Tumor Biol. (2016) 37:4791–4801
DOI 10.1007/s13277-015-4277-2

ORIGINAL ARTICLE

W346 inhibits cell growth, invasion, induces cycle arrest and potentiates apoptosis in human gastric cancer cells in vitro through the NF-κB signaling pathway

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Received: 26 July 2015 / Accepted: 19 October 2015 / Published online: 31 October 2015

Abstract The therapeutic agent selectively killing cancer cells is urgently needed for gastric cancer treatment. Curcumin has been investigated for its effect on the cancer treatment because of its significant therapeutic potential and safety profile. A synthetic unsymmetry mono-carbonyl compound termed W346 was developed from curcumin. In this study, we investigated the potential antineoplastic effect and mechanism of W346 against human gastric cancer cells. W346 suppressed the proliferation and invasion, blocked cell cycle arrest at G2/M phase, and increased apoptosis in gastric cancer cells, and it presented obviously improved anticancer activity than curcumin. Moreover, W346 effectively inhibited tumor necrosis factor (TNF-α)-induced NF-κB activation by suppressing IKK phosphorylation, inhibiting IκB-α degradation, and restraining the accumulation of NF-κB subunit p65 nuclear translocation. W346 also affected NF-κB-regulated downstream products involved in cycle arrest and apoptosis. In a word, W346 exhibited significantly improved anti-gastric cancer activity over curcumin by targeting NF-κB signaling pathway, and it is likely to be a promising starting point for the development of curcumin-based therapeutic agent.

Keywords W346 · Anticancer drug · Curcumin · Gastric cancer · Apoptosis · NF-κB

Introduction

Although the incidence of gastric cancer (GC) has decreased over the last decades, it is still one of the most common malignancies and has a high mortality rate worldwide. Most GC patients undergoing surgery are already at an advanced stage, the surgical treatment effect is not ideal. Thus, chemotherapy is still very important especially for patients with recurrence and metastasis [1]. Unfortunately, only few patients experience good response to chemotherapeutic drugs, mainly because they are resistant to chemotherapy [2]. Besides, currently available chemotherapeutic drugs for the treatment of GC are associated with numerous side effects. Therefore, more effective and tolerable treatment strategies and drugs with nontoxic and more selective for GC become eager.

There is a growing studies suggesting that natural occurring substances as dietary supplements can reduce cancer risk and have been reported to hold a significant role in the development of antitumor drugs [3]. Curcumin is a bioactive powder derived

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from the root of turmeric plant, which has been used as a condiment [4]. It has been well documented that curcumin possessed cytostatic effect on several cancer cell lines in vitro and also in vivo mouse tumor models [5, 6]. Most importantly, curcumin shows almost no or minor toxicity to normal cells. Curcumin exhibited its activity by regulating a variety of signaling pathways and molecular targets [7]. In currently, there has been considerable interest in curcumin's ability to inhibit nuclear factor κB (NF-κB) pathway activity [8, 9].

NF-κB is a transcription factors that control the expression of numerous genes involved in cell proliferation, inflammation, invasive, and apoptosis [10, 11]. In most resting cells, NF-κB is in an inactive state in the cytoplasm through binding to the endogenous specific inhibitor proteins called IκBs. Upon phosphorylation of the inhibitor by IκB kinase (IKK), NF-κB is released, leading to IκB-α phosphorylation and degradation, and allowed NF-κB subunit p65 translocate to the nucleus where it exerts its transcriptional activity [12]. Overexpression of NF-κB in tumor tissue has been previously observed in larger study cohorts of gastric, prostate, hepatocellular, and oral as well as colorectal carcinoma [13–15]. Thus, activated NF-κB was suggested as a therapeutic target for the treatment of tumors, and the NF-κB inhibition may be a useful method in antitumor therapy. In addition, curcumin has been shown to be a potent inhibitor of NF-κB activation in several cell types, and inhibition of NF-κB contributes to cell apoptosis induced by curcumin [7, 9].

However, due to its low bioavailability, poor absorption, and rapid metabolism, the therapeutic potential of curcumin is limited in clinical trials [16]. Structural curcumin analogs have been created to optimize the therapeutic effects of curcumin by increasing potency, increasing absorption, and slowing metabolism [17, 18]. MACs, the abbreviation of mono-carbonyl analogs of curcumin, which was displaced curcumin's β-diketone moiety with a single carbonyl group [19]. MACs have shown to exhibit a greater biological activity and metabolic stability than that of curcumin itself and are without increased toxicity [19]. In addition, MACs also have shown exhibit NF-κB inhibition activity as well as curcumin by suppressing IKK phosphorylation [20]. In our previous work, our team has designed and synthesized a series of MACs [21, 22]. In this study, we reported a novel MACs termed W346, and studied its antitumor activity in vitro and its effect on NF-κB signaling pathways.

Materials and methods

Chemical synthesis

The structure of curcumin and MACs was shown in Fig. 1a. The synthetic process of W346 was shown in Fig. 1b. The purification method was carried out by silica gel chromatography, and the purity of W346 was greater than 97 %. The structure of

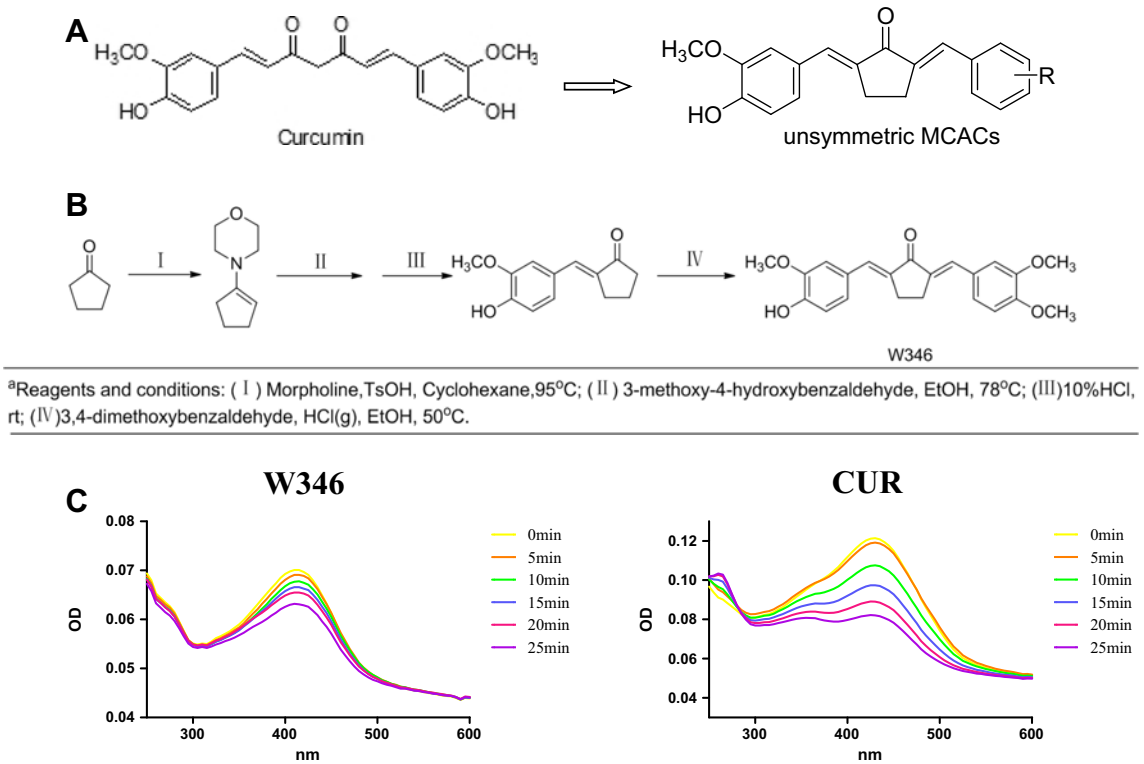


Fig. 1 W346 is more stable than curcumin in vitro. **a** The chemical structural design of curcumin (CUR) to unsymmetric mono-carbonyl analogs of curcumin (unsymmetric MACs). **b** The chemical synthesis reaction of W346. **c** UV-visible absorption spectra of W346 (*left*) and CUR (*right*)

