Quercetin Enhances the Antitumor Activity of Trichostatin A through Upregulation of p53 Protein Expression In Vitro and In Vivo

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Abstract
This study investigated the effects of quercetin on the anti-tumor effect of trichostatin A (TSA), a novel anticancer drug, in vitro and in vivo and the possible mechanisms of these effects in human lung cancer cells. We first showed that quercetin (5 µM) significantly increased the growth arrest and apoptosis in A549 cells (expressing wild-type p53) induced by 25 ng/mL of (122.5 µM) TSA at 48 h by about 25% and 101%, respectively. However, such enhancing effects of quercetin (5 µM) were not significant in TSA-exposed H1299 cells (a p53 null mutant) or were much lower than in A549 cells. In addition, quercetin significantly increased TSA-induced p53 expression in A549 cells. Transfection of p53 siRNA into A549 cells significantly but not completely diminished the enhancing effects of quercetin on TSA-induced apoptosis. Furthermore, we demonstrated that quercetin enhanced TSA-induced apoptosis through the mitochondrial pathway. Transfection of p53 siRNA abolished such enhancing effects of quercetin. However, quercetin increased the acetylation of histones H3 and H4 induced by TSA in A549 cells, even with p53 siRNA transfection as well as in H1299 cells. In a xenograft mouse model of lung cancer, quercetin enhanced the antitumor effect of TSA. Tumors from mice treated with TSA in combination with quercetin had higher p53 and apoptosis levels than did those from control and TSA-treated mice. These data indicate that regulation of the expression of p53 by quercetin plays an important role in enhancing TSA-induced apoptosis in A549 cells. However, p53-independent mechanisms may also contribute to the enhancing effect of quercetin.

Introduction
Trichostatin A (TSA) is a histone deacetylase inhibitor, which is a member of the promising class of anti-cancer drugs that selectively induce the differentiation and apoptosis of various transformed cells [1]. The accumulation of acetylated histones and nonhistone proteins is an important mechanism by which TSA affects the transcriptional patterns of many genes, including those associated with cell growth arrest and apoptosis [2,3]. Several studies have suggested that TSA may be a potential therapy for lung cancer [4–7] because it induces apoptosis of lung cancer cells by activating the death receptor and mitochondria-mediated pathways [4]. However, the toxicity of this drug, such as the cardiac hypertrophy effect, limits its application [8].

Several phytochemicals, such as quercetin, have been reported to prevent cancer development by themselves or by enhancing the effects of anti-cancer drugs [9–12]. Quercetin, a flavonoid, is ubiquitously found in various vegetarian foods, and research suggests it may act to prevent the development of cancers [13,14]. Quercetin may exert its anti-cancer effect through several mechanisms, including acting as an antioxidant, inducing apoptosis, acting as an anti-inflammatory agent, and modulating signaling pathways [15–18]. For example, a recent study showed that quercetin at a concentration of 80 µM induces mitochondria-mediated apoptosis in HeLa cells through the activation of p53 [17]. In addition, it has been demonstrated that quercetin significantly increases the anti-cancer effect of doxorubicin in breast cancer cells and reduces the cytotoxic side effects of doxorubicin in non-tumor cells [19]. Chen and Kang [20] found that quercetin (10–40 µM) in combination with TSA cooperatively induces cell death in human leukemia HL-60 cells. However, the combined effect of TSA and quercetin in human lung cancer cells is unclear. We hypothesized that quercetin would be an effective adjuvant to TSA treatment in lung cancer cells.

Thus, the aim of this study was to investigate the enhancing effects of quercetin on the antitumor effect of TSA in vitro and in vivo. In addition, we investigated the role of p53 in the enhancing effect of quercetin because it has been reported that quercetin exerts its antitumor effect through upregulation of p53 expression [21]. We used two human lung cancer cell lines, A549 and H1299, to address this issue. A549 cells express wild-type p53 protein, whereas H1299 cells are a p53 null mutant. Moreover, we used a xenograft mouse model to confirm the in vitro findings.
Materials and Methods

Ethics Statement
Animal care followed the guidelines of the National Research Council and all study protocols were approved by the Institutional Animal Care and Use Committee at Chung Shan Medical University.

Reagents
All chemicals used were reagent grade or higher. Quercetin and TSA were purchased from Sigma Chemical Co. (St. Louis, MO, USA). RPMI medium 1640, fetal bovine serum, trypsin, penicillin, streptomycin, sodium pyruvate, and nonessential amino acids were purchased from GIBCO/BRL (Rockville, MD, USA).

