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# POTENTIAL ANTICANCER ACTIVITY OF CURCUMIN ANALOGS CONTAINING SULFONE ON HUMAN CANCER CELLS

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**Abstract**: Three curcumin analogs (S1-S3) containing sulfone were investigated for their effects on human prostate cancer PC-3, colon cancer HT-29, lung cancer H1299 and pancreatic cancer BxPC-3 cells. The three compounds were approximately 16- to 96-fold more active than curcumin in these cell lines as determined by the MTT assay. The effects of these compounds on cell growth were further studied in prostate cancer PC-3 cells in both two dimensional (2D) and three dimensional (3D) cultures. S1-S3 strongly inhibited the growth and induced cell death in PC-3 cells, and the effects of these compounds were associated with suppression of nuclear factor kappa B (NF-κB) transcriptional activity. Moreover, treatment of PC-3 cells with all three compounds caused a decrease in the level of phosphorylated signal transducer and activator of transcription-3 (p-STAT3) (Tyr705), but not p-STAT3 (Ser727). Only S1 and S2 decreased the presence of phosphorylated Akt (p-Akt) in PC-3 cells. These curcumin analogs warrant further *in vivo* studies for anticancer activities in suitable animal models.

**Key words:** anticancer; curcumin analogs; NF-κB; sulfone; 3D cell culture

## INTRODUCTION

Numerous studies have shown that curcumin, a yellow natural compound, which is isolated from the rhizomes of the plant *Curcuma longa* (Mehta et al., 2014), can be used for preventing and treating various human diseases because of its multiple biological properties, including antioxidant (Ruby et al., 1995; Trujillo et al., 2013), anti-inflammatory (Ramsewak et al., 2000; Aggarwal et al., 2013), antiviral, antibacterial, antifungal and antitumor activities (Moghadamtousi et al., 2014; Prasad et al., 2014; Aggarwal et al., 2003). The effectiveness and safety of curcumin have been proven, but bioavailability of curcumin remains a major concern, as a relative high concentration of curcumin is needed to achieve its antitumor effects (Chen et al., 2012; Tuorkey, 2014; Li et al., 2014). The weakness of the pharmacokinetic profile of curcumin *in vivo* significantly inhibits its clinical application (Chuah et al., 2014). Much effort has been devoted to developing useful curcumin analogs to not only circumvent curcumin's low bioavailability but also to enhance its pharmacological capability. Various synthetic curcumin derivatives and curcumin analogs had been designed and evaluated for their biological activities (Wei et al., 2012; Zhou et al., 2013; Zhang et al., 2014).

Curcumin analogs containing sulfone have previously been studied (Rovnyak et al., 1982), but the pharmacological activity of this kind of curcumin analogs has received little attention. Until recently, Go et al. reported that curcumin analogs of this kind can strongly influence the activation of ER stress signaling pathways and apoptotic cell death in acute promyelocytic leukemic cells (Tan et al., 2014). Our study is

the first report investigating the effects of three curcumin analogs containing sulfone including (3Z,5Z)-3,5-bis-(4-methoxybenzylidene)-dihydro-2H-thiopyran-4-(3H)-one-1,1-di-oxide (S1) (Rovnyak et al., 1982), (3Z,5Z)-3,5-bis(3,4-dimethoxy-benzylidene) dihydro-2H-thiopyran-4(3H)-one-1,1-dioxide (S2) and (3Z,5Z)-3,5-bis-(3,4,5-trimethoxybenzyli-dene)-dihydro-2H-thiopyran-4(3H)-one-1,1-dioxide (S3) (Tan et al., 2014) (Fig. 1) against several cancer cell lines including prostate cancer PC-3 cells, colon cancer HT-29 cells, pancreatic cancer BxPC-3 cells and lung cancer H1299 cells.

We also employed a three dimensional (3D) culture model to observe the morphology and effects of curcumin and curcumin analogs on prostate cancer PC-3 cells. To further investigate the mechanism of these compounds, we determined the effect of these compounds on the activity of nuclear factor kappa B (NF-κB) in PC-3 cells using a luciferase reporter assay, as well as the levels of phosphorylated protein kinase B (p-Akt), phosphorylated signal transducer and activator of transcription-3 (STAT3) (Tyr705 and Ser727) by Western blotting analysis.