W346 ((2E,5E)-2-(3,4-dimethoxybenzylidene)-5-(4-hydroxy-3-methoxybenzylidene)cyclopentanone) was characterized by ESI-MS and ^{1}H-NMR. W346 was dissolved in DMSO solution in vitro studies.

Stability assay

W346 solution and phosphate buffer (pH 7.4) was mixed well then detected the OD values from 250 to 600 nm using microplate reader. Taking 5 min as intervals, the absorption curves were recorded for over 25 min.

Cell culture

Human gastric cancer cell lines SGC-7901, BGC-823, and MGC-803 were obtained from The Cell Bank of Chinese Academy of Sciences (Wuhan, China), KATO III was obtained from The Cell Bank of Chinese Academy of Sciences (Shanghai, China), and normal gastric mucosa epithelial cell line GES-1 was obtained from American Type Culture Collection (ATCC). All cells were routinely cultured in RPMI-1640 media (Gibco) supplemented with 10 % fetal calf serum (HyClone), 100 U/ml penicillin, and 100 mg/ml streptomycin (Gibco) in 5 % CO_2 at 37 °C. The cells used for our experiments were in the log phase of growth.

Chemical reagents

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), dimethyl sulfoxide (DMSO), and propidium iodide (PI) were purchased from Sigma; FITC Annexin V Apoptosis Detection Kit and basement membrane Matrigel were purchased from BD Biosciences; NF-κB activation, Nuclear Translocation Assay Kit was purchased from Beyotime; TNF-α (tumor necrosis factor α) was purchased from Univbio. Crystal violet staining solution was purchased from Beyotime.

Cell viability assay

The effect of W346 on cell proliferation was determined by the MTT assay. Briefly, the cells (5000 per well) were incubated with W346 or curcumin for indicated concentration (0.096, 0.48, 2.4, 12, 60 μM) in triplicate in a 96-well plate and then incubated for 72 h at 37 °C. An MTT solution was added to each well and incubated for 4 h. The crystals were solubilized with DMSO, and the absorbance of the cell suspension was measured at 490 nm using microplate reader.

Clonogenic assay

Tumor cells have a capability of unlimited division and form colonies. The SGC-7901 and BGC-823 cells were treated with W346 or curcumin. After 24 h incubation, cells were transferred to the normal medium and allowed to forming colonies. Colonies were stained with crystal violet staining solution and taken pictures manually after 7 days.

Matrigel invasion assay

The invasion abilities of tumor cells were evaluated in a 24-well plant. The upper surface of the chamber was coated with basement membrane Matrigel and air-dried. Cells were detached by trypsin and resuspended in serum-free medium. Medium containing 10 % FBS was applied to the lower chamber as a chemoattractant, and then cells contained with W346 or curcumin were seeded on the upper chamber at a concentration of 1×10^{6} cells/well in 200 μL of serum-free medium. After incubation for 24 h, the cells of lower surface of chamber were stained with crystal violet staining solution, after which cells on the upper side were removed completely with a cotton swab. The migrating cells on the lower surface of the membrane filter were caught with a light microscope.

Flow cytometric analysis

To determine the effect of compound on the cell cycle, cells were exposed to W346 or curcumin for 24 h. Thereafter, cells were washed, fixed with 75 % ethanol, and incubated for 1 h at −20 °C. Then cells were washed again with PBS and incubated in propidium iodide solution for 15 min. Then cells were analyzed with fluorescence-activated cell sorting (FACS).

For quantification of apoptotic cells, cells were exposed to W346 or curcumin for 24 h, harvested, washed with PBS, and centrifuged for 5 min at maximum speed (1000 rpm) at 4 °C. Then tumor cells were incubated with Annexin-V in the dark for 10 min, afterwards, propidium iodide (PI) was added. After centrifugation for 5 min, flow cytometric analysis was then performed using a FACSCalibur flow cytometer (BD Biosciences).

Preparation of nuclear and cytoplasmic extracts

Gastric cancer cells were treated with W346 or curcumin for 1 h and then were treated with the TNF-α for 1 h. The cell samples were lysed in buffer A, and splitted on ice for 10 min, then added buffer B. After incubation on ice for 1 min, samples were centrifuged for 5 min at maximum speed (16,000 rpm) in a microcentrifuge. The supernatants were collected as cytoplasmic extracts. The sediments were resuspended in buffer C and incubated on ice for 40 min. After centrifugation for 10 min, samples were centrifuged, and the supernatants were collected as nuclear extracts. Both extracts concentrations were determined by Bio-Rad Protein Assay and were stored at −80 °C.

Western blotting

Whole-cell lysates containing equal amounts of protein were electrophoresed by 10 or 12 % SDS-PAGE and transferred to a poly vinylidene difluoride (PVDF) membrane. Then the membranes were blocked with freshly 5 % nonfat milk in TBST for 90 min at room temperature, after that were incubated with specific antibodies (1:300 or 1:1000) in TBST with gentle shaking for overnight at 4 °C. After washing three times with TBST, the membranes were incubated with the corresponded secondary antibody (1:3000) for 1 h at room temperature and washed again. The blotting was visualized using chemiluminescence detection kit ECL-PLUS.

Antibodies were purchased from the following sources: primary antibodies for anti-caspase 3, anti-bax, anti-bcl-2, anti-p53, anti-mdm-2, anti-cdc-2, anti-cyclin B1, anti-p-Ikk, anti-Ikk, anti-p-IκB-α, anti-IκB-α, anti-p65, anti-Lamin B, and anti-GAPDH were all obtained from Santa Cruz Biotechnology, and anti-cleaved-caspase 3 was purchased from Cell Signaling Technology. Secondary antibody for goat anti-mouse IgG-HRP, donkey anti-rabbit IgG-HRP, and donkey anti-goat IgG-HRP was purchased from Santa Cruz Biotechnology.

Assessment of NF-κB nuclear translocation

NF-κB activation and nuclear translocation assay was performed according to the reagent manufacturer's instructions (Beyotime). Briefly, 24 h after being plated in six-well plate, cells were treated with W346 or curcumin for 1 h before treated with the TNF-α for 1 h, then fixed and blocked at room temperature. After incubation with rabbit anti-p65 NF-κB antibody overnight at 4 °C, cells were added with fluorescent secondary antibody. Eventually, the nuclear was dyed with DAPI. The fluorescence microscope was used to take pictures.

