h. Cell viability was analyzed by Sulforhodamine B (SRB, Sigma-Aldrich, USA) assay according to the manufacturer’s instructions.

**Western blotting.** Cells were washed twice with PBS and lysed in 100μl protein lysis buffer (Shanghai Beyotime Co., China). All cell lysates were centrifuged at 12,000 × g for 15 min by using a refrigerated centrifuge. Then the protein concentrations were analyzed by using bicinchoninic acid (BCA) protein assay kit (Beyotime Co, China). After SDS-PAGE at 4 °C for 2h, transferred to PVDF membranes (Millipore, USA). At room temperature, the membrane was blocked with 5% non-fat milk in TBST (TBS containing 0.05% Tween 20) for 1h. Thereafter the membrane was incubated with anti-PARP (Cell Signaling, Beverly, MA, USA) antibodies overnight at 4 °C, washed 3 times with TBST for 5 min. Subsequently incubated with secondary antibodies which are HRP-conjugated for 1h at room temperature. The membrane was incubated with HRP substrate for 4 min after washed 3 times with TBST and the fluorescence signals were detected by using X-ray films. The protein was quantified using Image J software.

**Hoechst 33258 staining.** A549 lung cancer cells grew on 24 well were stained with 10mg/ml Hoechst 33258 and avoid the light for 30 min at 37 °C after treatment with 0.1% DMSO or compounds 3c, 3d, 6c for 24h and 48h. Cells were washed with PBS then photographed by using an Olympus (Japan) BH-2 fluorescence microscope.

**Measurement of intracellular ROS.** As previously described, A549 lung cancer cells grew on 24 well were washed with RPMI-1640 medium for 5 min and incubated with 10μM 2′, 7′-dichlorodihydrofluorescein (DCHF, Sigma-Aldrich) at 37 °C for 30 min. After washed 3 times with PBS, it was photographed by utilizing an Olympus (Japan) BH-2 fluorescence microscope.

**Luciferase assay.** HeLa cells which contain Nrf-2-responsive/pGL4-3 × ARE-basic luciferase reporter vector were seeded onto 96-well plates and cultured overnight, then incubated with indolyl-chalcones derivatives (3d) at the different concentrations (1, 2.5, 5, 10μM) and times (12h or 24h). Luciferase activity of cells was examined by using Luciferase Reporter Gene Assay Kit (Beyotime, China) and normalized to cell viability measured by Sulforhodamine B (SRB) assay.

**Immunofluorescence Assay.** Immunofluorescence assay was performed as described. In brief, A549 lung cancer cells were fixd with 4% paraformaldehyde for 15 min and blocked with 3% normal donkey serum (Solarbio, SLO50) for 20 min at room temperature. Then, the cells were incubated with primary antibody (1:100) (Nrf-2, Proteintech, America) at 4 °C overnight and then corresponding secondary antibody (1:200) at 37 °C for 1h. Cells were washed 3 times with 0.1 M phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, and 2 mM KH$_2$PO$_4$). Cells were incubated with DAPI for 10 min and washed 3 times with PBS, then photographed by using confocal fluorescence microscopy Zeiss LSM700 (Germany).

**Quantitative real-time PCR.** Extraction of total RNA use of Trizol reagent (Invitrogen, USA). The reverse transcription step involved use of the PrimeScript RT reagent kit with gDNA Eraser (DRR047, TAKARA). The relative mRNA level of HO-1 was quantified by SYBR Premix Ex Taq (Tli RNaseH Plus) RT-PCR reactions. The expression of β-actin was used to normalize with a melting curve for each reaction. Primers for β-actin were:

- sense: GAAGTGTGACGTGGACATCC; antisense: CCGATCCACACGGAGTACTT.
- sense: TGCACATCCGTGCAGAGAAT; antisense: CTGGGTTCTGCTTGCTTGTTTCGC.

**In vivo tumor assay of chick embryo chorioallantoic membrane (CAM).** The fertilized chicken eggs were incubated at 37 °C with 60% relative humidity. On embryonic day 8, a silicone ring with a 5.5 mm inner diameter was placed on the CAM, and then 8 million A549 lung cancer cells in 20μl of medium were seeded into this silicone ring. Eggs were divided into four groups in which 5 eggs were contained. On day 3, every egg was treated with 3d at the concentrations of 100 or 200μM every 2 days, 5-FU (200μM) was as the positive control.
group. After treatment with 3d or 5-FU for 6 days, samples of the CAM and tumors were taken. The size of tumors were measured and the tumor volume calculation was performed as described in literature.

**TUNEL assay.**  TUNEL assay was performed according to the manufacturer’s instructions (Promega, USA) to detect DNA fragmentation of the tumor tissues. Then the apoptosis was assessed by utilizing laser scanning confocal microscopy Zeiss LSM700 (Germany).

**Angiogenesis assay of CAM.**  The fertilized chicken eggs were incubated at 37 °C with 60% relative humidity. On embryonic day 9, the gelatin sponge absorbed compound 3d (100 and 200 μM) or DMSO was placed on the CAM. The treatment with 3d or DMSO was performed every 2 days. After 6 days, the CAM zones including the gelatin sponge were taken out. The biomicroscopy image and quantitative analysis were performed by Image-Pro Plus.

**Flow cytometric analysis.**  A549 cancer cells were treated with compound 3d (2.5 μM) or DMSO for 48 h, then gathered by centrifugation at 400 g, 4 °C for 5 min. Cells were fixed with 75% ethanol, then stained with 2 mg/ml propidium iodide (PI) containing 1 mg/ml RNase A at 4 °C for 30 min. The stained cells were analyzed by flow cytometry (Amnis ImageStream Mark II, USA).

**Statistical analysis.**  All data were presented as means ± SEM from at least three independent experiments and analyzed by SPSS (Statistical Package for the Social Sciences) software. When p value was < 0.05, differences were recognized as statistically remarkable.

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Author Contributions
J.Y.M. contributed to the application of scientific funding and designed the experiments. B.X.Z. and D.S.S. provided guidance for approaches to the synthesis of a series of indolyl-chalcone derivatives (3a-3d, 6a-6e). X.Z. performed the experiments, analysed the data, prepared figures, and wrote the main manuscript. W.L.D. synthesized a series of indolyl-chalcone derivatives (3a-3d, 6a-6e). Y.D.G. performed the experiments. L.S. helped perform the analysis with constructive discussions. F.J. and Q.Y. supplied HeLa cells which were transfected with luciferase-based Nrf-2 reporter plasmid. All authors reviewed the manuscript.

Additional Information
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