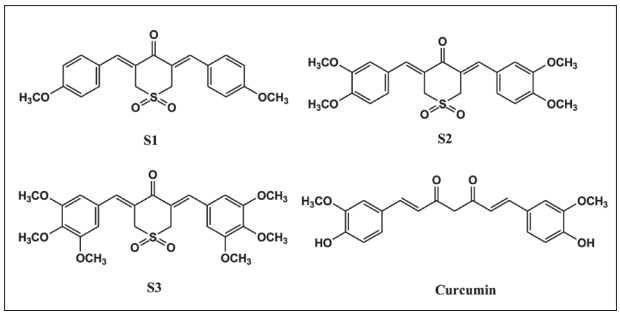



**Fig. 1.** Structure of curcumin and curcumin analogs containing sulfone.

## MATERIALS AND METHODS

### Cell culture and reagents

Human cancer PC-3, colon cancer HT-29, lung cancer H1299 and pancreatic cancer BxPC-3 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cell lines were all maintained in RPMI-1640 medium (Gibco, USA), which was supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) and penicillin (100 units/mL)-streptomycin (100 μg/mL). The cells were incubated at 37°C in a humidified atmosphere containing 5% $CO_2$. Curcumin and curcumin analogs were dissolved in DMSO, and the final concentration of DMSO in all experiments was 0.1%. Primary antibodies included Actin (C-2: sc-8432, Santa Cruz Biotechnology), p-Akt antibody (Cat. #9271, Cell Signaling), anti-phospho-STAT3 (Tyr705) clone EP2147Y, rabbit monoclonal (Cat. #04-1059, Millipore) and phospho-STAT3 (Ser727) antibodies (Cat. #9134, Cell Signaling). Secondary antibodies, all purchased from Santa Cruz Biotechnology, included goat anti-mouse IgG-HRP sc-2055 and goat anti-rabbit IgG-HRP sc-2004.

### MTT assay

PC-3, HT-29, H1299 and BxPC-3 human cancer cells were seeded at a density of $2.5\times10^4$ cells/mL (200 μL/well) in 96-well microplates and incubated for 24 h at 37°C. The cells were treated with various concentrations of different compounds. After 72 h of incubation, the medium was removed, and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) solution (5.5 mg/mL, 100 μL/well) was added to the wells. After 4 h of incubation, the MTT solution was removed and 100 μL of DMSO was added to each well. Plates were then swirled gently to facilitate formazan crystal solubilization. Finally, the absorbance was measured at 570 nm using a microplate reader (Tecan Infinite M200 Pro, Switzerland).

### Trypan blue assay

PC-3 cells were seeded at a density of $2.5\times10^4$ cells/mL in 35-mm tissue culture dishes (2.0 mL/dish) and incubated for 24 h. The cells were treated with curcumin or curcumin analogs for 72 h. Numbers of viable and dead cells were determined by trypan blue exclusion assay. In brief, 40 μL of cell suspension was mixed with 10 μL of 0.4% trypan blue solution for 2 min. Blue cells were counted as dead cells and the cells that did

not absorb dye were counted as live cells. The cells, including dead and alive cells, were then examined under a light microscope (Nikon Optiphot, Japan).

**Three dimensional (3D) cell culture**

PC-3 cells were mixed with Matrigel (Collaborative Research, Bedford, MA) on ice at a density of $0.5\times10^5$ cells/mL The Matrigel containing PC-3 cells was then placed in a 12-well plate (1.0 mL/well) and incubated at 37°C for 2 h to let the Matrigel solidify. Afterward, RPMI-1640 medium was added to each well on top of the gel. The cells were incubated for 24 h and then treated with curcumin or curcumin analogs once every other day. At day 10, the 3D cultures were examined under a microscope (Nikon Eclipse TE200, Japan) for the formation of tissue-like structures.