Statistical analysis

Student's *t* test was used for statistical analyses. Calculations were carried out with GraphPad software. All results with a $P<0.05$ were considered statistically significant.

Results

W346 was more stable than curcumin in vitro

The chemical stability of W346 and curcumin in phosphate buffer was analyzed using an absorption spectrum assay. As shown in Fig 1c, the UV-visible absorption intensity of the curcumin decreases significantly for it lost more than 40 % of its original intensity within 25 min, while W346 showed almost complete stability in 25 min of incubation period. This result suggested that W346 was much more stable than curcumin in vitro.

W346 inhibited proliferation of GC cells

The effects of W346 and curcumin on cell viability were evaluated by MTT assay in SGC-7901, BGC-823, MGC-803, KATOIII, and GES-1 cells. The cells were exposed to different concentrations of W346 or curcumin for 72 h. The IC_{50} values (50 % cell growth inhibitory concentrations) for the individual compound on cancer cells viability were determined. The IC_{50} values of W346 on GC cells were 8.1±1.1, 7.2±1.0, 14.0±0.5, and 16.9±0.9 μM, while that of curcumin were 20.4±2.4, 20.4±5.9, 22.0±3.5, and 21.2±1.5 μM, respectively. This result suggested that antiproliferative effect of W346 was more pronounced than curcumin, particularly in SGC-7901 and BGC-823. Moreover, IC_{50} of W346 on GES-1 was 29.4±1.9 μM, which was close to that of curcumin (28.9±3.2 μM). The compound W346 may have a target role to tumor cells.

W346 inhibited colony formation and invasion of GC cells

In accordance with the results of IC_{50}, SGC-7901 and BGC-823 cells were used for clonogenic assay to further validate the effect of W346 against proliferation. After treating with W346 or curcumin for 24 h, cells were replaced with fresh medium and allowed to forming colonies. The results showed W346 significantly inhibited cell colony formations in a dose-dependent manner, while curcumin at the same concentration had no significantly effect on the colonies (Fig. 3a, b).

Effects of W346 on cell migration of SGC-7901 and BGC-823 cells were further assessed by Matrigel invasion assay. SGC-7901 or BGC-823 cells were plated in the upper chamber containing a membrane, then cells were treated with W346 or curcumin for 24 h, and the number of cells on the underside of the membrane was assessed under light microscopy (Fig. 3c, d). Migration of SGC-7901 and BGC-823 cells was inhibited in a dose-dependent manner when treated with W346.

W346 induced G2/M arrest and potentiated apoptosis of GC cells

To examine the mechanisms by which W346 and curcumin inhibit the proliferation of SGC-7901 and BGC-823 cells, we examined and compared their effect on the rate of growth inhibition. Therefore, we determined the behavior of these cells in the various phases of the cell cycle by flow cytometric analysis. Results showed that there was a concentration-

dependent accumulation of cells in the G2/M-phase of the cell cycle in W346-treated group (Fig. 4a, b).

We future assessed the effect of W346 and curcumin on the induction of apoptosis in SGC-7901 and BGC-823 cells by flow cytometry. The results showed that W346 dose-dependently increases cells apoptotic rate after 24 h treatment, W346 at 20 μM induced about 30 % apoptosis, while curcumin at the same concentration only induced about 10 % apoptosis (Fig. 4c, d).

W346 inhibited NF-κB activation induced by TNF-α in GC cells by suppressing IKK, IκB-α phosphorylation and inhibiting degradation of IκB-α

Since curcumin is a potent inhibitor of NF-κB activation, whether W346 has the same access to this pathway? Activation of NF-κB triggered by TNF-α is achieved through blocking IKK activity and inhibiting IκB-α degradation, which results in the release and subsequent nuclear translocation of NF-κB [23, 24]. To determine whether W346 inhibited NF-κB activation induced by TNF-α was mediated by suppressing IKK, IκB-α phosphorylation and inhibiting IκB-α degradation, we performed a Western blotting analysis using the anti-p-IKK, anti-p-IκB-α, and anti-IκB-α antibody. Results showed that p-IKK and p-IκB-α were increased, and IκB-α was degraded after TNF-α treatment, while treating with W346 or curcumin before stimulated with TNF-α inhibited the increasing of p-IKK and p-IκB-α expression as well as inhibited the degradation of IκB-α expression induced by TNF-α in a dose-dependent manner (Fig. 5).

W346 inhibited the NF-κB activation induced by TNF-α in GC cells by inhibiting p65 nuclear translocation

Modifications of NF-κB main subunit p65 play an important role in NF-κB transcriptional activity [12]. Therefore, we examined the effect of W346 on the expression of p65 in both nuclear extracts and cytoplasmic extracts by Western blotting. Results showed that TNF-α stimulation caused p65 nuclear transposition, and nuclear p65 was decreased in W346 or curcumin-treated group (Fig. 6a). A similar inhibition, the p65 translocation induced by TNF-α was also observed in W346-treated BGC-823 cells (Fig. 6b).

Immunofluorescence was also used to detect p65 nuclear transferation by an NF-κB nuclear transfer detection kits. After treating with W346 or curcumin for 1 h before treating with the TNF-α for 1 h, cells were then processed for fluorescence microscopy. Results showed that W346 caused inhibition of p65 nuclear translocation induced by TNF-α as well as curcumin in SGC-7901 cells (Fig. 6c).

W346 suppressed the expression of NF-κB-regulated gene products involved in cycle arrest and apoptosis in SGC-7901 gastric cancer cells

NF-κB is known to regulate the expression of numerous proteins involved in cell cycle arrest and apoptosis. To investigate whether W346 induced G2/M arrest was due to the expression of NF-κB-regulated gene products, whole-cell protein extracts were prepared and analyzed by Western blotting with the specific antibodies. W346 blocked expression of mdm-2, cdc-2, and cyclinB 1 in a dose-dependent manner in SGC-7901 cells. On the contrary, the expression of p53 protein was increased after treating with W346 (Fig. 7a).

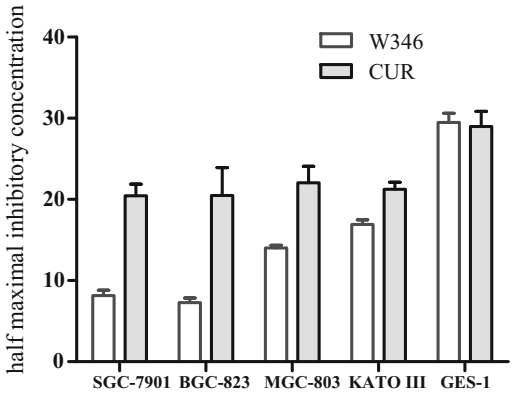
In addition, we also determined the effect of W346 on the NF-κB-dependent gene products that are involved in apoptosis. Western blotting analysis showed that the cleavage of caspase-3 and the expression of bax were enhanced, while the expression of bcl-2 was decreased when SGC-7901 cells were exposed to W346 (Fig. 7b).

Discussion

The prevalence and mortality of GC are still increasing despite our enhanced understanding of the pathogenesis of this disease, as well as establishment of improved therapeutic strategies for this malignancy. Chemotherapy constitutes an important treatment regimen for GC besides surgical resection [25]. However, the toxicity of drugs and the development of treatment resistance are two big challenges of currently used chemotherapy for GC. Thus, novel agents that are nontoxic, efficacious, and can significantly enhance the effects of existing chemotherapeutic drugs are urgently needed. Owning to lower toxicity of natural product, it is still important for chemotherapeutic drugs to design new medicine using natural medical as leading compounds [26, 27].