Cell Culture and Cell Growth Test
A549 cells and H1299 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Both cell lines were cultured in RPMI medium 1640 containing 10% (v/v) FBS, 0.37% (w/v) NaHCO₃, penicillin (100 units/mL), and streptomycin (100 μg/mL) at 37 °C in a humidified incubator under 5% CO₂ and 95% air. An equal number (2.5 × 10⁴/mL) of cells was incubated for 24 h before the various treatments. After being washed twice with PBS (pH 7.4, containing 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, and 8.1 mM Na₂HPO₄), the cells were incubated in fresh culture medium containing TSA (25 ng/mL equal to 82.5 nM) alone or in combination with quercetin. Stock solutions of ethanol-TSA (100 mg/mL) and ethanol-quercetin (20 mM) were freshly prepared before each experiment. The final solvent concentration in medium was <0.15%. The medium was replaced every day. Cell growth was mainly measured by MTT colorimetric assay.

Annexin V-FITC-propidium Iodide Assay
An Annexin V-FITC apoptosis detection kit (BD Pharmingen, San Diego, CA, USA) was used to determine the number of apoptotic cells. According to the manufacturer’s instructions, the treated cells were harvested after the indicated time, washed twice

Figure 1. Effects of trichostatin A (TSA) alone or in combination with quercetin on the growth (A) and apoptosis (B) of A549 cells and H1299 cells. The cells were incubated with TSA (25 ng/mL) alone or in combination with 2 or 5 μM quercetin (2Q and 5Q, respectively) for 24 h and 48 h. Values (means ± SD, n = 3) at the same time not sharing a common letter (a-c and A-C for 24 and 48 h, respectively) are significantly different (p<0.05). The growth of H1299 cells at 24 h among groups was not significantly different. # denotes a significant interaction between TSA and quercetin (two-way ANOVA, p<0.05). doi:10.1371/journal.pone.0054255.g001

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with ice-cold PBS and resuspended in 100 μL of binding buffer. Then an aqueous mixture of Annexin V-FITC and propidium iodide staining buffer was added and the mixture was incubated in the dark at 37°C for 15 min. Before flow cytometric analysis, 400 μL of binding buffer was added to each sample. A total of 100,000 events per sample were analyzed. Flow cytometric analysis was performed with a FACS Calibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) with WinMDI 2.8 software.

Western Blot Assay
The treated cells were harvested and lysed with 20% SDS containing 1 mM phenylmethyl sulfonyl fluoride. The lysate was sonicated for 1 min on ice followed by centrifugation at 12,000×g for 30 min at 4°C. Mitochondrial and cytosolic fractions were isolated by using the ProteoExtract® Cytosol/Mitochondria Fractionation Kit (Merck Millipore, Billerica, MA, USA). Then an amount of protein from the supernatant was resolved by SDS-PAGE and transferred onto a nitrocellulose membrane. After blocking with TBS buffer (20 mM Tris–HCl, 150 mM NaCl, pH 7.4) containing 5% nonfat milk, the membrane was incubated with antibodies against p53 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), Bax, Apaf-1, Bcl2 (Gene Tex, CA, USA), cytochrome c (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or acetyl histones H3/H4 (Upstate Biotechnology, Lake Placid, NY, USA) followed by horseradish peroxidase-conjugated secondary antibodies and then was visualized with an ECL chemiluminescence detection kit (PerkinElmer Life Sciences, Waltham, MA, USA). The relative density of the immunoreactive bands was quantified by using a luminescent image analyzer (LSA-100, Fujifilm, Japan).

Caspase-3 and Caspase-9 Activities
Caspase-3 and caspase-9 activities were measured by using colorimetric protease assay kits (Chemicon, Billerica, MA, USA) according to the manufacturer’s instructions. Protein concentrations of lysates were determined by the Lowry method [22]. An aliquot of cell lysates (70 μL) was incubated with the substrate of caspase-3 or caspase-9 at 37°C for 2 h. Samples were analyzed at 405 nm in a microplate reader (Molecular Devices, Sunnyvale, CA, USA).
CA, USA). The relative caspase activity of the control group was taken as 100.

Transfection of siRNA

A549 cells were transfected with 100 nM predesigned siRNA for p53 (NM_000546; sequence: forward, 5'-GACUCCAGUG-GUAUACCTT-3'; reverse, 5'-GUAGAUUACCACUGGA-GUCTT-3') by using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA, USA). After 24 h of incubation, a fresh medium containing test compounds was added for another 24 h. Non-targeted siRNA was used as a negative control.