**NF-κB dependent reporter gene expression assay**

The NF-κB-luciferase construct (Lenti NF-κB Reporter, Qiagen, Valencia, CA) was transfected into PC-3 cells by Lipofectamine™ 2000 (Invitrogen Life Tech, Grand Island, NY) following the manufacturer's instructions, and a single stable clone, PC-3/N, was used in the present study. The cells were seeded at a density of $2.5\times10^4$ cells/mL of medium in 12-well plates (1.0 mL/well) and incubated for 24 h. The cells were then treated with the different concentrations of curcumin or curcumin analogs for 24 h, and the NF-κB luciferase activities were determined by luciferase reporter assay kits (E1500, Promega Madsion WI) according the manufacturer's instructions. The protein concentrations of cell lysates were determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The NF-κB transcriptional activities were normalized against protein concentrations and expressed as the percentage of luciferase activity relative to the control cells.

**Western blotting**

PC-3 cells were seeded at a density of $1.0\times10^5$ cells/mL in a 100-mm dish (10.0 mL/dish) and incubated at 37°C for 24 h. The cells were treated with curcumin or curcumin analogs for 24 h. Thereafter, cells were washed with ice-cold PBS and lysed in 200 μL of lysis buffer (10 mM Tris-HCl, pH 7.4, 50 mM sodium chloride, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 100 μM sodium orthovanadate, 2 mM iodoacetic acid, 5 mM zinc chloride, 1 mM phenylmethylsulfonyl fluoride and 0.5% Triton X-100). The lysates were centrifuged at 13000×g for 15 min at 4°C. The protein concentration of whole cell lysates was determined with a Bio-Rad protein assay kit (Bio-Rad).

Equal amounts (22.5 μg) of protein were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membrane. The membrane was washed three times with Tris-buffered saline (TBS) for 10 min and once with TBS containing 0.05% Tween 20 (TBST) for 10 min. The membrane was incubated in blocking buffer for 1 h. Then the membrane was cut and separately incubated at 4°C overnight with specific primary antibody against actin (C-2), p-Akt and p-STAT3 (Tyr705 and Ser727) (all primary antibodies were diluted 1:1000). Following removal of the primary antibody, the membranes were washed three times with TBS and once with TBST at room temperature. The membranes were subjected to fluorochrome-conjugated secondary antibody at a dilution of 1:5000 for 1 h at room temperature without light. Membranes were washed four times with TBST. Immunoreactivity was detected using SuperSignal West Femto Luminol/Enhancer solution (Thermo Scientific) and analyzed by Quantity One software (Bio-Rad). Relative quantification of protein markers compared to actin was analyzed using Image Studio Lite Ver 4.0 software.

**Statistical analysis**

Data were expressed as means±SEM. Student's t-test and one-way ANOVA were used to determine the statistical significance of differences between the test samples and controls. A p value $<0.05$ was considered statistically significant.

## RESULTS

### Effects of curcumin and curcumin analogs on different human cancer cell lines

The effects of curcumin and its analogs (S1-S3) on the growth of PC-3, HT-29, H1299 and BxPC-3 cells were determined using MTT assay. The resulting $IC_{50}$ values are presented in Table. 1. The $IC_{50}$ values of S1-S3 ranged from 0.72 μM to 1.73 μM on PC-3 cells, 0.19 μM to 0.38 μM on HT-29 cells, 0.46 μM to 1.24 μM on H1299 cells and 0.29 μM to 1.01 μM on BxPC-3 cells. The results showed that S1-S3 were approximately 16- to 96-fold more active than curcumin. Both inhibition of proliferation and cytotoxicity can result in a decrease in the MTT assay. Therefore, we assessed the number of viable cells and dead cells using the trypan blue exclusion assay. Since our laboratory has an experimental system for the prostate cancer PC-3 cells, we used this cell line for further studies.

**Table 1**. Inhibitory effect of curcumin and curcumin analogs on the growth of different cells.

| Compounds | $IC_{50}$ (μM) | | | |
|---|---|---|---|---|
| | HT-29 | H1299 | PC-3 | BxPC-3 |
| Curcumin | 18.39±0.35 | 19.87±0.94 | 21.64±1.83 | 18.25±1.27 |
| S1 | 0.19±0.14** | 1.24±0.08** | 1.73±0.26** | 1.01±0.11** |
| S2 | 0.38±0.15** | 0.58±0.04** | 0.85±0.10** | 0.32±0.08** |
| S3 | 0.29±0.09** | 0.46±0.01** | 0.72±0.17** | 0.29±0.09** |

HT-29, H1299, PC-3 and BxPC-3 cells were seeded at a density of $2.5\times10^4$ cells/mL in 96-well plates and incubated 24 h, respectively, after 72 h with various concentrations of test samples. MTT solution (100 μL, 5 mg/mL) was added to the wells and further incubated for 4 h at 37°C. Thereafter, the medium was removed and DMSO was added. Measured with microplate reader at a wavelength of 570 nm. Data are shown as means±SEM of three independent experiments. Significantly different from the curcumin *p<0.05, **p<0.01.