Curcumin is one of the most widely characterized of the phytochemicals, which has been widely used in adjuvant therapy for centuries owing to its anti-inflammatory, antioxidant, and anticarcinogenic properties [4]. Especially for its antitumor property, still has been the subject of a great deal of interest. Increasing evidence indicated that curcumin has anticancer effect against different types of human tumor cells, including of esophageal cancer cells, colon cancer cells, and lung cancer cells [9, 28, 29]. Unfortunately, therapeutic potential of curcumin is limited by its relatively poor cellular bioavailability [16]. Chemical structure modification of curcumin is an effective approach for optimizing the therapeutic effects of curcumin [18]. By displacing β-diketone structure of curcumin with a single carbonyl group, we can get monocarbonyl analogs of curcumin (MACs) [19]. Consequently, our team has designed and synthesized a series of MACs in our previous work. In the subsequent study, we found one of

A



B

half maximal inhibitory concentration（μM）

	W346	CUR
SGC-7901	8.1±1.1	20.4±2.4
BGC-823	7.2±1.0	20.4±5.9
MGC-803	14.0±0.5	22.0±3.5
KATO III	16.9±0.9	21.2±1.5
GES-1	29.4±1.9	28.9±3.2

Fig. 2 W346 inhibited proliferation of gastric cancer cells. **a**, **b** Gastric cancer cell lines SGC-7901, BGC-823, MGC-803, KATOIII, and normal gastric mucosa epithelial cell line GES-1 were treated with various concentrations of W346 or CUR (0.096, 0.48, 2.4, 12, 60 μM) for 72 h. Cell viability was determined using MTT assay. The IC_{50} values were taken as the concentration that caused 50 % inhibition of cell proliferation and were calculated by GraphPad statistical software

MACs, W346, was much more stable than curcumin in vitro (Fig 1c). We further studied its antitumor activity in GC cells. We discovered that W346 presented obviously improved antiproliferation activity than the parent compound curcumin in two GC cell lines, SGC-7901 and BGC-823. However, their inhibitory effect on GES-1 was considerable weak (Fig. 2). This may suggest that W346 has a target role to tumor cells.

In a variety of tumor cells, the anticancer effect of curcumin was identified through inhibiting the invasive potential, interfering with the cell cycle, and inducing apoptosis of cancers [28, 30, 31]. Potential of W346 on cell migration on SGC-7901 and BGC-823 cells was firstly assessed. Just as we had expected, migration of tumor cells was inhibited in a dose-dependent manner when treated with W346, and the effect of W346 was stronger than curcumin (Fig. 3). As study has reported the antitumor property of curcumin is partly due to the arrest of cancer cells in G2/M cell cycle phase [32], so we secondly determined the behavior of W346 in the cell cycle arrest. Flow cytometric analysis exhibited an increased accumulation of cells in the G2/M-phase of the cell cycle in W346-treated group (Fig. 4). Finally, the induction of apoptosis by W346 was determined. The number of early and late period apoptotic cells was increased in W346-treated group in a dose-dependent manner, and it was significantly higher than those of curcumin-treated cells (Fig. 4).

It has been demonstrated that NF-κB plays a pivotal role in the development of tumor through multiple signaling pathways, and aberrant activation of NF-κB has been observed in variety of cancer types [11, 15]. Constitutively activation of NF-κB not only promote tumor growth, but also is associated with harmful clinicopathological features of cancer patients, such as lymphatic metastasis, vascular invasion, bad response or resistance to chemotherapeutic agents, tumor recurrence, and poor treatment outcome [33]. A prominent mechanism linking NF-κB signaling to cancer progression is the abrogation of apoptosis. Docetaxel and capecitabine are two effective chemotherapeutic drugs in the treatment of GC in clinic. Study has showed docetaxel induced NF-κB activation in GC cells [34]. A combination of docetaxel and NF-κB decoy, a NF-κB inhibitor, enhanced tumor cell death [34].

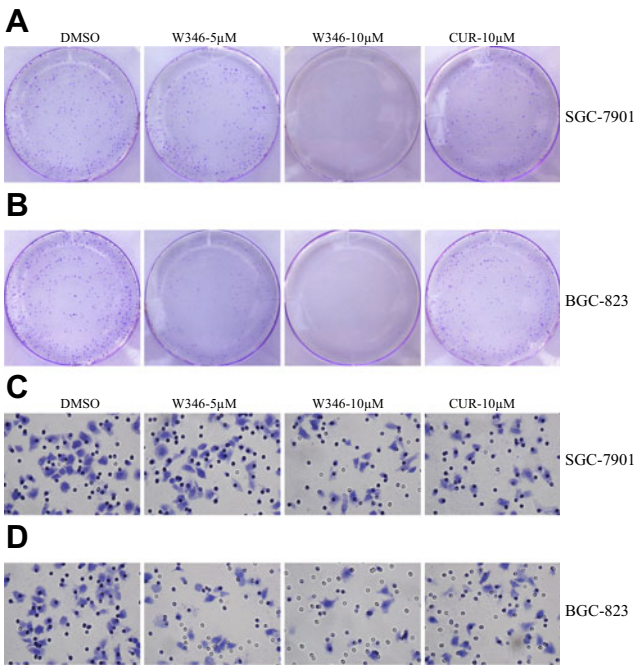


Fig. 3 W346 inhibited colony formation and invasion of gastric cancer cells. **a** SGC-7901 or **b** BGC-823 cells were treated with DMSO or W346 (5 or 10 μM) or CUR (10 μM). After 24 h incubation, cells were transferred to the normal medium and allowed to forming colonies. Colonies were stained with crystal violet and taken pictures manually after 7 days. **c** SGC-7901 or **d** BGC-823 cells were treated with DMSO or W346 (5 or 10 μM) or CUR (10 μM) for 24 h. The migrating cells on the lower surface of the membrane filter were stained by crystal violet and caught with a light microscope