Tumor Cell Xenograft Mouse Model

Thirty male nude mice (4 to 6 weeks of age) were obtained from the National Laboratory Animal Center (Taipei, Taiwan). The animals were housed in specific pathogen-free conditions with an alternating 12-hour light:dark cycle. After being acclimated for 1 week, the animals were subcutaneously injected in the right flank with A549 cells at a dose of 5 × 10⁶ cells (in 200 μL of matrigel; BD Biosciences, Franklin Lakes, NJ, USA). After 24 h of incubation, a fresh medium containing test compounds was added for another 24 h. Non-targeted siRNA was used as a negative control.

Figure 3. Effects of trichostatin A (TSA) alone or in combination with quercetin on Bax, Apaf-1 and Bcl-2 protein expression in A549 cells without or with p53-silencing (A); cytochrome c levels in cytosol and mitochondria in A549 cells without p53-silencing (B). The A549 cells were transfected with or without p53 siRNA before incubation with TSA (25 ng/mL) alone or in combination with 5 μM quercetin (5Q) for 18 h. Values (means ± SD, n = 3) among the groups without p53-silencing not sharing a common letter (a-c) are significantly different (p<0.05). Values in p53-silenced cells with asterisks (*) are significantly different from in p53-normal cells with the same treatment (p<0.05). doi:10.1371/journal.pone.0054255.g003

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access to a standard rodent diet (Lab 5001, Purina Mills, St. Louis, MO) and water during the study. During the 4-month experimental period, the body weights of the mice were recorded weekly. There were no significant differences in body weight among the groups in the study (data not shown).

Expression of p53 and Apoptosis in Tumor Tissue

After the mice were sacrificed, tumors were excised to determine p53 expression and apoptosis. Half of the tumor tissue from each mouse was stored in 10% formalin and the other half was stored at 2 \degree C. Then the tissues stored in formalin were embedded in paraffin, sectioned, and subjected to immunohistochemical staining for p53 expression using the streptavidin-peroxidase technique as described previously [25]. The frozen tumor tissues (0.03 g) were homogenized with 300 \mu L lysis buffer (10% Trion-100, 0.1% SDS, 0.5% sodium deoxycholate) containing 1 mM phenylmethyl sulfonyl fluoride and centrifuged at 12,000 \times g for 30 min at 4 \degree C. Then protein (250 \mu g) from the supernatant was used for p53 Western blot assay according the method described above. In addition, the levels of apoptosis of tumor tissue stored in formalin were detected using the Terminal Transferase dUTP Nick End Labeling (TUNEL) system following the manufacturer’s protocol (Chemicon, Billerica, MA, USA). The assay detects DNA fragmentation by terminal deoxynucleotidyl transferase. The enzyme catalyzes the addition of dUTPs that are secondarily labeled with a marker. The images of TUNEL and immunohistochemical staining were determined by microscopy at 6400 magnification.

Statistical Analysis

Values are expressed as means ± SD. We used one-way factorial analysis of variance followed by Duncan’s multiple range test for comparisons of group means or Student’s t test for two-group comparisons. A two-way ANOVA was performed to test the interaction between TSA and quercetin on cell growth, apoptosis, and the expression of p53 and acetyl histone in A549 cells. Differences were considered statistically significant at \( p < 0.05 \). In addition, we determined the combination index in apoptosis and the expression of p53 and acetyl histone using the method described by Mai et al. [10] to confirm the nature of the combined effect of TSA and quercetin. The combination index is calculated as the ratio of observed value/expected; a value >1 indicates a synergistic effect, while a ratio <1 indicates a less than additive or an antagonistic effect. Whereas, the expected value of combined treatment is calculated as [(observed TSA-treatment value/control value) + (observed quercetin-treatment value/control value)] × (control value).

Results

Quercetin has Different Effects on TSA-induced Cell Growth Arrest and Apoptosis in A549 Cells and in H1299 Cells

