

UC Irvine

UC Irvine Previously Published Works

Title

A novel chalcone derivative S17 induces apoptosis through ROS dependent DR5 up-regulation in gastric cancer cells.

Permalink

<https://escholarship.org/uc/item/1zn3h39s>

Journal

Scientific reports, 7(1)

ISSN

2045-2322

Authors

Zhang, Saiyang
Li, Tingyu
Zhang, Li
et al.

Publication Date

2017-08-01

DOI

10.1038/s41598-017-10400-3

Peer reviewed

SCIENTIFIC REPORTS



OPEN

A novel chalcone derivative S17 induces apoptosis through ROS dependent DR5 up-regulation in gastric cancer cells

Saiyang Zhang^{1,2}, Tingyu Li¹, Li Zhang¹, Xiangyu Wang¹, Hangqi Dong¹, Lili Li¹, Dongjun Fu¹, Yongchun Li¹, Xiaolin Zi³, Hong-Min Liu¹, Yanbing Zhang¹, Hongde Xu¹ & Cheng-Yun Jin¹

A new series of etherification chalcone derivatives were designed and synthesized through Willimison etherification and Claisen-Schmidt condensation. Among them, compound 2-c which was given chemical name of *S17*, has been successfully screened out as the most potent one on gastric cancer cell line (MGC803) through the investigation for their effects against the growth of five cancer cell lines (EC109, HepG2, MCF7, MGC803, SKNSH). *S17* exhibited strong anti-proliferative activity on other two gastric cancer cells (HGC27 and SGC7901), but less cytotoxicity to non-malignant gastric epithelial cells GES1. *S17* potently killed gastric cancer cells with causing modulation of Bcl-2 family proteins and activation of caspase 9/3 cascade. *S17* also up-regulated DR5 expression and DR5 knockdown partially reversed *S17*-induced apoptosis, caspase activation and MMP decrease. *S17* robustly induced generation of ROS with Keap/Nrf2 pathway activated and the application of ROS scavenger N-acetyl cysteine (NAC) completely blocked these effects by *S17* in MGC803 cells. Intraperitoneal administration of *S17* significantly inhibited the growth of MGC803 cells *in vivo* in a xenograft mouse model without observed toxicity. These results indicated that *S17* is a leadbrominated chalcone derivative and deserves further investigation for prevention and treatment of gastric cancer.

Gastric cancer is a kind of occurring commonly cancer in gastrointestinal tract cancer¹. In recent years, attention has been focused on the anti-cancer properties of natural products, which play an important role in the prevention of cancers². As an important candidates of the subclasses of the flavonoid family, chalcone derivatives are the precursors of the flavones in the biosynthesis of flavonoids and a large amount of which have been applied as anti-platelet, anti-inflammatory, anti-allergic, antimicrobial, antioxidant or anti-tumor agent^{3,4}. The most classical and general synthetic route of chalcone derivatives was the Claisen-Schmidt condensation among the reported ones⁵. Chalcone and its derivatives display a wide range of important pharmacological activities and have a huge importance in medicinal chemistry⁶. As reported, chalcone, coumarins and flavanones from the exudate of *Angelica keiskei* have chemopreventive effects⁷. Isobavachalcone exhibits anti-proliferative effects towards several human cancer cells through blocking of Akt signaling⁸. A chalcone panduratin A isolated from *Kaempferia pandurata* induce apoptosis and cell cycle arrest in androgen-independent human prostate cancer cells PC3 and DU145⁹. These observations suggested that naturally-occurring chalcone can be further optimized through synthesis of their derivatives as new anti-cancer agents to effectively treat certain cancers.

Cell apoptosis, or programmed cell death acted as one of the most important manner in regulation of carcinogenesis¹⁰. In the initial of apoptotic process, it triggers an activation of apoptotic signaling program leading to cell death rather than kills cells directly. Reactive oxygen species (ROS), a cellular metabolite which regulates multiple cancer-related signalling pathways appears to be an important regulatory signal of cell apoptosis¹¹. Nowadays, it is

¹School of Pharmaceutical Sciences, Key Laboratory of State Ministry of Education, Key Laboratory of Henan province for Drug Quality Control and Evaluation, Collaborative Innovation Center of New Drug Research and Safety Evaluation, Zhengzhou University, 100 Kexue Avenue, Zhengzhou, Henan, 450001, China. ²School of Basic Medicinal Science, Zhengzhou University, 100 Kexue Avenue, Zhengzhou, Henan, 450001, China. ³Departments of Urology, Pharmacology and Pharmaceutical Sciences, University of California, Irvine, Orange, USA. Saiyang Zhang and Tingyu Li contributed equally to this work. Correspondence and requests for materials should be addressed to H.X. (email: xhd1220@zzu.edu.cn) or C.-Y.J. (email: cyyjin@zzu.edu.cn)

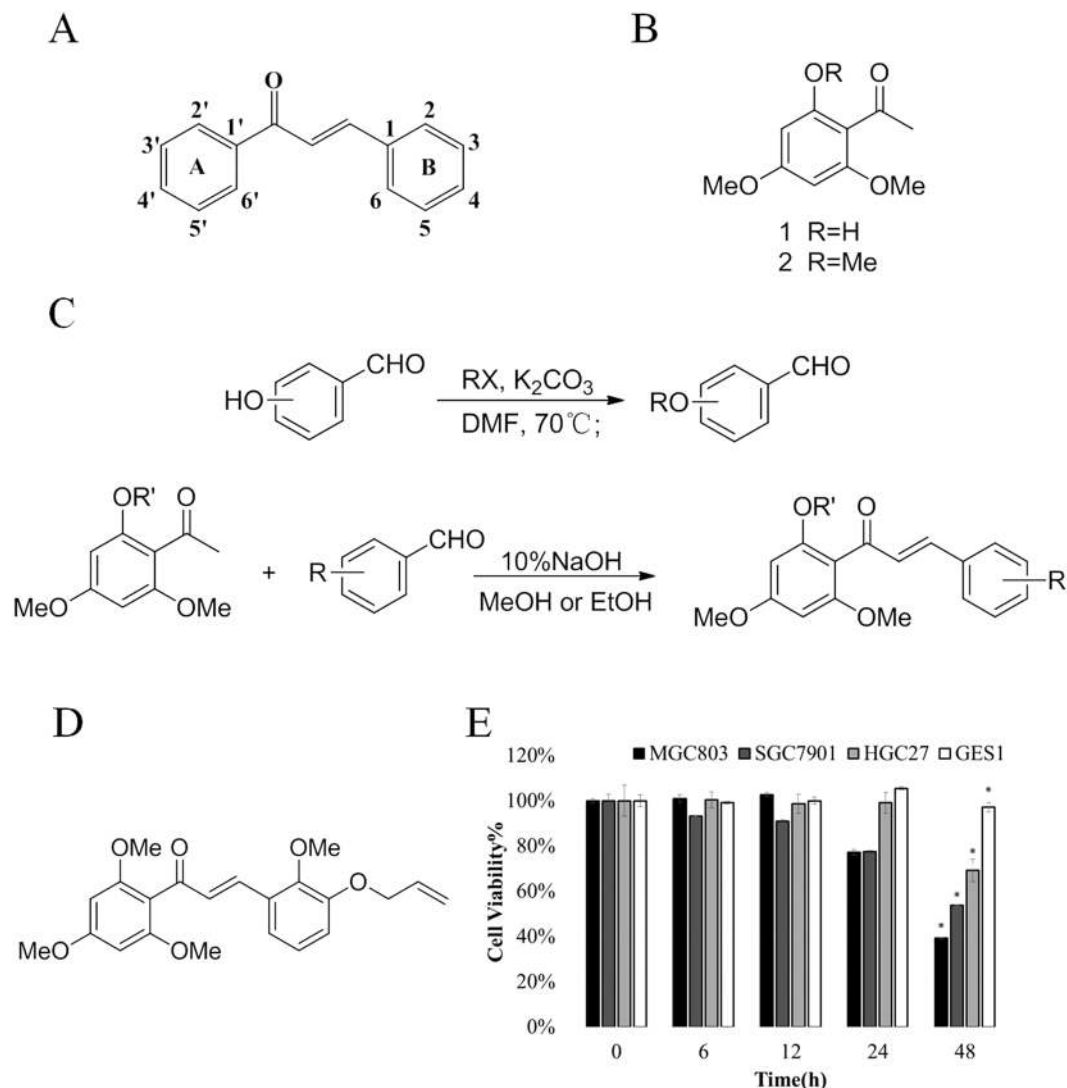


Figure 1. **S17** inhibited cell proliferation in gastric cancer cells. **(A)** Basic structure of chalcone. **(B,C)** Synthetic of analogues of Flavokawain A. **(D)** Structure of **S17**. **(E)** The cytotoxicity of **S17** by MTT assay. The cells were treated with **S17** (10 μ M) at indicated time points. * $p < 0.05$ vs. untreated group.

significantly recognized that ROS are involved in the function of antitumor, because high levels of ROS cause cell damage by oxidation and nitration of macromolecules including RNA, DNA, lipids, and proteins, as well as cause DNA damage and apoptosis^{12,13}. SL4, a chalcone-based compound, induces apoptosis by activation of the ROS/MAPK signaling pathway in human cancer cells *in vitro*¹⁴ and in another study, a new synthetic 2'-hydroxy-2,4,6-trimethoxy-5',6'-naphthochalcone induces cell apoptosis and cycle arrest of human colon cancer cells¹⁵. In this study, a series of etherified chalcone derivatives were rationally designed and their antitumor activities evaluated.