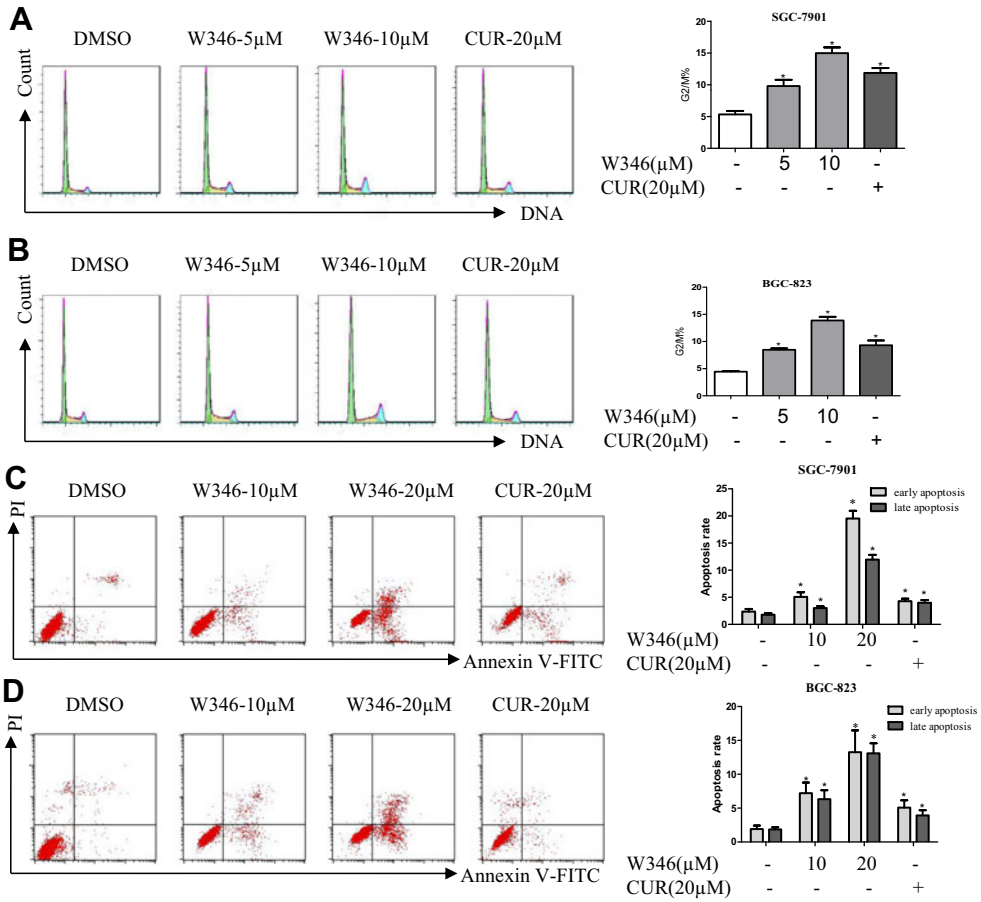


Fig. 4 W346 induced cycle arrest and apoptosis of gastric cancer cells. **a** SGC-7901 or **b** BGC-823 cells were exposed to DMSO or W346 (5 or 10 μM) or CUR (20 μM) for 24 h, then gathered all cells for staining with propidium iodide. Histogram illustrated the rate of G2/M phase from FACS analysis of three separate treatments. *$P<0.05$ compared with DMSO group. **c** SGC-7901 or **d** BGC-823 cells were exposed to DMSO or W346 (10 or 20 μM) or CUR (20 μM) for 24 h. Apoptosis were assessed by Annexin V/PI staining. Histogram illustrating of the rate of apoptosis cells from FACS analysis of three separate treatments. *$P<0.05$ compared with DMSO group

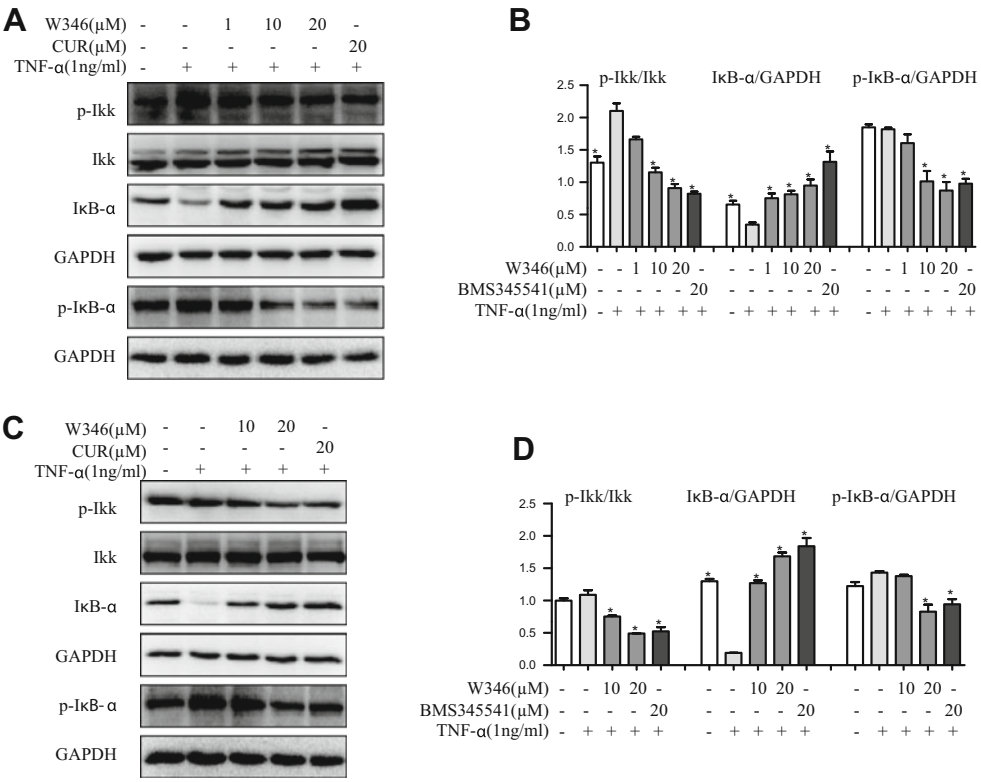


Fig. 5 W346 inhibited NF-κB activation induced by TNF-α in gastric cancer cells by suppressing IKK, IκB-α phosphorylation and inhibiting degradation of IκB-α. **a** SGC-7901 cells were pretreated with DMSO (−) or W346 (1, 10, or 20 μM) or CUR (20 μM) for 60 min, then exposed to TNF-α (1 ng/ml) for 15 min. The whole-cell extracts were analyzed by Western blotting for the expressions of p-IKK, p-IκB-α, and IκB-α. The intensity of immunoblots digitized by ImageJ software was normalized to IKK and GAPDH, respectively. **b** Extracts from BGC-823 cells were analyzed by Western blotting for p-IKK, p-IκB-α, and IκB-α expression. *$P<0.05$ compared with TNF-α stimulation group