TSA significantly inhibited the growth of A549 cells (Fig. 1A), and quercetin significantly enhanced the cell-growth-arrest effect of TSA in a dose-dependent manner. Quercetin at a concentration of 5 \mu M significantly increased the suppressing effect of TSA by about 25% at both 24 h and 48 h. In H1299 cells, TSA alone slightly rather than significantly (\( p < 0.05 \)) induced cell growth arrest at 48 h, and the addition of quercetin only slightly increased the effect of TSA. Quercetin alone did not significantly affect cell growth at 24 and 48 h (Fig. 1A), however, it induced cell-growth-arrest at 72 h by about 15% (\( p < 0.05 \)) in both A549 and H1299 cells. Furthermore, we determined the enhancing effect of quercetin (5 \mu M) on TSA-induced apoptosis in both cell lines. Quercetin significantly and markedly increased TSA-induced apoptosis in A549 cells. The apoptosis level changed from 13% to 28% at 48 h. Even though quercetin also increased the TSA-induced apoptosis in H1299 cells, the apoptosis level only changed from 4% to 6% (Fig. 1B). The combined effect of TSA and
Quercetin on apoptosis at 48 h in both A549 and H1299 cells was synergistic (two-way ANOVA, \( p < 0.001 \) and \( p = 0.006 \), respectively; combination index = 2.0 and 1.6, respectively). Quercetin alone did not induce apoptosis even at 72 h (data not shown).

**Quercetin Increases p53 Protein Expression in A549 Cells**

A549 cells express wild-type p53 protein, whereas H1299 cells are a p53 null mutant. To investigate whether p53 plays a role in the different effects of quercetin in the different cell lines, we determined the expression of p53 in A549 cells exposed to TSA alone or in combination with 5 \( \mu \)M quercetin. As shown in Fig. 2A, after incubation for 12 h, TSA alone did not increase the expression of p53 protein; quercetin alone increased p53 expression by 48% compared with the control group; whereas TSA in combination with quercetin significantly increased p53 expression by 69%. The combined effect of TSA and quercetin was synergistic (two-way ANOVA, \( p < 0.001 \); combination index = 1.3). To confirm the role of p53 in the enhancing effect of quercetin on TSA-induced apoptosis, we blocked p53 protein expression by transfection of p53 siRNA into A549 cells. The results showed that p53 siRNA markedly reduced the protein expression of p53 in A549 cells (Fig. 2B). Furthermore, the enhancing effect of quercetin on TSA-induced apoptosis at 24 h (data not shown) and 48 h was significantly diminished by transfection with p53 siRNA (Fig. 2C). However, p53 silencing did not completely inhibit the enhancing effect of quercetin on TSA-induced apoptosis in A549 cells, suggesting that the p53-independent pathway also contributes to the combined effect.

**Quercetin Enhances TSA-induced Apoptosis through the Mitochondrial Pathway in A549 Cells**

To study the pathway contributing to the enhancing effect of quercetin on TSA-induced apoptosis in A549 cells, we performed a preliminary microarray assay after A549 cells were treated for 48 h. We found that compared to TSA alone, TSA in combination with quercetin increased the expression of several...
mitochondria-associated pro-apoptosis genes including Apaf-1 and caspase-9; whereas Bcl-2 expression in the groups with TSA alone or in combination with quercetin exposure were lower than that in the control group (data not shown). Further Western blot assay in cells with various treatments for 18 h confirmed this finding. As shown in Fig. 3A, TSA in combination with quercetin rather than TSA alone significantly increased the levels of Bax and Apaf-1 proteins compared with those in the control group. TSA alone and in combination with quercetin similarly suppressed the levels of Bcl-2 protein compared with the control group. TSA alone and in combination with quercetin similarly suppressed the levels of Bcl-2 protein compared with the control group. p53 siRNA transfection suppressed TSA + quercetin-induced Bax and Apaf-1 protein expression (Fig. 3A). The effect of p53 siRNA on TSA alone or TSA + quercetin-induced Bcl-2 expression was not significant. Furthermore, TSA in combination with quercetin rather than TSA alone markedly induced the release of cytochrome c into the cytosol after treatment for 24 h (Fig. 3B).

In addition, we used ELISA kits to determine the activities of caspase-9 and caspase-3 in A549 cells with various treatments for 24 h. Consistent with the above results, TSA slightly rather than significantly increased caspase-9 and caspase-3 activities, whereas quercetin significantly enhanced the TSA-induced activation of caspase-9 and caspase-3 by 27% and 113%, respectively (Fig. 4). Silencing p53 expression also suppressed the enhancing effect of quercetin on the TSA-induced activation of caspase-9 and caspase-3. These data indicated quercetin + TSA induced apoptosis at least in part through the mitochondria apoptosis pathway.