Among them **S17** which was designed and synthesized for the first time exhibited strong cytotoxic effect against gastric cancer cells. We discussed the mechanism of **S17** on gastric cancer cell MGC803 with reactive oxygen species (ROS) causing apoptosis via mitochondria apoptotic pathway and through upregulation of DR5. DR5 knockdown indeed partially reversed the mitochondrial membrane potential decrease and apoptosis. At the same time the increasing ROS activated the Nrf2/HO-1 axis in a short time. We also evaluated antitumor activity of **S17** in a MGC803 tumor bearing xenograft mice model *in vivo*. The significant antitumor effects of **S17** have been confirmed both *in the vitro* and *vivo* experiments.

Results

S17 showed significant inhibition of proliferation of human gastric cancer cells (MGC803, HGC27 and SGC7901) with minimal toxicity to non-malignant human gastric epithelial cells GES-1. Etherification on ring A and B occurs relatively infrequently (Fig. 1A). Chalcone derivatives with multiple methoxy substituted on ring A and B have never been reported. Their anticancer activities haven't been elaborated. Therefore, a new series of etherification chalcone derivatives were designed and synthesized through Willimison etherification and Claisen-Schmidt condensation (Fig. 1B,C). Based on the screening results of the

synthesized compounds for inhibiting the growth of five cancer cell lines, **S17** was prioritized to perform further experiment for evaluating its anti-cancer potential in gastric cancer (Fig. 1D). Furthermore, the IC₅₀ value of **S17** for MGC803 is $6.754 \pm 0.830 \mu\text{M}$, SGC7901 is $9.285 \pm 0.968 \mu\text{M}$ and HGC27 is $12.292 \pm 1.090 \mu\text{M}$, exhibiting better cytotoxicity than other cell lines. Therefore, we chose **S17** and gastric cancer cells for the further experiment.

To evaluate the effects of **S17** on human gastric cancer cells, three gastric cancer cell lines (MGC803, HGC27 and SGC7901) and human gastric epithelial cells (GES1) were incubated with **S17** (10 μM) for indicated time points, and then the effects of **S17** on reducing cell viabilities were measured by an MTT assay. As shown in Fig. 1E, following treatment with **S17**, the viability of the gastric cancer cells decreased in a time-dependent manner. MGC803 cells showed the most sensitivity to **S17** treatment, causing 60.77% viability reduction at 48 h in related to control treatment. However, it is almost no toxicity to human gastric epithelial cells (GES1) (Fig. 1E). Taken together, these results suggested that **S17** has selective cytotoxicity against gastric cancer cells *versus* normal human gastric epithelial cells.

S17 induced caspase-dependent apoptosis of MGC803 cells. Further experiments were conducted to determine whether the inhibition of **S17** on the viability of gastric cancer cells was the result of apoptotic cell death. In Fig. 2A, we evaluated the apoptotic induction of **S17** on MGC803 with Annexin V and PI staining and found that the numbers of Annexin V positive cells showed gradually increase in a time-dependent manner from 12.0% to 58.7% in MGC803, whereas control treatment only resulted in 5.0% Annexin V positive staining cells. Morphological changes of MGC803 cells were then determined using DAPI staining, as shown in Fig. 2B, a significant number of cells with chromatin condensation were observed in cells treated with **S17**, whereas these features were not observed in control cells. Caspases are known as key executioners of apoptosis through the cleavage of various cellular substrates¹⁶. To further investigate whether **S17**-induced apoptosis is associated with the caspase cascade leading to proteolytic cleavage of poly(ADP-ribose) polymerases-1 (PARP-1) in MGC803 cells¹⁷, we assessed cleavage of caspases (3, 8 and 9) and PARP-1. As shown in Fig. 2C, Western blot analyses revealed that treatment of MGC803 cells with **S17** (10 μM) down-regulated the levels of pro-caspase3, pro-caspase9 and pro-PARP-1 proteins accompanied by an increase in levels of cleaved caspase3, 8, 9 and PARP-1 proteins compared to the control treatment in MGC803 cells.

To further evaluate the significance of caspase activation in **S17**-induced apoptosis, MGC803 cells were pretreated with z-VAD-fmk (100 μM) for 1 h, followed by treatment with 10 μM **S17** for 48h¹⁸. As shown in Fig. 2D, Pretreatment with z-VAD-fmk efficiently attenuated **S17**-induced apoptosis from 54.5% to 23.3% according to flow cytometry analysis. Taken together, these results suggest that **S17**-induced apoptosis is dependent of caspase activation in MGC803 cells.

S17-induced apoptosis involves the modulation of Bcl-2 and IAP family proteins, attenuation of Mitochondrial membrane potential (MMP/ $\Delta\Psi$) in MGC803 Cells. Mitochondria play an essential role in cell apoptosis pathway^{19,20} and the mitochondria-dependent apoptotic pathway is regulated by the Bcl-2 family of pro- and anti-apoptotic proteins^{21,22}. To determine the role of the mitochondria in **S17**-induced apoptosis of gastric cancer cells, the change of MMP ($\Delta\Psi$) was measured. As shown in Fig. 3A, **S17** significantly reduced the MMP level in a time-dependent manner in MGC803 cells. And pretreatment with z-VAD-fmk (the pan-Caspase inhibitor) which can block the process of apoptosis significantly reversed **S17**-induced decrease of the MMP level as shown in Fig. 3B. The role of the Bcl-2 and the IAP family proteins was determined by western blotting to explore the possible mechanisms involved in **S17**-induced apoptosis in MGC-803 cells. As shown in Fig. 3C, in comparison with the control cells, **S17** treatment of MGC803 cells led to a significant increase in the protein levels of Bim_{EL}, Bax, t-Bid and a reduction in the protein levels of XIAP in a time-dependent manner, whereas the levels of anti-apoptotic Bcl-2, Bcl-xL and death receptor family protein DR4 were not changed. These results revealed that the mitochondrial pathway plays an important role in **S17** induced apoptosis in gastric cancer cells.

S17-induced apoptosis is related to Death Receptor 5. Apoptosis usually happens following the death ligand binding to corresponding receptors such as DR4 and DR5²³. And we found that the death receptor DR5 expression level was obviously increased after treatment of 10 μM **S17** for 24 hours while DR4 wasn't influenced as shown in Fig. 4A. And this was confirmed by analysis of expression of DR5 in MGC803 cells by flow cytometry as shown in Fig. 4B. Then we knocked down the protein DR5 in MGC803 cells. The cell death induced by **S17** were attenuated significantly as shown in Fig. 4C,D. We also detected that the cleavage of Caspase3 and PARP-1 decreased to the modest degree as shown in Fig. 4E. Interestingly we also found that **S17**-induced MMP decrease was partially reversed in DR5^{-/-} MGC803 cells as shown in Fig. 4F. These results together revealed that DR5 is associated with **S17**-induced apoptosis and mitochondria pathway.