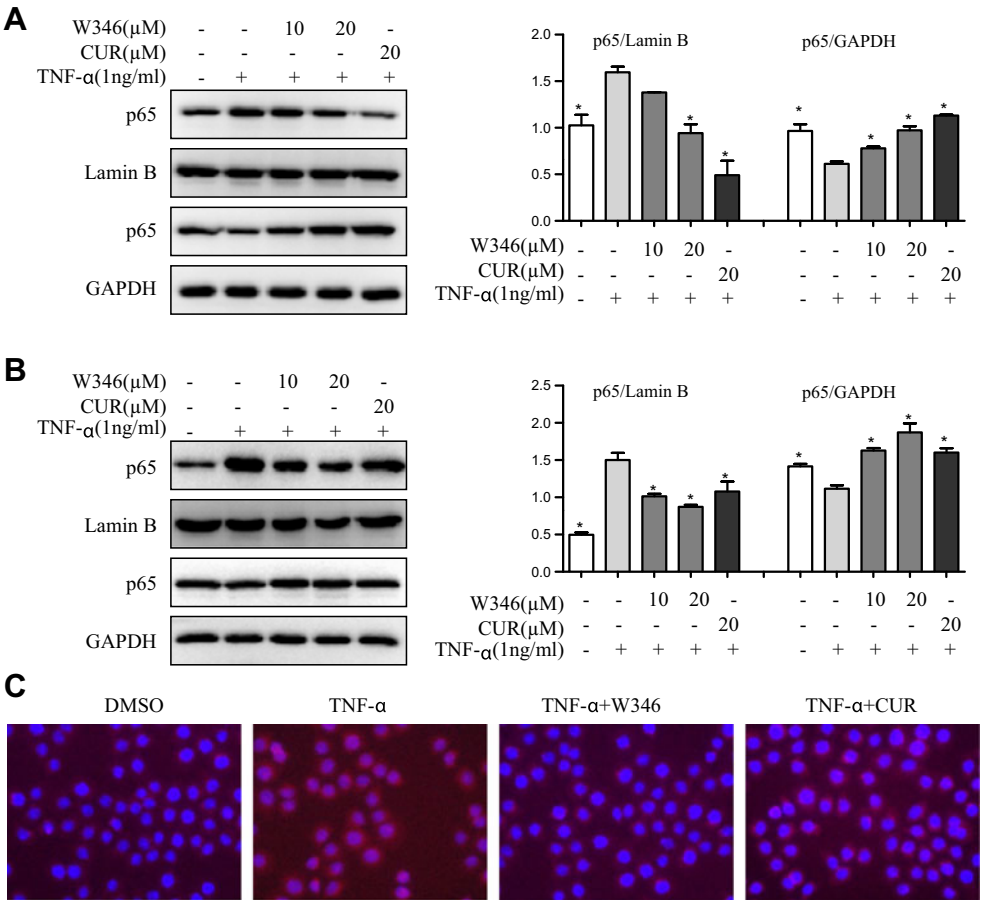


Fig. 6 W346 inhibited the NF-κB activation induced by TNF-α in gastric cancer cells by inhibiting p65 nuclear translocation. **a** SGC-7901 cells were treated with DMSO (−) or W346 (10 or 20 μM) or CUR (20 μM) for 1 h, then stimulated with TNF-α (1 ng/ml) for another 1 h, extracted the proteins of nuclear and cytoplasm, respectively, and the levels of p65 were measured by Western blotting. The intensity of nuclear p65 was normalized to Lamin B, and the intensity of cytoplasm p65 was normalized to GAPDH. **b** After the isolation of nuclear and cytoplasm extracts, the extracts of BGC-823 cells were analyzed by Western blotting for p65 expression, respectively. *$P<0.05$ compared with TNF-α stimulation group. **c** SGC-7901 cells were treated with DMSO (−) or W346 (20 μM) or CUR (20 μM) for 60 min, then stimulated with TNF-α (1 ng/ml) for 60 min. NF-κB subcellular localization was verified by immunofluorescence staining with p65 antibody

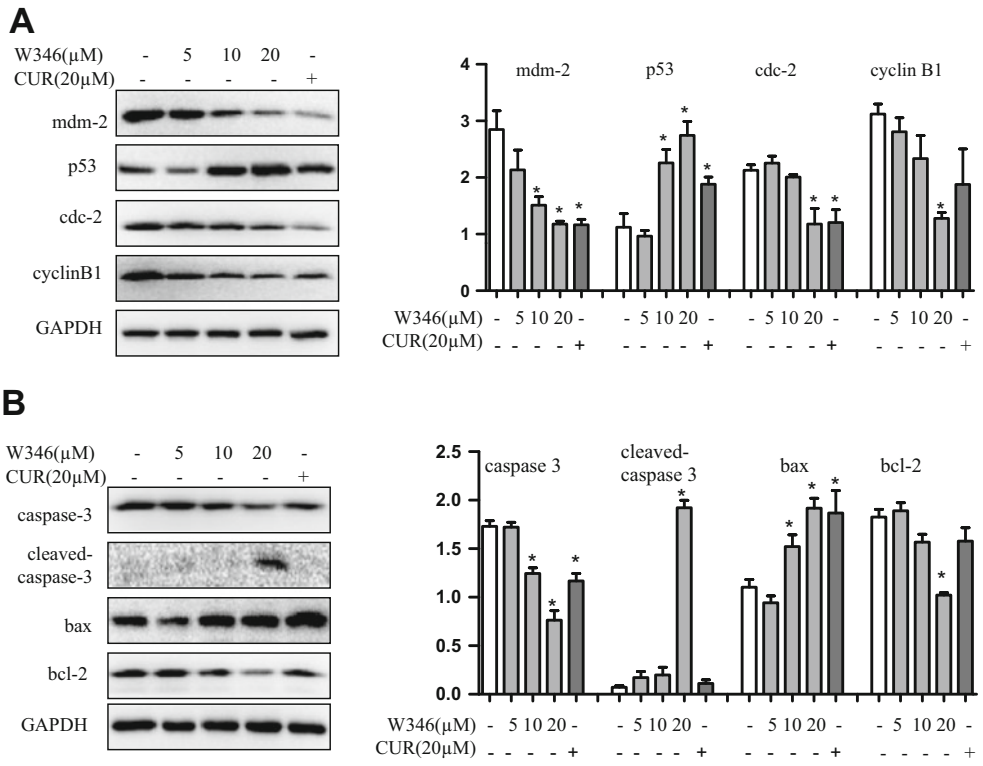


Fig. 7 W346 suppressed the expression of NF-κB-regulated gene products involved in cycle arrest and apoptosis in SGC-7901 gastric cancer cells. **a** Extracts from SGC-7901 cells treated with DMSO (−) or W346 (5, 10, or 20 μM) or CUR (20 μM) for 24 h were analyzed by Western blotting for the expressions of mdm-2, p53, cdc-2, and cyclin B1. GAPDH was used for equal protein. *$P<0.05$ compared with DMSO group. **b** Extracts from SGC-7901 cells were analyzed by Western blotting for the expressions of caspase 3, cleaved-caspase 3, bax, and bcl-2. GAPDH was used for equal protein. *$P<0.05$ compared with DMSO group

Moreover, γ-tocotrienol potentiated the effect of capecitabine through suppression of NF-κB activation and NF-κB-regulated markers of proliferation, invasion, angiogenesis, and metastasis in vivo and vitro [35]. Chemotherapeutic agent, helicobacter pylori, and some other inducers such as tumor necrosis factor (TNF-α), all of them were potent activator of NF-kB in GC [36, 37]. Whereas natural chemopreventive agents suppressed it, indicating a strong link between tumor biology and the anticancer effects of natural compounds. Numerous phytochemicals, including curcumin, have been reported that its antitumor effect was associated with inhibition of NF-κB activity [9, 38]. For instance, parthenolide could antagonize Taxol-mediated nuclear NF-κB nuclear translocation and activation and bcl-xl upregulation by selectively targeting IKK activity in A549 cells [39]. Curcumin suppressed NF-κB activation in Helicobacter pylori-infected rats [40]. Inhibition of NF-κB activation by curcumin shed some lights on the mechanism of W346-induced GC cells death. Based to this conclusion, we examined the inhibitory activity of W346 on NF-κB pathway. We found that W346 inhibited NF-κB activation induced by TNF-α by suppressing IKK phosphorylation and inhibiting degradation of IκB-α in GC cells (Fig. 5). Herein, we showed that W346 inhibited the accumulation of p65 nuclear transposition as well as curcumin (Fig. 6).