Quercetin Enhances TSA-induced Histone Acetylation

We investigated p53-independent pathways by which quercetin enhanced the effects of TSA since the p53-dependent pathway can not completely explain the combined effects on lung cancer cells. Because an increase in histone acetylation is one of the major mechanisms by which TSA induces apoptosis in various cancer cells [2,3], we determined the effect of quercetin on TSA-induced acetylation of histones. The results showed that TSA alone significantly increased the acetylation of histones H3 and H4 at 24 h and 48 h (Fig. 5). Quercetin significantly enhanced the TSA-induced acetylation of histones H3 and H4 by about 50%–250% at 24 h and 48 h (Fig. 5A). The enhancing effect of quercetin was synergistic (two-way ANOVA, p<0.05; the combination index = 1.4 and 3.0 for histone H3; 1.5 and 2.8 for histone H4 at 24 and 48 h, respectively). In addition, silencing p53 expression did not affect the enhancing effect of quercetin on TSA-induced histone acetylation (Fig. 5B). Furthermore, we found that the effects of TSA alone or in combination with quercetin on the expression of acetylated histones H3 and H4 in H1299 cells were similar to those in A549 cells, that is, quercetin also enhanced TSA-induced histones H3 and H4 acetylation in H1299 cells (Fig. 6). The combined effect was synergistic (two-way ANOVA, p<0.05; the combination index = 2.5 and 3.5 for histone H3; 1.4 and 2.7 for histone H4 at 24 and 48 h, respectively). These findings suggest that quercetin enhanced TSA-induced histone acetylation.
Acetylation by p53-independent mechanisms and this may contribute to the enhancing effect of quercetin on apoptosis.

Quercetin Enhances the Anticancer Effect of TSA in the Xenograft Tumor Model

To determine the potency of quercetin in enhancing the anticancer effect of TSA in vivo, we performed experiments with an A549 xenograft tumor model in nude mice. We found that a high dose (1 mg/kg body wt) of TSA was of borderline significance (t-test, p = 0.07) in inhibiting tumor growth in A549 tumor-bearing nude mice, whereas a low dose (0.5 mg/kg body wt) of TSA and quercetin alone had no effect (t-test, p > 0.05). However, treatment with the low dose of TSA in combination with quercetin (administered through intraperitoneal injection) significantly inhibited tumor growth in A549 tumor-bearing nude mice (t-test, p = 0.0003, Fig. 7A). The antitumor effect of combined treatment was better than or similar to that of the high dose of TSA. In addition, histological assessments and Western blot assay also indicated that quercetin increased low-dose TSA-induced p53 expression in tumor tissue (Fig. 7B and C). Similar to our in vitro study, quercetin slightly increased the expression of p53. The level of apoptosis in tumor tissue from the combined treatment group was higher than in tissue from the control group or the group treated with each single compound (Fig. 7D).

Discussion

Growing evidence suggests that the combination of phytochemicals with anticancer drugs may be a strategy for cancer therapy [10,26,27]. However, little has been reported about the combined effect of quercetin and TSA, except that quercetin in combination with TSA cooperatively induces cell death in human leukemia cells [20]. Similarly, in the present study, we first demonstrated that quercetin at a physiological dose (2 or 5 mM) enhanced TSA (25 ng/mL)-induced cell growth inhibition and apoptosis in A549 cells. Quercetin also increased the antitumor effect of TSA in H1299 cells at 48 h, however, the enhancing effect of quercetin on A549 cells (containing the wild type p53 gene) was markedly stronger than on H1299 cells (p53 null mutant). Compared to the TSA alone group, the increase in apoptosis induced by TSA in combination with quercetin seemed more marked than the decrease in cell growth (Fig. 1). This is due to apoptosis being expressed as the percentage of total cells. The percentage was low...
in the control and TSA alone group. Thus, the change induced by TSA+quercetin seemed marked. In fact, the increased numbers of apoptotic cells were lower than the decreased number in cell growth, suggesting that except for apoptosis, the inhibition of cell growth induced by TSA in combination with quercetin could also be due to the cell-growth-arrest effect or other modes of death.