S17 induces generation of ROS in MGC803 cells and activation of keap1/Nrf2 pathway. The generation of intracellular ROS has been shown to be related to the induction of apoptosis in various cell types²⁴. We therefore measured ROS production in MGC803 cells by flow cytometry analysis of cellular DCFH-DA fluorescence intensity. As shown in Fig. 5A, treatment of MGC803 cells with 10 μM of **S17** resulted in a time-dependent increase of ROS generation. Since elevated ROS activates Nrf2 and its subsequent nuclear-translocation and increases the downstream proteins expression²⁵, as shown in Fig. 5B,C, we confirmed the increasing expression of proteins like p-Nrf2, HO-1 and NQO1 and the immunofluorescence assay also showed accumulation of Nrf2 around the nuclear and on the chromatin. However, we also noted that Nrf2 expression dramatically decreased if not vanished and NQO1 expression also reached its peak at 3 h after **S17** treatment indicating Nrf2 didn't happen accompanied with **S17** all the time. These results together suggest **S17**

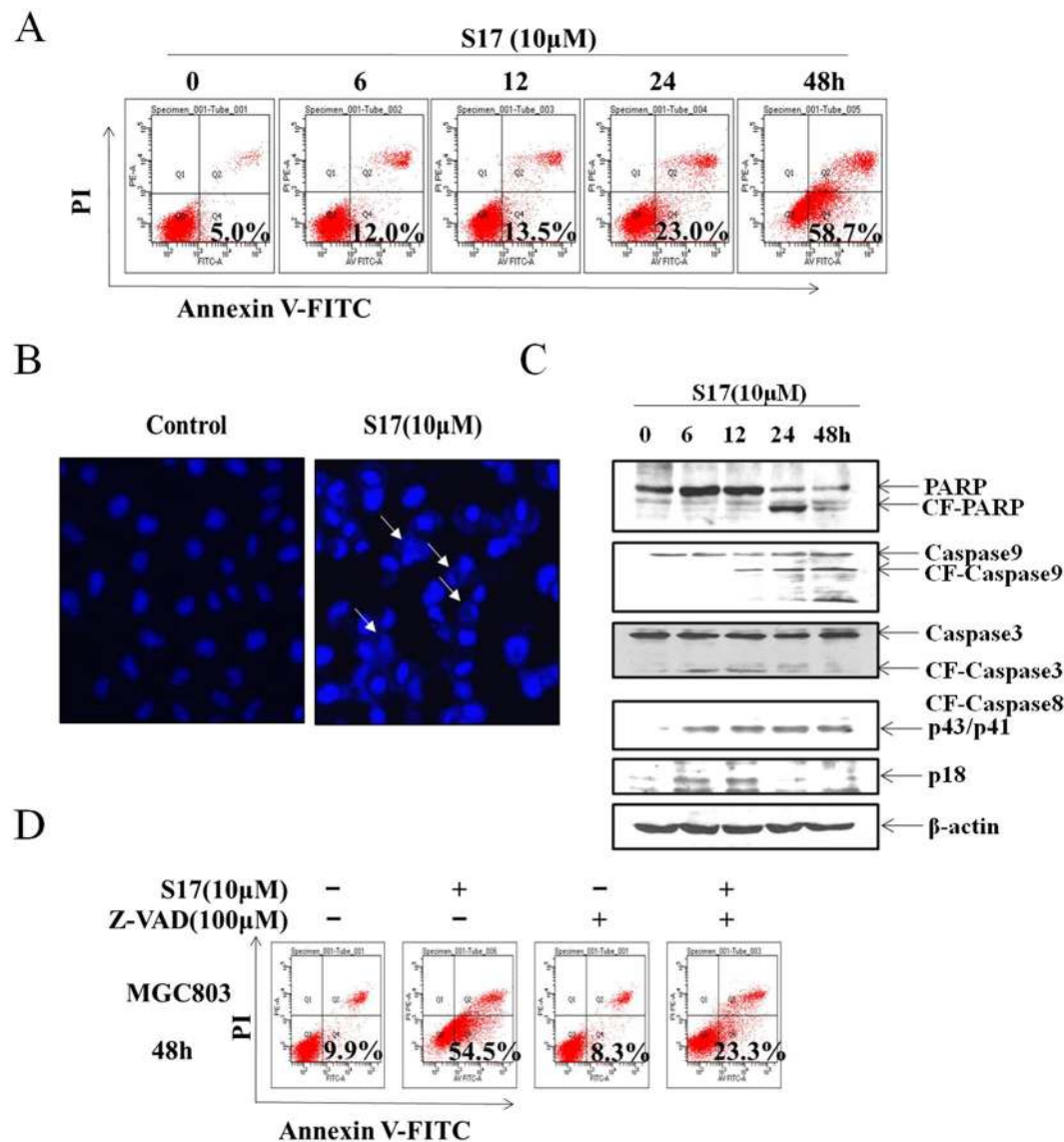


Figure 2. *S17* induced apoptosis in MGC803 cells. (A) *S17* induced cell apoptosis of MGC803 cells in a time-dependent manner by Flow Cytometer analysis. (B) MGC803 cells were stained with DAPI solution. Stained nuclei were then observed under a fluorescence microscope using a blue filter (magnification, 400X). The arrow was indicating chromatin condensation. (C) Western blotting assay showed the effects of *S17*(10 μ M) on the expression CF-PARP, CF- caspase3, CF- caspase8 and CF-caspase9. (D) Flow Cytometric analysis demonstrated the effect of the inhibitor of caspases (Z-VAD-fmk, 100 μ M) on *S17*(10 μ M)-induced cell apoptosis of MGC803 cells for 48 h.

indeed induced the generation of ROS and the activation of keap1/Nrf2 pathway was meant to protect cells from the injury by ROS, although it didn't reverse *S17*-induced cell death.

***S17*-induced Apoptosis is associated with the generation of ROS.** Next, to determine whether *S17*-induced ROS production was attributable to the cell inhibition and apoptosis induction, the cells were pretreatment with NAC for 1 h and co-incubated with 10 μ M of *S17* for a further 48 h. Figure 6A,B revealed that pretreatment with NAC almost completely attenuated *S17*-induced cell inhibition in three gastric cancer cells and completely attenuated apoptosis in MGC803 cells. Blocking the generation of ROS by pretreating MGC803 cells with NAC prevented *S17*-induced modulation of Bcl-2 and IAP family proteins, activation of caspases, cleavage of PARP-1, up-regulation of DR5 and also abrogated the decrease of MMP level. And in SGC7901 cells, NAC also decreased the expression levels of Bax, DR5 and CF-PARP proteins induced by *S17*, which were consistent with the effect of NAC on MGC803 (Fig. 6C,D). In order to demonstrate how *S17* induces lethal ROS generation in gastric cancer cells, MGC803 cells were pretreated 1 h with the different functional ROS inhibitors include Catalase (CAT), Butylhydroxyanisole (BHA), GKT137831 (GKT), Rotenone (ROT), Neohesperidin (NEO), and Apocynin (APO). Figure 6E exhibited that the cytotoxicity of *S17* was significantly attenuated by BHA and CAT

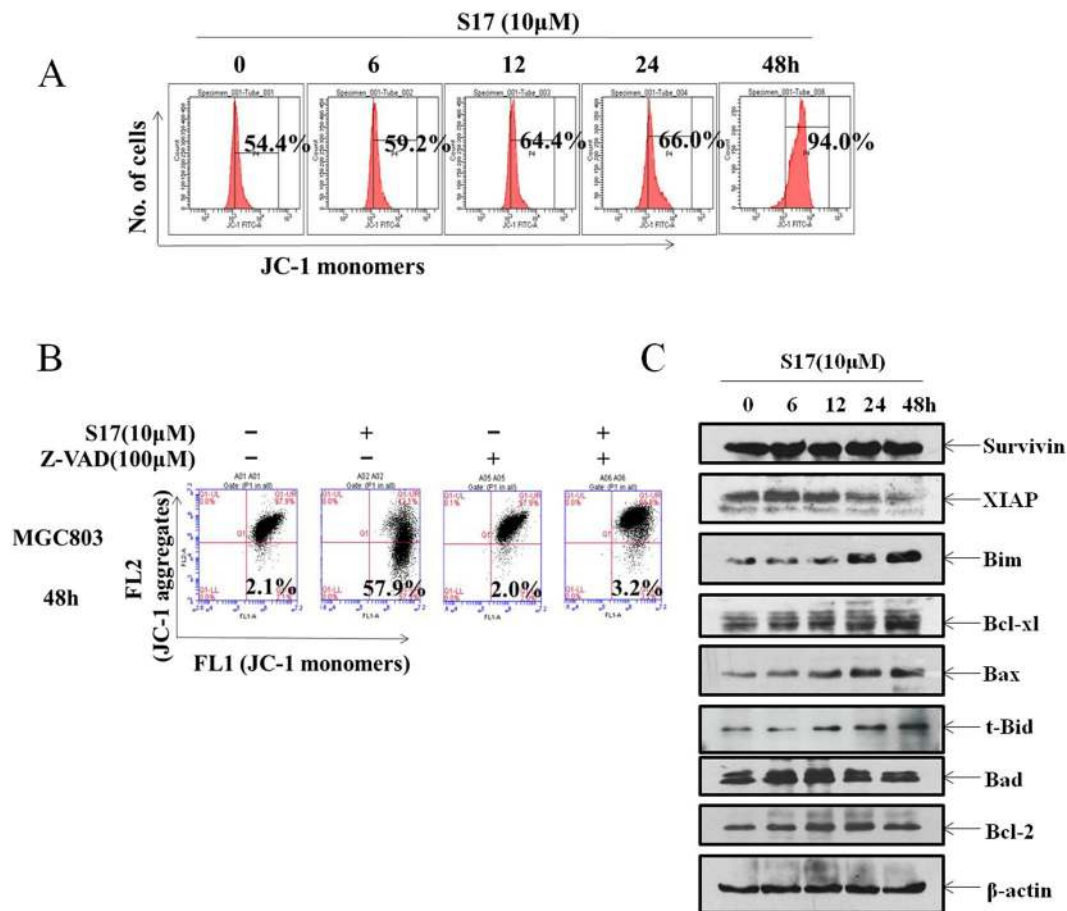


Figure 3. *S17*-induced apoptosis involves Mitochondrial pathway in MGC803 Cells. (A) *S17* decreased the membrane potential ($\Delta\Psi$) of mitochondria. Membrane potential was measured by JC-1 dye retention using Flow Cytometry. (B) Flow Cytometric analysis demonstrated the effect of the inhibitor of caspases (Z-VAD-fmk, 100 μ M) on *S17* (10 μ M)-induced decrease of the mitochondria membrane potential ($\Delta\Psi$) of MGC803 cells for 48 h. (C) The protein levels of Bax, Bim_{EL}, Bcl-2, t-Bid, Bcl-XL, Bad, XIAP and Survivin were determined by Western blotting assay.