In experiments using the trypan blue exclusion assays, we found that treatment of PC-3 cells with S1-S3 decreased the number of viable cells in a concentration-dependent manner (Fig. 2A). Treatment of PC-3 cells with the compounds also resulted in a concentration-dependent increase in the number of dead cells (Fig. 2B). All compounds were much more active than curcumin, and S3 showed the strongest activity.

### Effects of curcumin and curcumin analogs on PC-3 cells in 2D and 3D cultures

The 3D cell culture model was used to determine the effects of curcumin and curcumin analogs on the growth and formation of 3D structures of PC-3 cells. The morphology of PC-3 cells treated with curcumin or curcumin analogs in the 3D culture system were examined and compared with conventional 2D monolayer culture. As shown in Fig. 3, S1-S3 showed strong inhibitory effects at a concentration of 1.0 μM (Fig. 3C, E and G) and 2.5 μM (Fig. 3D, F and H) in 2D culture, compared to control PC-3 cells or treatment with curcumin (Fig. 3A, B). As for the 3D culture, control PC-3 cells or treatment with curcumin formed a tissue-like morphology in extra cellular matrix gel (Fig. 4A, B), while treatment with S1-S3 at a concentration of 2.5 μM showed inhibitory effects on the growth of tissue-like structures (Fig. 4D, F and H). However, these compounds had no obvious effects at the concentration of 1.0 μM except for S3 (Fig. 4C, E and G).

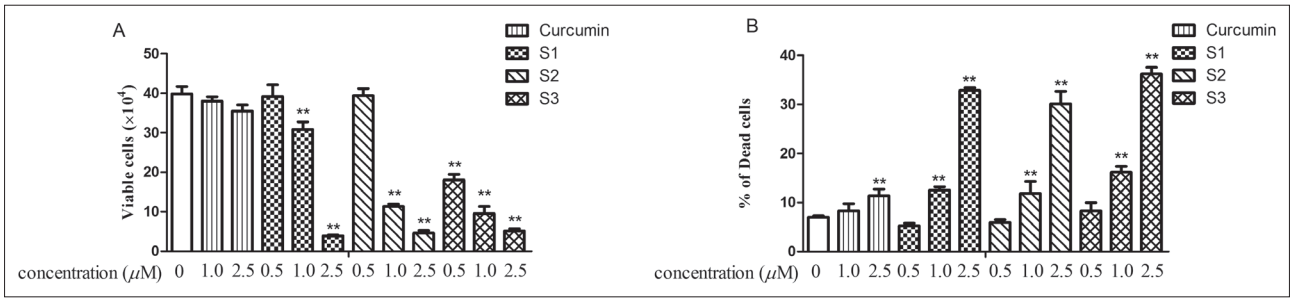



**Fig. 2.** Effect of curcumin and curcumin analogs on the growth of PC-3 cells. (A) Viable cells; (B) % of dead cells. PC-3 cells were treated with curcumin or curcumin analogs for 72 h. The number of viable cells and dead cells were determined by the trypan blue assay. Data are shown as means±SEM of three independent experiments. Significance of results compared to the control groups *p<0.05, **p<0.01.