The difference in sensitivity of A549 cells and H1299 cells to combined treatment suggests an association of p53 protein with the different effects of the combined treatment. The p53 protein, which is a known potent tumor suppressor, is maintained at low levels in unstressed cells [28]. In stimulated cells, p53 is increased and acts as a transcription factor to upregulate the expression of many genes, including cell-growth-arrest and apoptosis associated genes [29]. Several studies [17,21,30] have shown that quercetin itself suppresses cell growth and induces apoptosis in cancer cells. Consistent with these findings, in the present study, we found that quercetin significantly increased p53 expression in A549 cells whether they were exposed to TSA or not. However, TSA+ quercetin significantly induced apoptosis after 24 h while quercetin only induced cell-growth-arrest up to 72 h. This may be due to the differences in p53 expression levels, the qualitative status of p53, and other cellular contexts, which have been suggested to influence p53 to stimulate cell cycle arrest or apoptosis [31,32]. Furthermore, transfection of p53 siRNA into A549 cells markedly diminished the enhancing effect of quercetin on TSA-induced apoptosis, indicating that a p53-dependent pathway plays an important role in the enhancing effect of quercetin on TSA-induced apoptosis. The precise mechanisms by which quercetin increased p53 expression in the present study remain unclear. However, a study performed on melanoma cells showed that quercetin induces phase II detoxification enzymes, nicotinamide adenine dinucleotide phosphate:quinone oxidoreductase 1, which quercetin induces phase II detoxification enzymes, nicotinamide adenine dinucleotide phosphate:quinone oxidoreductase 1, which

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Furthermore, using A549 tumor-bearing nude mice, we demonstrated that quercetin administered through intraperitoneal injection also enhanced the anticancer effect of TSA (0.5 mg/kg body wt) in vivo. The efficiency of the combined treatment was better than or similar to that of the high dose of TSA (1 mg/kg body wt). Quercetin also increased TSA-induced p53 expression and apoptosis in tumor tissue, which suggests that p53 may also play an important role in the enhancing effect of quercetin in vivo. A recent study showed that 50 mg/kg of quercetin (given by intraperitoneal injection 3 times) enhanced the antitumor effect of cisplatin in combination with gemcitabine [39] in vitro and in vivo. The study indicated that the suppression of Hsp27 expression, which is induced by chemotherapy and enhances the chemoresistance of lung cancer cells, contributes to the enhancing effect of quercetin. These findings and ours suggest that quercetin has potential as an adjuvant to chemotherapy and may reduce the dose of anticancer drugs and thereby reduce the toxicity of chemotherapy [11].

It has been demonstrated that the anticancer effect of TSA in lung cancer cells is associated with the induction of p53 protein and both death receptor- and mitochondria-mediated apoptosis pathways [4,40]. Studies have shown that TSA at 100 nM upregulates the ratio of Bax/Bcl-2, the activity of caspase-9, and the release of cytochrome c, three major components of the mitochondrial apoptosis pathway. However, we found that TSA at 25 ng/mL (25.5 nM) did not affect p53 protein expression, the ratio of Bax/Bcl-2, caspase-9 activity and the release of cytochrome c. This lack of effect may have been due to the lower dose and short incubation time we used because in our preliminary study we found that 25 ng/mL of TSA alone did not increase the expression of p53 protein, which induces apoptosis typically followed mitochondria pathway [41], until 48 h of incubation (data not shown). Our data suggests that TSA at 25 ng/ml induced apoptosis in A549 firstly through death-receptor pathway (or others) rather than mitochondria pathway.

Except for the p53-dependent pathway, p53-independent pathways could also be involved in the mechanisms underlying the enhancing effect of quercetin in both A549 and H1299 cells. Although the precise mechanisms are unclear, our results showed that quercetin increased the TSA-induced acetylation of histones H3 and H4 in A549 cells with or without p53 expression as well as in H1299 cells, p53 null cells, suggesting that this pathway may contribute to the p53-independent mechanisms. Studies have shown that histone deacetylase inhibitors induce histone acetylation, which in turn lead to cell apoptosis by p53-independent mechanisms [42-44]. For example, histone acetylation of specificity protein 1 binding sites on the p21 promoter, an important cell cycle and apoptosis-associated gene [38,45], induces apoptosis; histone acetylation increases chromatin relaxation and enhances the accessibility of DNA to apoptotic endonucleases [46]. Our study also showed that TSA alone induced apoptosis of A549 cells at 24 h accompanied by an increase in acetylation of histones H3 and H4 rather than p53 protein expression. Thus, we postulate that histone acetylation may also account for the enhancing effect of quercetin on TSA-induced apoptosis in A549 cells. In agreement with our hypothesis, a recent study showed that histone hyperacetylation is involved in the human leukemia cell death induced by quercetin (75–100 μM) [47]. The p53-independent mechanisms associated with the effects of quercetin warrant further investigation.

In conclusion, our in vitro study demonstrated that quercetin enhanced the TSA-induced apoptosis in human lung cancer cells through p53-dependent and p53-independent pathways. We also confirmed the enhancing effect of quercetin in tumor-bearing
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Author Contributions
Conceived and designed the experiments: SLY. Performed the experiments: STC NCY CSH. Analyzed the data: STC JWL SLY. Contributed reagents/materials/analysis tools: NCV JW. Wrote the paper: STC SLY.