compared with the rest of ROS inhibitors. These data suggested that the generation of ROS is required for the *S17*-induced apoptosis in gastric cancer cells.

***S17* inhibits *in vivo* tumor growth in the xenograft nude mouse model of MGC803 cells.** There is no significant difference in mean body weights over time between control and *S17* treated groups (Fig. 7A) (Student's t test; $P > 0.05$). The growth rate of MGC803 xenograft tumors from mice which were treated with *S17* was lower than those from the vehicle control-treated mice. (Fig. 7B). The mean of wet tumor weights in the *S17* treated mice was about 60% less than that of the control treated mice (Student's t test; $P < 0.01$) (Fig. 7C). In order to determine whether xenograft tumor cells treated with *S17* underwent ROS-mediated apoptosis, immunohistochemical assay was performed to determine changes of cleaved-caspase3, cleaved-caspase8 proteins expression and the levels of ROS in xenograft tumors treated with *S17*. As shown in Fig. 7D, *S17* treatment led to an increase in expression of cleaved caspase 3, cleaved caspase 8 and ROS. These results demonstrated the anti-tumor activity and the apoptotic effect of *S17* against gastric cancer cells *in vivo*.

Discussion

Carcinogenesis is a complex process which consists of a series proliferative signaling, including evasion of growth suppression, damaged extracellular matrix (ECM) components, resistance to cell death, uncontrolled proliferation as well as increasing invasion and metastasis of cancer cells^{26, 27}. Thus polypharmacological approaches for developing anti-tumour drugs attract much interests of investigator. In recent years, naturally occurring botanicals and their derivatives are attracting considerable attention as cancer chemopreventive agents^{28, 29}. Chalcones which belong to a specific class of flavonoids frequently occur in fruits, vegetables and beverages (tea, coffee, beer, wine and fruit drinks)³⁰, and a new brominated chalcone derivative *H72* in our previous work has been proved to effectively induce apoptosis in gastric cancer cells³¹.

In this study, we have demonstrated that *S17*, a novel chalcone derivative, selectively inhibits the growth of gastric cancer cells MGC803 by inducing cell apoptosis through ROS-mitochondria apoptotic pathway. *S17* induced

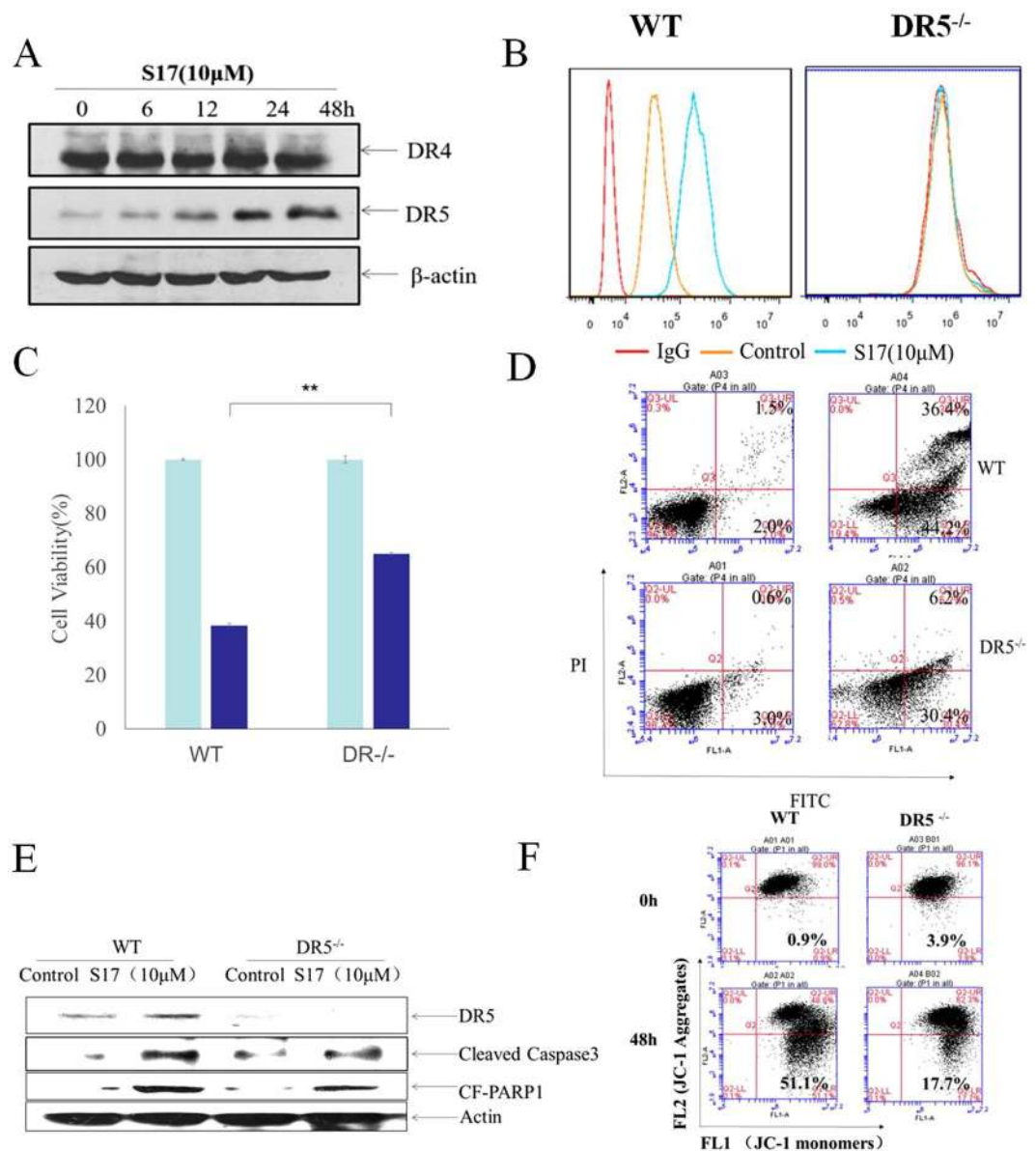


Figure 4. *S17* induced cell apoptosis of MGC803 cells involved with extrinsic pathway. Both wild type and DR5^{-/-} MGC803 cells were treated of 10 μM *S17* for indicated time. (A) *S17* increases DRs protein expression in MGC803 cells at a time-dependent manner. (B) DR5 protein expressions in MGC803 cells were determined by flow cytometer analysis after *S17* incubation for 24 hours. (C) Cell viabilities were measured by MTT assay after 30 hours *S17* incubation. ***p* < 0.01 vs. untreated group. (D) The ratios of apoptotic cells were determined by Flow Cytometric analysis after *S17* incubation for 48 hours. (E) The expression levels of apoptosis-related proteins were tested by western blotting assay after *S17* incubation for 24 hours. (F) Flow Cytometric analysis demonstrates MMP($\Delta\Psi$) decrease after 48 hours treatment of *S17*.

robust ROS obviously detected by flow cytometer analysis in MGC803 cells. It led to the apoptosis through DR5 and mitochondrial apoptotic pathway.

Since mitochondrial respiration are the main source of ROS, mitochondria are considered to be the major target of ROS damaging and increase in ROS level can damage mitochondrial membrane and result in apoptosis by oxidizing mitochondrial pores, thereby disrupting the mitochondrial membrane potential(MMP)^{32,33}. In this study, we demonstrated the ability of *S17* to induce mitochondrial dysfunction, by detecting the MMP.