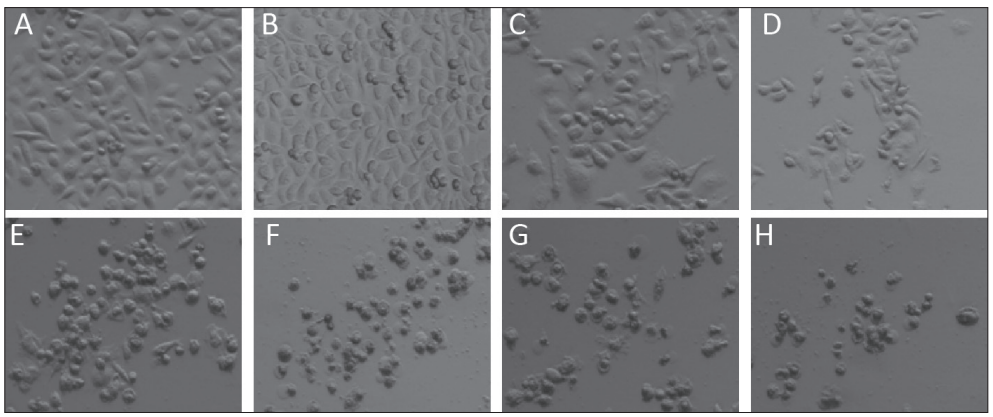



**Fig. 3.** Morphology of PC-3 cells treated with curcumin or curcumin analogs in the 2D cells culture system. PC-3 cells were treated with curcumin or curcumin analogs for 48 h, and then checked the morphology of the cultures in a microscope. (A) control; (B) curcumin; (C) **S1**-1.0 μM; (D) **S1**-2.5 μM; (E) **S2**-1.0 μM; (F) **S2**-2.5 μM; (G) **S3**-1.0 μM; (H) **S3**-2.5 μM.

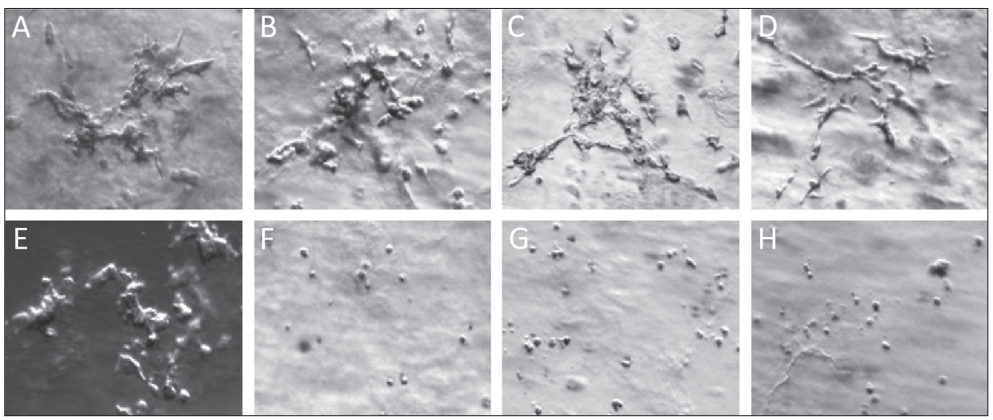



**Fig. 4.** Morphology of PC-3 cells treated with curcumin and curcumin analogs in the 3D cells culture system. PC-3 cells were cultured in Matrigel and treated with curcumin or curcumin analogs once every other day. At day 10, the 3D cultures were examined under a microscope for the formation of issue-like structure. (A) control; (B) curcumin; (C) **S1** – 1.0 μM; (D) **S1** – 2.5 μM; (E) **S2** – 1.0 μM; (F) **S2** – 2.5 μM; (G) **S3** – 1.0 μM; (H) **S3** – 2.5 μM.

### Effects of curcumin and curcumin analogs on NF-κB activation

The effects of curcumin and curcumin analogs on activation of NF-κB were determined by a luciferase reporter gene expression assay. As shown in Fig. 5, treatments of PC-3 cells with curcumin analogs S1-S3 caused a significant inhibitory effect on the luciferase activity at the concentration of 1.0 μM, resulting in 19.41%, 67.87% and 71.57% inhibition of the luciferase activity, respectively. With a higher concentration of 2.5 μM, S1-S3 caused 50.4%, 91.95% and 96.37% inhibitions of the luciferase activity, respectively. There were good correlations between the inhibition of NF-κB activity and cell growth inhibition of PC-3 cells at concentrations of both 1.0 μM and 2.5 μM.