Various lines of evidence suggested that the activation of transcription factor NF-κB regulates the expression of numerous proteins involved in cell cycle arrest and apoptosis. Curcumin also suppressed NF-κB-regulated gene products such as bcl-2 [29, 41, 42]. Furthermore, curcumin reversed chemoresistance of doxorubicin by downregulating the NF-κB-regulated anti-apoptotic gene products in GC cells [43]. In our previous study, we have found W346 blocked cell cycle in the G2/M-phase and significantly induced apoptosis by flow cytometric analysis. So, we then test the changes of cycle and apoptosis related proteins expression. In accordance with flow cytometric analysis, G2/M phase related proteins also changed in a dose-dependent manner after treating with W346. Moreover, Western blotting analysis showed that W346 inhibits the expression of anti-apoptotic protein bcl-2 and promote pro-apoptotic protein bax expression, thus, promote cell apoptosis. At the same time, caspase-3 was activated in SGC-7901 cells after treating with W346, W346 was more efficacious than curcumin in cleavage of caspase-3 (Fig. 7).

Currently, a great deal of research effort is being devoted to the development of curcumin analogs, such as EF24, B19, and AC17. However, none of them successfully enter clinical application [20, 44, 45]. In this study, we reported that curcumin analogs W346 suppressed the proliferation and invasion of GC cell lines, and the inhibitory effects were correlated with the cell cycle arrest at G2/M phase and apoptosis induction, and it presented obviously improved anticancer activity than the parent compound curcumin. Furthermore, we showed W346 inhibited NF-κB activation induced by TNF-α, and it also suppressed the expression of NF-κB-regulated proteins involved in cycle arrest and apoptosis. Our study indicated that W346 might be a potent and promising NF-κB inhibitor to combat GC and deserves further evaluation. Further studies are also needed to clarify its bioavailability and antitumor in animal models.

Compliance with ethical standards

Conflicts of interest None

Funding This work was supported by the National Natural Science Foundation of China (Grant No. 81272462), the Zhejiang Province Natural Science Fund of China (Grant Nos. LY14H160044, Y13H300005), and the Technology Foundation for Medical Science of Zhejiang Province (Grant No. 2012KYA129).

References

1. Wagner AD, Grothe W, Haerting J, Kleber G, Grothey A, Fleig WE. Chemotherapy in advanced gastric cancer: a systematic review and meta-analysis based on aggregate data. J Clin Oncol Off J Am Soc Clin Oncol. 2006;24:2903–9.
2. Kim HK, Choi IJ, Kim CG, Kim HS, Oshima A, Michalowski A, et al. A gene expression signature of acquired chemoresistance to cisplatin and fluorouracil combination chemotherapy in gastric cancer patients. PLoS One. 2011;6, e16694.
3. Gupta SC, Kim JH, Prasad S, Aggarwal BB. Regulation of survival, proliferation, invasion, angiogenesis, and metastasis of tumor cells through modulation of inflammatory pathways by nutraceuticals. Cancer Metastasis Rev. 2010;29:405–34.
4. Hatcher H, Planalp R, Cho J, Torti FM, Torti SV. Curcumin: from ancient medicine to current clinical trials. Cell Mol Life Sci CMLS. 2008;65:1631–52.
5. Kunnumakkara AB, Guha S, Krishnan S, Diagaradjane P, Gelovani J, Aggarwal BB. Curcumin potentiates antitumor activity of gemcitabine in an orthotopic model of pancreatic cancer through suppression of proliferation, angiogenesis, and inhibition of nuclear factor-kappaB-regulated gene products. Cancer Res. 2007;67:3853–61.
6. Prakobwong S, Gupta SC, Kim JH, Sung B, Pinlaor P, Hiraku Y, et al. Curcumin suppresses proliferation and induces apoptosis in human biliary cancer cells through modulation of multiple cell signaling pathways. Carcinogenesis. 2011;32:1372–80.
7. Kunnumakkara AB, Anand P, Aggarwal BB. Curcumin inhibits proliferation, invasion, angiogenesis and metastasis of different cancers through interaction with multiple cell signaling proteins. Cancer Lett. 2008;269:199–225.
8. Kamat AM, Tharakan ST, Sung B, Aggarwal BB. Curcumin potentiates the antitumor effects of bacillus Calmette-Guerin against bladder cancer through the downregulation of NF-kappaB and upregulation of TRAIL receptors. Cancer Res. 2009;69:8958–66.
9. Shakibaei M, Mobasheri A, Lueders C, Busch F, Shayan P, Goel A. Curcumin enhances the effect of chemotherapy against colorectal cancer cells by inhibition of NF-kappaB and Src protein kinase signaling pathways. PLoS One. 2013;8, e57218.