References
1. Henderson C, Mizuza M, Paroni G, Maestro R, Schneider C, et al. (2003) Role of caspases, Bid, and p53 in the apoptotic response triggered by histone deacetylase inhibitors trichostatin-A (TSA) and suberoylanilide hydroxamic acid (SAHA). J Biol Chem. 278: 12579–12589.

2. Dokmanovic M, Marks PA (2003) Prospects: histone deacetylase inhibitors. J Cell Biochem 96: 293–304.

3. Scu HO, Jin HO, Woo SH, Kim YS, An S, et al. (2011) Histone deacetylase inhibitors sensitize human non-small cell lung cancer cells to ionizing radiation through acetyl p53-mediated c-myc down-regulation. J Thorac Oncol 6: 1313–1319.

4. Kim HR, Kim EJ, Yang SH, Jeong ET, Park C, et al. (2006) Trichostatin A induces apoptosis in lung cancer cells via simultaneous activation of the death receptor and mitochondrial pathway. Exp Mol Med 38: 616–624.

5. Platta CS, Greenblatt DY, Kunnimalaiyaan M, Chen H (2007) The HDAC inhibitor trichostatin A inhibits growth of small cell lung cancer cells. J Surg Res 142: 219–226.

6. Chang J, Yargoe DS, Gillan MC, Peryon M, Modi B, et al. (2012) Differential response of cancer cells to HDAC inhibitors trichostatin A and depudeptide. Br J Cancer 106: 116–123.

7. Wang X, Li G, Wang A, Zhang Z, Merch R, et al. (2011) Combined histone deacetylase and cyclooxygenase inhibition achieves enhanced antiangiogenic effects in lung cancer cells. Mol Carcinog [Epub ahead of print].

8. Karagiani TC, Lin AJ, Vereris K, Chang I, Tang M, et al. (2010) Trichostatin A accentuates doxorubicin-induced hypertrophy in cardiac myocytes. Aging (Albany NY) 2: 659–668.

9. Gantmayer M, Ocker M, Zopp S, Leitner S, Hahn EG, et al. (2004) A quadruple therapy synergistically blocks proliferation and promotes apoptosis of hematopoietic cells. Oncol Rep 11: 943–950.

10. Mai Z, Blackburn GL, Zhou JR (2007) Soy phytochemicals synergistically enhance the preventive effect of tamoxifen on the growth of estrogen-dependent human breast cancer in mice. Carcinogenesis 28: 1217–1223.

11. Samuel T, Faddalla K, Mosley L, Katoori V, Turner T, et al. (2012) Dual-mode interaction between quercetin and DNA-damaging drugs in cancer cells. Anticancer Res 32: 61–71.

12. Shu L, Khor TO, Lee JH, Boyanapalli SS, Huang Y, et al. (2011) Epigenetic CpG demethylation of the promoter and reactivation of the expression of Neurogl by curcumin in prostate LNCaP cells, AAPS J 13: 156–159.

13. Lasonow DW, Brugil MS (2008) Antioxidants and cancer: part 5. Altern Med Rev 13: 196–208.

14. Xavier CP, Lima CF, Rohde M, Pereira-Wilson C (2011) Quercetin enhances 5-fluorouracil-induced apoptosis in MIB cell colorectal cancer cells through p53 modulation. Cancer Chemother Pharmacol 68: 143–157.

15. Pilal M (1996) Retinoid related molecules: new promises against lung and breast cancer. Acta Biomed 77: 118–123.

16. Divis D, Di Tommaso S, Salvenini S, Marramone G, Crisci R (2006) Diet and tissue distribution of quercetin in rats and pigs. J Nutr Biochem 17: 395–398.

17. Vidya Priyadarsini R, Senthil Murugan R, Maitreyi S, Ramalingam K, et al. (2010) Histone deacetylase inhibitor, is associated with inhibition of cyclooxygenase-2 activity in human non-small cell lung cancer cells. Int J Oncol 27: 473–479.

18. Ashcroft M, Vonsden KH (1999) Regulation of p53 stability. Oncogene 18: 7637–43.

19. Ross WP, Kaina B (2012) DNA damage-induced apoptosis: from specific DNA lesions to the DNA damage response and apoptosis. Cancer Letters 2012 [Epub ahead of print].