In addition, ROS inhibitors with different function were utilized to investigate how *S17* induces lethal ROS generation in MGC803 cells. Butylhydroxyanisole (an antioxidant acts as a radical-scavenger) and catalase (H₂O₂ enzyme) decreased the death of MGC803, due to oxygen free radicals and H₂O₂ are mainly derived from mitochondrial aerobic respiration and the metabolic process, and is the byproduct source of mitochondrial electron transport chain (ETC, includes complex members I-IV)³⁴. We utilized Rotenone (an irreversible inhibitor of complex I) and Apocynin (a selective NADPH-oxidase inhibitor) to detect whether the generation of ROS is associated with ETC, but Rotenone and Apocynin failed to reverse the cytotoxicity of *S17*, ETC may be unrelated with

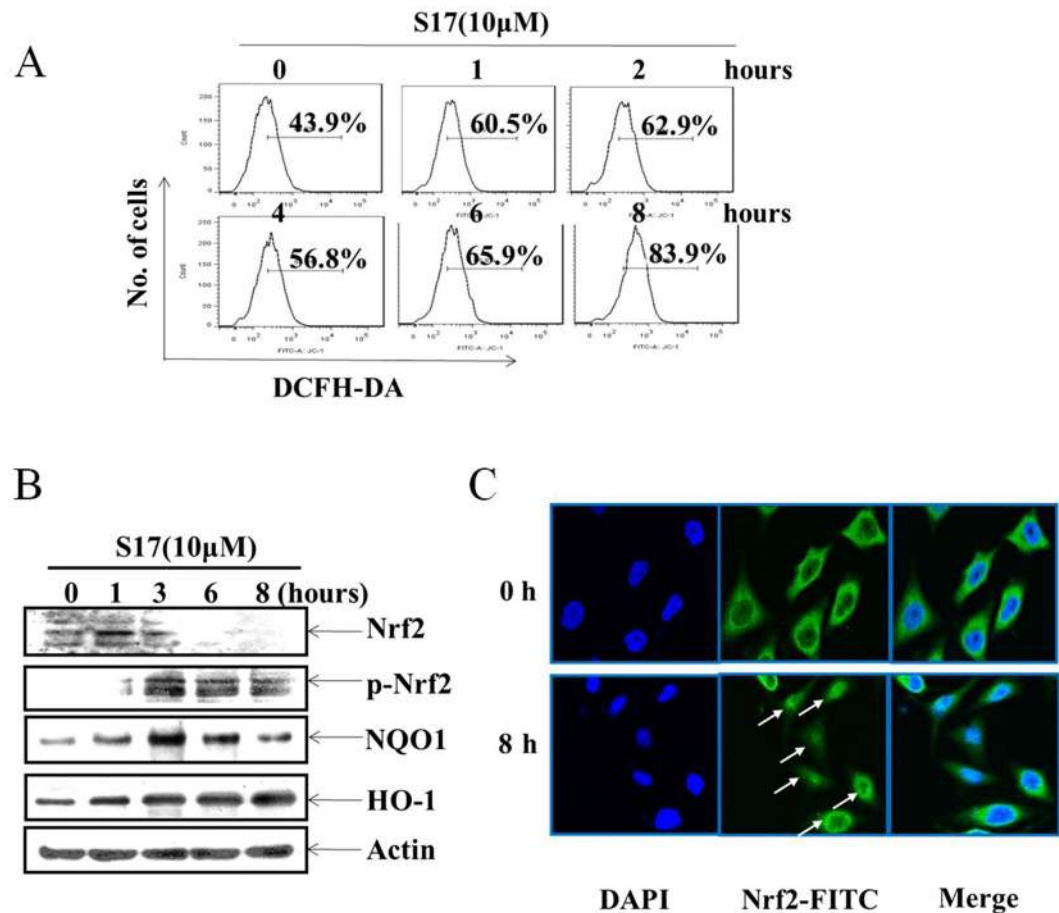


Figure 5. *S17* induced ROS elevating in gastric cancer cells. (A) Measurement of ROS. MGC803 cells were treated with *S17* (10 μ M) for 0,1,2,4,6,8 hours. The level of ROS was measured by DCFH-DA with Flow Cytometry. (B) Western blotting demonstrated *S17* (10 μ M)-induced protein expression changes at indicated time points. (C) MGC803 cells were treated with *S17* (10 μ M) for 8 hours. The treated and untreated samples are stained with Nrf2 antibody (Green) and DAPI (Blue) (magnification, 400X). The arrow was indicating Nrf2 nuclear-translocation.

the generation of ROS induced by *S17*. However, ROS from mitochondria are not all derived from mitochondrial electron transport chain, mitochondrial H₂O₂ also available from monoamine oxidase, and generated from oxidation of cytochrome *c* directly by the oxidoreductase p66shc³⁵, which may be as the target for *S17* to induce apoptosis and deserve to be further investigated. Furthermore, the associated mechanisms of Neohesperidin (an antioxidant activity in scavenging the DPPH radical) and GKT137831 (dual Nox1/Nox4 inhibitor) could be excluded, however, how *S17* induces lethal ROS generation in gastric cancer cells MGC803 needs to be further investigated.

The mitochondrial associated apoptosis pathway involves mitochondrial outer membrane permeabilization followed by release of cytochrome *c* into the cytosol, which induces a caspase cascade culminating in cell death^{36,37}. On the other hand, in the cytosol, cytochrome *c* triggers activation of initiator caspase 9 could then activate the effector caspase 3 to contribute to activation the mitochondrial apoptosis pathway. Executioner caspase-3 is responsible for cleaving its substrates, such as poly ADP-ribose polymerase 1 (PARP-1), subsequently inducing apoptosis^{38,39}. Western blot analysis showed that caspase 9, caspase 8, caspase 3 and PARP-1 were all activated in a time-dependent manner by treatment with *S17* in MGC803 cells (Fig. 3A), and the activated caspase 8 can make Bid cleaved into t-Bid triggering the mitochondria apoptotic pathway, elucidating the interplay between extrinsic and intrinsic apoptosis pathway induced by *S17* is essential. Mechanistically, *S17* activated caspases and modulated XIAP and Bcl-2 member proteins involved in mitochondrial apoptotic pathway *in vitro*. Inhibitors of apoptosis (IAP) proteins are endogenous inhibitors of apoptosis⁴⁰, and the best characterized of these are XIAP and survivin. They respectively inhibit the activation of caspase-9 and caspase-3, thereby negatively regulating apoptosis. Our results showed that the decreased expression of XIAP, which might contribute to the reason of caspase-9 activation induced by *S17*. However, there was no change in survivin after treatment with *S17*, indicating that activation of caspase-3 by *S17* was mediated by upstream signaling. Bcl-2 family proteins are important regulators of mitochondrial apoptosis approach, which govern MMP and can be either pro-apoptotic, such as Bax, or anti-apoptotic such as and Bcl-2, and Bcl-xL. Even if caspase proteins are generally required in apoptosis