10. Van Waes C. Nuclear factor-kappaB in development, prevention, and therapy of cancer. Clin Cancer Res An Off J Am Assoc Cancer Res. 2007;13:1076–82.
11. Karin M. Nuclear factor-kappaB in cancer development and progression. Nature. 2006;441:431–6.
12. Vermeulen L, De Wilde G, Notebaert S, Vanden Berghe W, Haegeman G. Regulation of the transcriptional activity of the nuclear factor-kappaB p65 subunit. Biochem Pharmacol. 2002;64: 963–70.
13. Li L, Aggarwal BB, Shishodia S, Abbruzzese J, Kurzrock R. Nuclear factor-kappaB and IkappaB kinase are constitutively active in human pancreatic cells, and their down-regulation by curcumin (diferuloylmethane) is associated with the suppression of proliferation and the induction of apoptosis. Cancer. 2004;101:2351–62.
14. Weichert W, Boehm M, Gekeler V, Bahra M, Langrehr J, Neuhaus P, et al. High expression of RelA/p65 is associated with activation of nuclear factor-kappaB-dependent signaling in pancreatic cancer and marks a patient population with poor prognosis. Br J Cancer. 2007;97:523–30.
15. Sakamoto K, Maeda S, Hikiba Y, Nakagawa H, Hayakawa Y, Shibata W, et al. Constitutive NF-kappaB activation in colorectal carcinoma plays a key role in angiogenesis, promoting tumor growth. Clin Cancer Res An Off J Am Assoc Cancer Res. 2009;15:2248–58.
16. Anand P, Kunnumakkara AB, Newman RA, Aggarwal BB. Bioavailability of curcumin: problems and promises. Mol Pharm. 2007;4:807–18.
17. Qiu X, Du Y, Lou B, Zuo Y, Shao W, Huo Y, et al. Synthesis and identification of new 4-arylidene curcumin analogues as potential anticancer agents targeting nuclear factor-kappaB signaling pathway. J Med Chem. 2010;53:8260–73.
18. Liang G, Shao L, Wang Y, Zhao C, Chu Y, Xiao J, et al. Exploration and synthesis of curcumin analogues with improved structural stability both in vitro and in vivo as cytotoxic agents. Bioorg Med Chem. 2009;17:2623–31.
19. Zhao C, Liu Z, Liang G. Promising curcumin-based drug design: mono-carbonyl analogues of curcumin (MACs). Curr Pharm Des. 2013;19:2114–35.
20. Kasinski AL, Du Y, Thomas SL, Zhao J, Sun SY, Khuri FR, et al. Inhibition of IkappaB kinase-nuclear factor-kappaB signaling pathway by 3,5-bis (2-flurobenzylidene) piperidin-4-one (EF24), a novel monoketone analog of curcumin. Mol Pharmacol. 2008;74:654–61.
21. Weng Q, Fu L, Chen G, Hui J, Song J, Feng J, et al. Design, synthesis, and anticancer evaluation of long-chain alkoxylated mono-carbonyl analogues of curcumin. Eur J Med Chem. 2015;103:44–55.
22. Liang G, Li X, Chen L, Yang S, Wu X, Studer E, et al. Synthesis and anti-inflammatory activities of mono-carbonyl analogues of curcumin. Bioorg Med Chem Lett. 2008;18:1525–9.
23. Young AM, Campbell EC, Lynch S, Dunn MH, Powis SJ, Suckling J. Regional susceptibility to TNF-alpha induction of murine brain inflammation via classical IKK/NF-kappaB signalling. PLoS One. 2012;7, e39049.
24. Luo JL, Maeda S, Hsu LC, Yagita H, Karin M. Inhibition of NF-kappaB in cancer cells converts inflammation-induced tumor growth mediated by TNFalpha to TRAIL-mediated tumor regression. Cancer Cell. 2004;6:297–305.
25. Cervantes A, Rosello S, Roda D, Rodriguez-Braun E. The treatment of advanced gastric cancer: current strategies and future perspectives. Ann Oncol Off J Eur Soc Med Oncol / ESMO. 2008;19 Suppl 5:v103–7.
26. Rasool M, Malik A, Arooj M, Manan A, Qazi MH, Kamal MA, Sheikh IA, Gan SH, Asif M, Naseer MI. Roles of Natural Compounds from Medicinal Plants in Cancer Treatment: Structure and Mode of Action at Molecular Level. Medicinal chemistry. 2015.
27. Cecarini V, Cuccioloni M, Mozzicafreddo M, Bonfili L, Angeletti M, Eleuteri AM. Targeting proteasomes with natural occurring compounds in cancer treatment. Curr Cancer Drug Targets. 2011;11:307–24.
28. Yang CL, Liu YY, Ma YG, Xue YX, Liu DG, Ren Y, et al. Curcumin blocks small cell lung cancer cells migration, invasion, angiogenesis, cell cycle and neoplasia through Janus kinase-STAT3 signalling pathway. PLoS One. 2012;7, e37960.
29. O'Sullivan-Coyne G, O'Sullivan GC, O'Donovan TR, Piwocka K, McKenna SL. Curcumin induces apoptosis-independent death in oesophageal cancer cells. Br J Cancer. 2009;101:1585–95.
30. Lee DS, Lee MK, Kim JH. Curcumin induces cell cycle arrest and apoptosis in human osteosarcoma (HOS) cells. Anticancer Res. 2009;29:5039–44.
31. Cai XZ, Wang J, Li XD, Wang GL, Liu FN, Cheng MS, et al. Curcumin suppresses proliferation and invasion in human gastric cancer cells by downregulation of PAK1 activity and cyclin D1 expression. Cancer Biol Ther. 2009;8:1360–8.
32. Weir NM, Selvendiran K, Kutala VK, Tong L, Vishwanath S, Rajaram M, et al. Curcumin induces G2/M arrest and apoptosis in cisplatin-resistant human ovarian cancer cells by modulating Akt and p38 MAPK. Cancer Biol Ther. 2007;6:178–84.
33. Izzo JG, Malhotra U, Wu TT, Ensor J, Luthra R, Lee JH, et al. Association of activated transcription factor nuclear factor kappab with chemoradiation resistance and poor outcome in esophageal carcinoma. J Clin Oncol Off J Am Soc Clin Oncol. 2006;24:748–54.
34. Nakahara C, Nakamura K, Yamanaka N, Baba E, Wada M, Matsunaga H, et al. Cyclosporin-A enhances docetaxel-induced apoptosis through inhibition of nuclear factor-kappaB activation in human gastric carcinoma cells. Clin Cancer Res Off J Am Assoc Cancer Res. 2003;9:5409–16.
35. Manu KA, Shanmugam MK, Ramachandran L, Li F, Fong CW, Kumar AP, et al. First evidence that gamma-tocotrienol inhibits the growth of human gastric cancer and chemosensitizes it to capecitabine in a xenograft mouse model through the modulation of NF-kappaB pathway. Clin Cancer Res Off J Am Assoc Cancer Res. 2012;18:2220–9.
36. Maeda S, Yoshida H, Ogura K, Mitsuno Y, Hirata Y, Yamaji Y, et al. H. pylori activates NF-kappaB through a signaling pathway involving IkappaB kinases, NF-kappaB-inducing kinase, TRAF2, and TRAF6 in gastric cancer cells. Gastroenterology. 2000;119:97–108.
37. Ueda M, Kokura S, Imamoto E, Naito Y, Handa O, Takagi T, et al. Blocking of NF-kappaB activation enhances the tumor necrosis factor alpha-induced apoptosis of a human gastric cancer cell line. Cancer Lett. 2003;193:177–82.
38. Sohma I, Fujiwara Y, Sugita Y, Yoshioka A, Shirakawa M, Moon JH, et al. Parthenolide, an NF-kappaB inhibitor, suppresses tumor growth and enhances response to chemotherapy in gastric cancer. Cancer Genomics Proteomics. 2011;8:39–47.
39. Zhang D, Qiu L, Jin X, Guo Z, Guo C. Nuclear factor-kappaB inhibition by parthenolide potentiates the efficacy of Taxol in non-small cell lung cancer in vitro and in vivo. Mol Cancer Res MCR. 2009;7:1139–49.
40. Sintara K, Thong-Ngam D, Patumraj S, Klaikeaw N, Chatsuwan T. Curcumin suppresses gastric NF-kappaB activation and macromolecular leakage in Helicobacter pylori-infected rats. World J Gastroenterol WJG. 2010;16:4039–46.
41. Kunnumakkara AB, Diagaradjane P, Guha S, Deorukhkar A, Shentu S, Aggarwal BB, et al. Curcumin sensitizes human colorectal cancer xenografts in nude mice to gamma-radiation by targeting nuclear factor-kappaB-regulated gene products. Clin Cancer Res Off J Am Assoc Cancer Res. 2008;14:2128–36.
42. Notarbartolo M, Poma P, Perri D, Dusonchet L, Cervello M, D'Alessandro N. Antitumor effects of curcumin, alone or in combination with cisplatin or doxorubicin, on human hepatic cancer cells. Analysis of their possible relationship to changes in NF-kB

activation levels and in IAP gene expression. Cancer Lett. 2005;224:53–65.
43. Yu LL, Wu JG, Dai N, Yu HG, Si JM. Curcumin reverses chemoresistance of human gastric cancer cells by downregulating the NF-kappaB transcription factor. Oncol Rep. 2011;26:1197–203.
44. Zhou B, Zuo Y, Li B, Wang H, Liu H, Wang X, et al. Deubiquitinase inhibition of 19S regulatory particles by 4-arylidene curcumin analog AC17 causes NF-kappaB inhibition and p53 reactivation in human lung cancer cells. Mol Cancer Ther. 2013;12:1381–92.
45. Wang Y, Xiao J, Zhou H, Yang S, Wu X, Jiang C, et al. A novel monocarbonyl analogue of curcumin, (1E, 4E)-1,5-bis (2, 3-dimethoxyphenyl) penta-1,4-dien-3-one, induced cancer cell H460 apoptosis via activation of endoplasmic reticulum stress signaling pathway. J Med Chem. 2011;54:3768–78.