### Effects of curcumin and curcumin analogs on levels of p-Akt, and p-STAT3 on PC-3 cells

Expressions of p-Akt, p-STAT3 were determined by the Western blotting analysis. In these experiments, PC-3 cells were treated with curcumin or curcumin analogs at a concentration of 1.0 μM for 24 h. The levels

of p-Akt, p-STAT3 (Tyr705 and Ser727) were analyzed by optical density measurements and normalized for actin. As shown in Fig. 6, the three curcumin analogs all decreased the expression of p-STAT3 (Tyr705) but not p-STAT3 (Ser727), while only S1 and S2 decreased the expression of p-Akt on PC-3 cells.

## DISCUSSION

Earlier studies synthesized and characterized curcumin analogs containing sulfone **S1**-**S3** (Rovnyak et al., 1982; Tan et al., 2014). Although a recent study showed that this class of curcumin analogs induced apoptotic cell death in acute promyelocytic leukemic cells, their activities on prostate and other cancer cells were not reported. In the present study, we investigated the effects of three curcumin analogs (**S1**-**S3**; Fig. 1) against human prostate cancer PC-3, colon cancer HT-29, lung cancer H1299 and pancreatic cancer BxPC-3 cells. Results from the MTT assay indicated that these compounds were approximately 16- to 96-fold more active than curcumin. Further studies in PC-3 cells using the trypan blue exclusion assay showed that S1-S3 decreased the number of viable cells and increased the number of dead cells. This result indicated that these compounds had both growth inhibition and cytotoxic effects on the cells. It is reasonable to assume that the effects of these compounds on PC-3, HT-29, H1299 and BxPC-3 cells as determined by the MTT assay were from both growth inhibition and cytotoxicity. Future studies are needed to determine the cytotoxicity of these compounds in normal cells and animals.

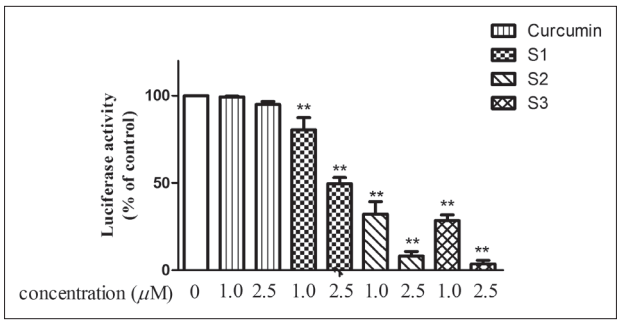
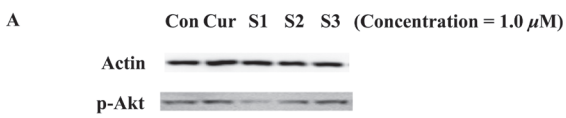



**Fig. 5.** Effects of curcumin and curcumin analogs on NF-κB transcriptional activity in PC-3/N cells. PC-3/N cells were treated with curcumin or curcumin analogs for 24 h. NF-κB transcriptional activity was determined by a luciferase reporter gene assay. Data are shown as means±SEM of three independent experiments. Significance of results compared to the control groups *p<0.05, **p<0.01.




B (Concentration = 1.0 μM)

| | Con | Cur | S1 | S2 | S3 |
|---|---|---|---|---|---|
| Actin | 1.00 | 1.20 | 1.02 | 0.99 | 0.98 |
| p-Akt | 1.00 | 1.11 | 0.38 | 0.72 | 1.07 |
| p-Stat-3 (Ser727) | 1.00 | 1.24 | 1.01 | 0.89 | 1.08 |
| p-Stat-3 (Tyr705) | 1.00 | 0.97 | 0.75 | 0.51 | 0.80 |

**Fig. 6.** Effect of curcumin or curcumin analogs on Ser727) protein expression in p-Akt, p-Stat3 (Tyr705 and Ser727) PC-3 cells. PC-3 cells were treated with curcumin or curcumin analogs for 24 h. The levels of p-Akt and p-Stat3 were determined by Western blot analysis.