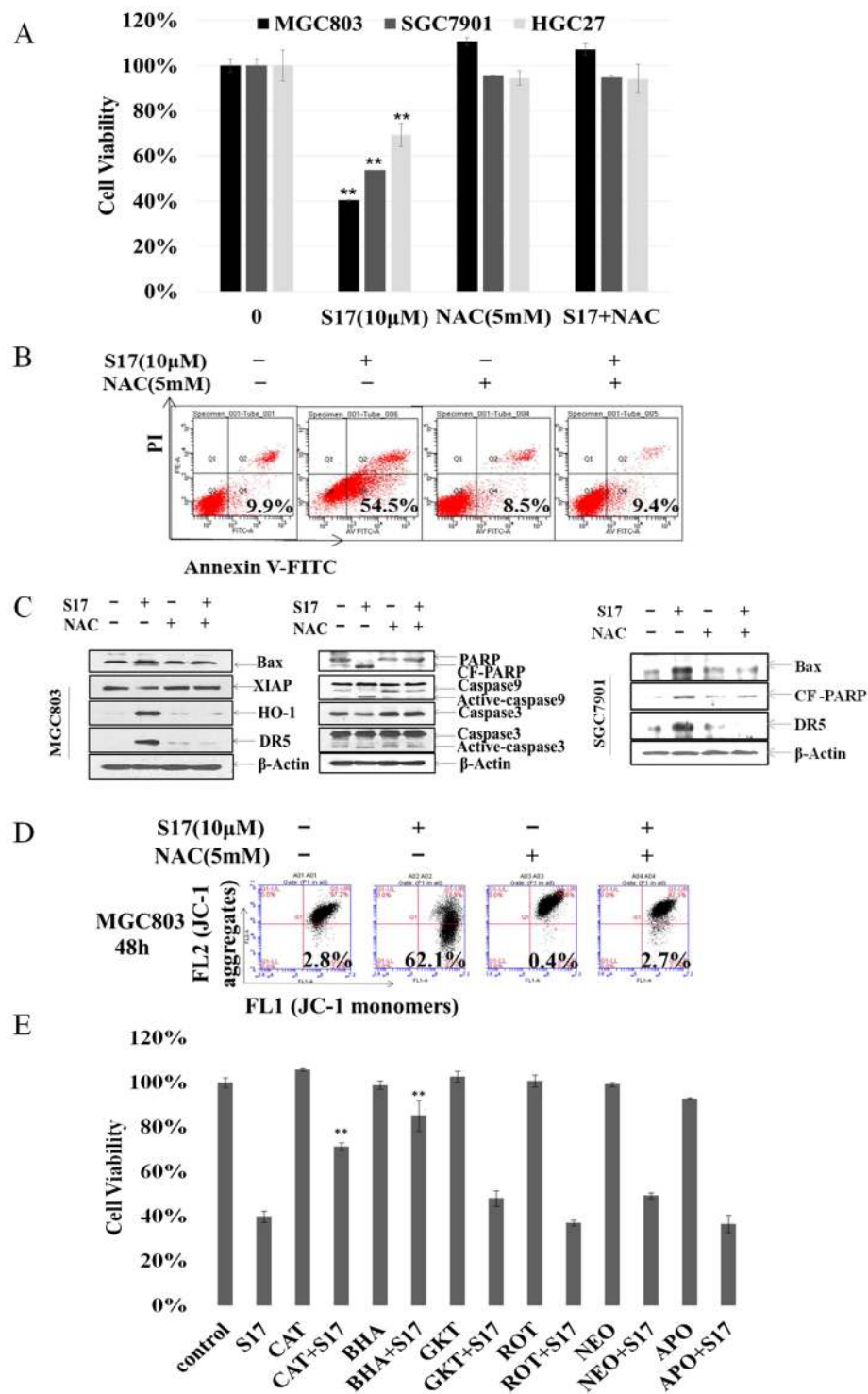


Figure 6. *S17* induced apoptosis is related with elevating ROS level in gastric cancer cells. (A) MTT assay demonstrated the effect of NAC (5 mM) on *S17*-induced cell death of MGC803, HGC27 and SGC7901 cells at 48 h. ** $p < 0.01$ vs untreated group. (B) Flow Cytometric analysis showed the effects of NAC (5 mM) on *S17*(10 μ M)-induced cell apoptosis of MGC803 cells. (C) Western blotting demonstrated the effect of NAC (5 mM) on *S17*(10 μ M)-induced protein expression changes at 48 h. (D) Flow Cytometric analysis showed the effect of NAC (5 mM) on *S17*(10 μ M)-induced loss of membrane potential ($\Delta\Psi$) in MGC803 cell mitochondria at 48 h. (E) MTT assay demonstrated the effect of different ROS inhibitors (CAT (250 μ M), BHA (15 μ M), GKT (200 μ M), ROT (125 nM), NEO (200 μ M) and APO (10 μ M)) on *S17* (10 μ M)-induced cell death of MGC803 at 48 h. ** $p < 0.01$ vs *S17* treatment group.

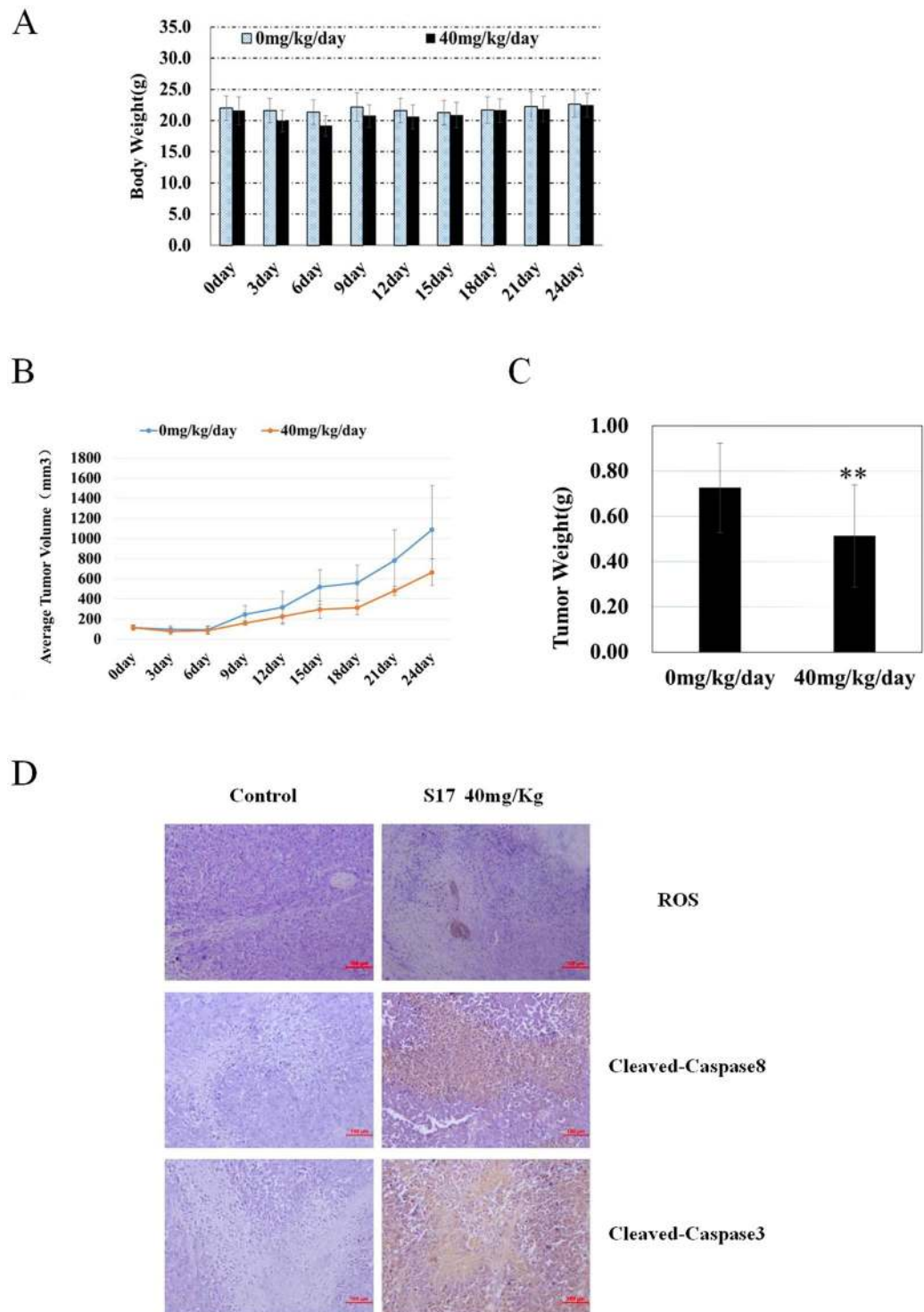


Figure 7. *S17* inhibited tumor growth in MGC803 xenograft model. Mice were respectively treated or not treated with *S17*. (A) The body weight-time bar charts. Statistical analyses demonstrated that the average volume (B) and weight (C) of MGC803 xenografts from *S17*-treated mice were significantly reduced. $**P < 0.01$ vs Control group. Treatment was initiated when the average size of the tumor reached 100 mm^3 . The test group were treated with indicated dosage in $20 \mu\text{l}$ of DMSO of *S17* every day and the control group received injection of DMSO alone. The mice ($n = 5$ per group) were treated for 21 days. (D) Immunohistochemical assays were performed in xenograft tumors to demonstrate the expression levels of cleaved caspase3, cleaved caspase8 and ROS induced by 40 mg/kg *S17*.

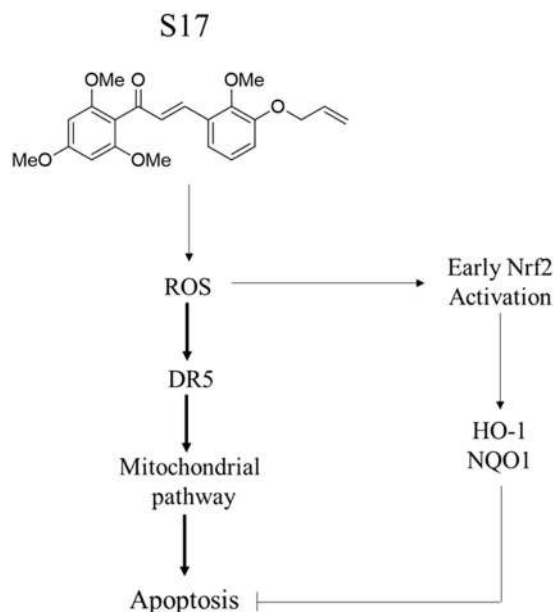


Figure 8. Summary of *S17*-induced apoptosis through ROS dependent DR5 up-regulation and Nrf2-mediated relief in gastric cancer cells.

process, Since z-VAD-fmk didn't completely reversed *S17*-induced apoptosis, we supposed there was another caspase-independent way involved in it. Thus, drugs which use ROS-mitochondrial apoptosis approaches in cancer cells may prove to be valuable anti-cancer therapeutics.