20. Chou CC, Yang JS, Lu HF, Ip SW, Lo C, et al. (2010) Quercetin-mediated cell cycle arrest and apoptosis involving activation of a caspase cascade through the mitochondrial pathway in human breast cancer MCF-7 cells. Arch Pharm Res 33: 1101–1119.

21. Haupt S, Berger M, Goldberg Z, Haupt Y (2003) Apoptosis - the p53 network. J Cell Sci 116: 4077–4085.

22. Zuckerman W, Wolnykic N, Sionov RV, Haupt S, Haupt Y (2009) Tumor suppression by p53: the importance of apoptosis and cellular senescence. J Pathol 219: 3–15.

23. Nyakas CD, Rittmaster R, Sridharan T, Gill A, et al. (2010) Protein suppression by p53: the importance of apoptosis and cellular senescence. J Pathol 219: 3–15.

24. Chen J, Kang JH (2005) Quercetin and trichostatin A cooperatively kill human myocytes. Aging (Albany NY) 2: 659–668.

25. Kaplowitz N, Pyron RC, Chinsinh C, et al. (2003) Role of NADPH oxidase in the activation of caspase-9 in p53-dependent apoptosis and tumor inhibition. Science 284: 156–159.

26. Merker M, Ghosh S, Remon J, Michel BB, et al. (1998) Histone deacetylase inhibitors trichostatin-A (TSA) and suberoylanilide hydroxamic acid (SAHA). J Biol Chem. 273: 12579–12589.

27. Chiu RH, Davisson JR, Keefe DL, et al. (2003) Histone deacetylase inhibitors sensitize human non-small cell lung cancer cells to ionizing radiation through acetyl p53-mediated c-myc down-regulation. J Thorac Oncol 6: 1313–1319.

28. Chen J, Kang JH (2005) Quercetin and trichostatin A cooperatively kill human myocytes. Aging (Albany NY) 2: 659–668.

29. Falso M, Pernollet JC, Pando E, et al. (2007) Inhibition of caspase-9 and caspase-7 by quercetin. FEBS Lett 581: 863–867.

30. Zuckerman W, Wolnykic N, Sionov RV, Haupt S, Haupt Y (2009) Tumor suppression by p53: the importance of apoptosis and cellular senescence. J Pathol 219: 3–15.

31. Haupt S, Berger M, Goldberg Z, Haupt Y (2003) Apoptosis - the p53 network. J Cell Sci 116: 4077–4085.

32. Zuckerman W, Wolnykic N, Sionov RV, Haupt S, Haupt Y (2009) Tumor suppression by p53: the importance of apoptosis and cellular senescence. J Pathol 219: 3–15.

33. Thangamani T, Sittapathy S, Lanza-Jacobs S, Wachshaber PR, Limesand KH, et al. (2007) Quercetin selectively inhibits bioreduction and enhances apoptosis in melanoma cells that overexpress tyrosinase. Nutr Cancer 59: 258–68.

34. Beniston RG, Morgan IM, O’Brien V, Campo MS (2001) Quercetin, E7 and p53 in papillomavirus oncoprotein cell transformation. Carcinogenesis 22: 1069–76.

35. Reed JC, Jurcenkiemc JM, Matuyama S (1998) Bcl-2 family proteins and mitochondrial. Biochim Biophys Acta 1366: 127–137.

36. de Boer VC, Dihal AA, van der Woude H, Arts IC, Wolffram S, et al (2005) Histone deacetylase inhibitor MS-275 and the CDK-inhibitor CYC-202 inhibit cell cycle arrest and apoptosis involving activation of a caspase cascade through the mitochondrial pathway in human breast cancer MCF-7 cells. Arch Pharm Res 33: 1101–1119.

37. Haupt S, Berger M, Goldberg Z, Haupt Y (2003) Apoptosis - the p53 network. J Cell Sci 116: 4077–4085.

38. Miyahita T, Reed JC (1995) Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell 20: 293–299.

39. Bouvard V, Zaitchouk T, Vacher M, Canivet M, et al. (2000) Tissue and cell-specific expression of the p53-target gene: bax, fas, mdm2 and p21, before and following ionizing irradiation in mice. Oncogene 19: 649–660.

40. Choi YJ, Lin JH, Huang WS, Hou TW, Su K, et al. (2011) Chemoresistance of lung cancer stemlike cells depends on activation of Hsp27. Cancer 117: 1516–1528.

41. Haupt S, Berger M, Goldberg Z, Haupt Y (2003) Apoptosis - the p53 network. J Cell Sci 116: 4077–4085.