Compared to a conventional 2D monolayer cell culture, the 3D culture system had advantages in that it can mimic the structural architecture and different function of the tumor tissues (Weiswald et al., 2015; Rothan et al., 2014). It is well known that cell-cell interactions and cell-matrix interactions within a 3D environment are important to the physiological function and response of cancer cells to anticancer agents (Liu et al., 2011; Tsunoda et al., 2014; Akeda et al., 2009). In the present study, we determined the effects of curcumin and S1-S3 on PC-3 cells both in 2D and 3D cell cultures. We found that PC-3 cells formed 3D tissue-like morphology in extra cellular matrix Matrigel. Compared to the control group, the tissue-like structure did not change much when the cells treated with a single compound at the concentration of 1.0 μM, except for S3. When treated with S1-S3 at a higher concentration (2.5 μM), the tissue-like structures dispersed. This result indicates that S1-S3 may inhibit the formation of tumor structures and have useful anticancer activities.

A growing body of evidence has proved the important role of NF-κB in immune response and inflamma-

tion (Terlizzi et al., 2014; Jing et al., 2014; Marusawa et al., 2014). NF-κB not only regulates the expression of most anti-apoptotic gene products associated with the survival of the tumor, but also regulates the gene products linked with the proliferation, invasion, angiogenesis and metastasis of tumors (Liu et al., 2011; Zhang et al., 2014). Extensive research showed that curcumin exerts a wide range of antitumor effects through modulation of significant signaling pathways, including the NF-κB pathway (Oyagbemi et al., 2009; Chen et al., 2008; Kunnumakkara et al., 2008). To further determine the mechanism of growth inhibition on PC-3 cells, we also investigated the effect of S1-S3 on the activation of NF-κB through an NF-κB-luciferase reporter gene expression assay. We found that treatment with S1-S3 strongly inhibited NF-κB activity at concentrations of both 1.0 μM and 2.5 μM in comparison to curcumin. This result suggests that inhibition of NF-κB activity may contribute to the inhibitory effect on cell growth induced by S1-S3.

Previous studies have demonstrated that STAT3 activation was associated with cell proliferation, inhibition of apoptosis and cellular transformation (Kamran et al., 2013; Yu et al., 2014; Kluge et al., 2011), and Akt was shown to play a major role in a variety of tumors because Akt activation not only inhibits apoptosis of cells and is thus involved in cell survival pathways, but induces protein synthesis pathways (Liang et al., 2015; Mollazadeh et al., 2015; Almhanna et al., 2011). In addition, curcumin can suppress NF-κB activation by an Akt-dependent or Akt-independent inhibition of IKK (Lin et al., 2007; Aggarwal et al., 2006). In the present study, our results indicated that the effects on PC-3 cells of curcumin analogs containing sulfone were associated with a decrease in p-Akt and p-STAT3. S1-S3 can decrease the expression of p-STAT3 (Tyr705) but not p-STAT3 (Ser727). In addition, S1 and S2 can decrease the expression of p-Akt, but S3 had no effects. Possibly S3 exerts its antitumor effects through other pathways.

In summary, we found that curcumin analogs S1-S3 containing sulfone strongly inhibited the growth of human prostate cancer PC-3, colon cancer HT-29, lung cancer H1299 and pancreatic cancer BxPC-3 cells. In addition, the compounds S1-S3 had strong effects on growth inhibition of PC-3 cells not only in 2D culture but also in 3D culture system. We further found that the potent effects of S1-S3 on PC-3 cells were associated with their inhibition of NF-κB transcriptional activity, indicating that the NF-κB pathway may be involved in the growth inhibition induced by these compounds. Moreover, our results showed that S1-S3 can decrease the level of p-STAT3 (Tyr705) but not p-STAT3 (Ser727). This result suggests the phosphorylation in tyrosine 705 is important for the effect of S1-S3 in PC-3 cells. Decreases in the amount of p-Akt in PC-3 cells treated with S1 and S2 indicate that suppression of Akt was involved in the effects of S1 and S2 but not in S3. Our studies indicate that these curcumin analogs warrant further *in vivo* studies for anticancer activities in suitable animal models.

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**Authors' contribution:** Conception and design: XZ, ZD, QZ; experiments: DL, YL, HW, CZ; analysis and interpretation: QZ, HH, YH, data collection: CZ, XC; writing the article: QZ, XZ, ZD.

**Conflict of interest disclosure:** The authors declare that there is no conflict of interest regarding the publication of this paper.

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