We detected that DR5 increased greatly after *S17* treatment. To investigate the role of DR5 in the regulation of *S17*-induced cell apoptosis. According to our results, MMP decrease was significantly reversed in DR5^{-/-} cells comparing to that in wild type MGC803 cells after 10 μ M *S17* treatment for 48 hours (Fig. 4F), indicating that DR5-related apoptotic pathway activated mitochondria pathway as was elaborated that caspase 8 makes Bid cleaved into t-Bid triggering the mitochondria apoptotic pathway⁴¹.

In addition, we found that the elevating level of ROS reflectively activated Nrf2 and promoted its translocation into nuclear and increased antioxidant enzymes expression such as HO-1, NQO1. Similarly as reported 15d-PGJ₂, a stable PGD₂ degradation product, causing intracellular redox imbalance in p53-deficient MG-63 OS cells induced p38 activation and Akt phosphorylation within 4 h followed by Nrf2 translocation into nuclear which acted as a protective pathway⁴². In addition, compared with *H72* in our previous work³¹, Nrf2 activation is a special mechanism and the apoptotic effects of *S17* on gastric cancer cell lines may be better than *H72* if the Nrf2 pathway be blocked. Nrf2/HO-1 signaling indeed play protection effect on the cells in spite of it didn't reverse apoptosis, Furthermore, our results showed Nrf2 decreased and NQO1 reached its peak at 3 h after *S17* treatment but HO-1 increased all the time. The underlying reason was until unknown and need further investigation.

This study showed that a novel chalcone derivate, *S17*, inhibited tumor growth *in vivo*. Moreover, we found that *S17* reduced tumor burdens in MGC803 xenografted mice without gross toxicity. In the MGC803 xenograft model, *S17* showed good antitumor activity and less toxicity for gastric cancer cells. Furthermore, immunohistochemical analysis showed *S17* treatment led to the increased levels of cleaved caspase 3 and cleaved caspase 8 in xenografted tumors. More importantly, the levels of ROS also were increased induced by *S17* compared with control, indicating the anti-tumor activity of *S17* against gastric cancer cells in xenograft tumors via ROS-mediated apoptosis manners.

Overall, *S17* may be a novel lead compound as a cancer drug candidate with polypharmacological properties. Therefore for our research work, we made a summary that *S17*-induced apoptosis through ROS dependent DR5 up-regulation and Nrf2-mediated relief in gastric cancer cells (Fig. 8), and we found *S17* of new synthesized chalcone is a promising candidate in drug discovery.

Methods

Reagents and chemicals. Fetal bovine serum (FBS), RPMI-1640, and penicillin-streptomycin were purchased from HyClone (Victoria, Australia). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), N-acetyl-L-cysteine (NAC) and JC-1 fluorescent dye (Sigma-Aldrich, St Louis, MO). Butylhydroxyanisole, Rotenone and Apocynin were purchased from MCE. GKT137831, Neohesperidin and z-VAD-fmk were purchased from Selleck. FITC/Annexin V Apoptosis Detection Kit (BestBio, Shanghai, China), Catalase and 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) was purchased from Beyotime Biotechnology (Shanghai, China). Antibodies specific for β -actin (sc-1615, goat, 1:1000), Bim (sc-11425, rabbit, 1:1000), Bax (sc-493, rabbit, 1:1000), Bad (sc-8044, mouse, 1:1000), caspase-3 (sc-7272, rabbit, 1:1000), caspase-9 (sc-7885, rabbit, 1:800), poly (ADP-ribose) polymerase-1 (PARP-1) (sc-7150, rabbit, 1:1000), DR4 (sc-7863, rabbit, 1:1000), DR5 (sc-65314, mouse, 1:1000) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies

specific for Bid (#2002, rabbit, 1:1000), Bcl-xL (#2764, rabbit, 1:1000), XIAP (#14334, rabbit, 1:1000) and cleaved caspase-8 (#9496, rabbit, 1:1000) were purchased from Cell Signaling Technology (Danvers, MA). The antibody specific for survivin (ab76424, rabbit, 1:2000) was purchased from Abcam (Cambridge, MA). Anti-DR5 (ab1675, goat, 1:500) antibody for flow cytometer were purchased from Abcam (Cambridge, MA). Peroxidase-labeled anti-goat (1:5000), anti-rabbit (1:5000) and anti-mouse (1:5000) polyclonal immunoglobulins were purchased from Bioss (Shanghai, China). The enhanced chemiluminescence (ECL) kit was purchased from Thermo Fisher (Waltham, MA).

Chemosynthesis. The synthetic method of the etherification chalcone derivatives was shown in Fig. 1B,C. Substituted benzaldehydes, which were synthesized by willimison etherification, reacted with 2-hydroxyl-4,6-dimethoxyl acetophenone (Compound 1 Fig. 1B) or 2,4,6-trimethoxyl acetophenone (Compound 2 Fig. 1B) by Claisen-Schmidt condensation reaction to afford a novel series of flavokawain A derivatives. The chemical structure of **S17** was identified using $^1\text{H-NMR}$ and $^{13}\text{C-NMR}$.

S17 was dissolved in DMSO to 10 mM stock solution and stored at room temperature. For each experiment, the stock solution were diluted in the culture medium to obtain the desired concentration. The final DMSO content in cell culture was $\leq 0.5\%$ (v/v), which was found to be nontoxic to cells.

Cell lines and cultures. EC109 (human esophagus cancer), HepG2 (human hepatoma) cells, MCF7 (human breast cancer) cells, SKNSH (human neuroblastoma), MGC803 (human gastric cancer), HGC27 (human gastric cancer), SGC7901 (human gastric cancer) and GES1 (human gastric epithelial cell) were purchased from the American Type Culture Collection (Manassas, VA), and cultured at 37°C in an atmosphere containing $5\% \text{CO}_2$, with RPMI1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100U/ml penicillin and 0.1mg/ml streptomycin. MGC803 of DR5^{-/-} cell line were cultured by our group through a lentiviral system and the primer sequence is 5'-GCAAGUCUUUACUGUGGAA-3'.

Cell proliferation. The cells were seeded into a 96-well plate at a density of 4,000 ($100 \mu\text{l}$) cells per well for 24 h, followed by compounds added ($200 \mu\text{l}$) to the respective wells in the indicated final concentrations at different times. Next, $20 \mu\text{l}$ of 5mg/ml MTT per well was added to the medium, and the cells were incubated for 4 h at 37°C and $5\% \text{CO}_2$. After removing the culture medium, $150 \mu\text{l}$ of DMSO was added to dissolve the formazan crystals. The absorbance was read by enzyme labeling instrument with 570nm wavelength measurement. The viability of treated cells for 0 hour was set as 100% , and the viability in the other groups was calculated by comparing the optical density reading with the control. The IC_{50} values were calculated using nonlinear regression analysis.

Nuclear staining with DAPI. Morphological changes of nuclei were visualized following DAPI staining. Following incubation with $10 \mu\text{M}$ **S17** for 24 h, cell were fixed with 4% paraformaldehyde for 10 min at room temperature. Fixed cells were washed with PBS, and stained with $2 \mu\text{g/ml}$ DAPI solution (dissolved with PBS containing 0.1% TritonX-100) for 40 min at room temperature. Cells were washed two more times with PBS and analyzed via a fluorescence microscope.

Apoptosis analysis. MGC-803 cells were seeded at 1.5×10^5 cells/well in 6-well plates and cultured for 24 h. Next, the cells were exposed of **S17** ($10 \mu\text{M}$) to different times. After that, cells were collected and washed with PBS twice, and incubated with fluorescein isothiocyanate (FITC)-conjugated Annexin V and PI using FITC Annexin-V/PI apoptosis kit following the step-by-step protocol provided by the manufacturer. The cell apoptosis was detected by flow cytometer. Annexin V⁺/PI⁻ cells were considered as early apoptotic while Annexin V⁺/PI⁺ cells as late apoptotic/necrotic.

Analysis of DR5 expression by flow cytometer. 5×10^5 MGC803 cells were harvested, fixed by 1% PA (paraformaldehyde) and washed three times with PBS. Washed cells were blocked in 1X PBS/ 10% normal goat serum for 30 min at 4°C to eliminate the non-specific protein-protein interactions followed by the antibody (at $1:200$ dilution) for 45 min. Goat anti-mouse Ig G was used at $1:500$ dilution for 30 min at 4°C . Then cells were washed three times by PBST and detected by flow cytometer.

Measurement of loss of mitochondrial membrane potential (MMP, $\Delta\Psi$). JC-1 probe was used as a measure of loss of MMP. Briefly, Cells were seeded at 1.5×10^5 /well in 10% FBS RPMI-1640 into 6-well plates, and treated with **S17** ($10 \mu\text{M}$) for indicated times. Then JC-1 ($2.5 \mu\text{g/ml}$), were added to incubate with an equal volume of cell suspension at 37°C for 10 min and rinsed twice with PBS. The concentration of retained JC-1 dye was determined by a flow cytometer.

Measurement of ROS. The levels of intracellular reactive oxygen species (ROS) were determined using 2,7-dichlorodihydro fluorescent diacetate (DCFH-DA), as described previously. MGC-803 cells were plated into a 6 well plate for 24 h prior to the experiment. On the following day, cells were subjected to **S17** ($10 \mu\text{M}$) for a certain range of time. Following treatment, cells were incubated with 20mM DCFH-DA dissolved in cell-free medium at 37°C for 30 min in dark. After incubating, the cells were washed by PBS, trypsinized, and collected by centrifugation, and then washed three times with PBS. ROS generation was assessed in fluorescence intensity (FL-1, 530nm) using a flow cytometer. Control cells were subjected to the same manipulation, except for treatment with **S17**.

Western blotting analysis. MGC-803 cells (1×10^6) were cultured in each 100mm plates. After treatment with **S17** ($10 \mu\text{M}$) for indicated times, cells were collected and lysed with ice-cold lysis buffer (Beyotime, Shanghai, China). After centrifugation at $12,000 \text{rpm/min}$ for 30 min, protein concentrations of the lysates were determined by the micro-BCA protein assay kit. The total cellular protein extracts were boiled with loading buffer

and separated by SDS-PAGE and transferred to nitrocellulose membrane. After blocking with 5% skimmed milk in PBS with 0.1% Tween-20 for 2 h, the membranes were incubated with appropriate antibodies overnight at 4 °C, followed by HRP conjugated anti-mouse, anti-goat or anti-rabbit secondary antibodies. The detection of specific proteins was carried out with an ECL Western blotting kit according to the recommended procedure.

Immunofluorescence analysis of Nrf2. MGC803 cells were treated with 10 μ M of **S17** for 8 h. Cells were fixed with 4% paraformaldehyde in PBS for 30 min, permeabilized with 0.1% Triton X-100, and blocked with 10% normal goat serum for 30 min. Incubation with primary antibodies against Nrf2 was done overnight at 4 °C. After washing, cells were exposed to FITC-conjugated antibody (goat-anti-rabbit Ig (H + L)-FITC). After washing, the nuclei were visualized with 2 μ g/ml DAPI solution (dissolved with PBS) and added 10 min before imaging. The confocal microscopy (Nikon) was used for co-localization analysis.

Immunohistochemistry (IHC). Paraffin-embedded tissue sections were dewaxed and rehydrated, washed by PBS, following the antigen retrieval, endogenous peroxidase were blocked by 3% H₂O₂ for 20 min, and normal goat serum was used to block non-specific binding sites for 20 min, the sections were then incubated with cleaved caspase3 and cleaved caspase 8 antibody diluted 1:50 in PBS at 4 °C overnight. Peroxidase-conjugated anti-rabbit antibodies were used for secondary detection, the reaction was revealed with diaminobenzidine (DAB). And ROS was detected according to ROS Immunohistochemistry kit direction (Y-J biological company, Shanghai, China). Sections were counterstained with hematoxylin. Images were acquired on a microscope (Nikon).

Tumor xenograft growth assay *in vivo*. Animals were treated according to protocols established by the ethics committee of Zhengzhou University and the experiments *in vivo* were conducted according to the approved guidelines and approved by the ethics committee of Zhengzhou University. Thirty male nude mice (5 weeks-old) were purchased from the Chinese Academy of Sciences (Beijing, China). Cells were digested and resuspended with PBS at a density of 2.5×10^7 cells/ml. Cell suspension (200 μ l) was subcutaneously injected into the nude mice on the backside. Tumor growth was monitored by tumor volume which was measured with calipers and calculated according to the formula, $V = 0.5 \times (\text{length} \times \text{width}^2)$. Finally, tumors were harvested after 21 days (21 injections), body weight, tumor volume and tumor weight were measured.

Statistical Analysis. The data are expressed as means \pm SD. Significant differences between the groups were determined using the unpaired Student's t-test. * and ** respectively represent $p < 0.05$ and $p < 0.01$.

References

- Overby, A., Zhao, C. M., Bones, A. M. & Chen, D. Naturally occurring phenethyl isothiocyanate-induced inhibition of gastric cancer cell growth by disruption of microtubules. *J Gastroen Hepatol* **29**, 99–106, doi:10.1111/jgh.12732 (2014).
- Li, J. *et al.* Natural therapeutic agents for neurodegenerative diseases from a traditional herbal medicine *Pongamia pinnata* (L.) Pierre. *Bioorg Med Chem Lett* **25**, 53–58, doi:10.1016/j.bmcl.2014.11.015 (2015).
- Radhakrishnan, S. K., Shimmon, R. G., Conn, C. & Baker, A. T. Evaluation of Novel Chalcone Oximes as Inhibitors of Tyrosinase and Melanin Formation in B16 Cells. *Archiv der Pharmazie* (2015).
- Wani, Z. A. *et al.* A novel quinazolinone chalcone derivative induces mitochondrial dependent apoptosis and inhibits PI3K/Akt/mTOR signaling pathway in human colon cancer HCT-116 cells. *Food Chem Toxicol* **87**, 1–11, doi:10.1016/j.fct.2015.11.016 (2015).
- Matos, M. J., Vazquez-Rodriguez, S., Uriarte, E. & Santana, L. Potential pharmacological uses of chalcones: a patent review (from June 2011–2014). *Expert Opin Ther Pat* **25**, 351–366, doi:10.1517/13543776.2014.995627 (2015).
- Mahapatra, D. K., Bharti, S. K. & Asati, V. Anti-cancer chalcones: Structural and molecular target perspectives. *Eur J Med Chem* **98**, 69–114, doi:10.1016/j.ejmech.2015.05.004 (2015).
- Akihisa, T. *et al.* Chalcones, coumarins, and flavanones from the exudate of *Angelica keiskei* and their chemopreventive effects. *Cancer Letters* **201**, 133–137, doi:10.1016/S0304-3835(03)00466-X (2003).
- Jing, H. *et al.* Abrogation of Akt signaling by Isobavachalcone contributes to its anti-proliferative effects towards human cancer cells. *Cancer Lett* **294**, 167–177, doi:10.1016/j.canlet.2010.01.035 (2010).
- Yun, J. M., Kweon, M. H., Kwon, H., Hwang, J. K. & Mukhtar, H. Induction of apoptosis and cell cycle arrest by a chalcone panduratin A isolated from *Kaempferia pandurata* in androgen-independent human prostate cancer cells PC3 and DU145. *Carcinogenesis* **27**, 1454–1464, doi:10.1093/carcin/bgi348 (2006).
- Hikita, H. *et al.* Bak deficiency inhibits liver carcinogenesis: A causal link between apoptosis and carcinogenesis. *J Hepatol* **57**, 92–100, doi:10.1016/j.jhep.2012.01.027 (2012).
- Ralph, S. J., Rodriguez-Enriquez, S., Neuzil, J. & Moreno-Sanchez, R. Bioenergetic pathways in tumor mitochondria as targets for cancer therapy and the importance of the ROS-induced apoptotic trigger. *Mol Aspects Med* **31**, 29–59, doi:10.1016/j.mam.2009.12.006 (2010).
- O'Donovan, P. *et al.* Azathioprine and UVA light generate mutagenic oxidative DNA damage. *Science* **309**, 1871–1874, doi:10.1126/science.1114233 (2005).
- Waszczak, C. *et al.* Sulfenome mining in *Arabidopsis thaliana*. *P Natl Acad Sci USA* **111**, 11545–11550, doi:10.1073/pnas.1411607111 (2014).
- Wang, L. H. *et al.* SL4, a chalcone-based compound, induces apoptosis in human cancer cells by activation of the ROS/MAPK signalling pathway. *Cell Prolif* **48**, 718–728, doi:10.1111/cpr.12226 (2015).
- Lee, J. M. *et al.* A new synthetic 2'-hydroxy-2,4,6-trimethoxy-5',6'-naphthochalcone induces G2/M cell cycle arrest and apoptosis by disrupting the microtubular network of human colon cancer cells. *Cancer Lett* **354**, 348–354, doi:10.1016/j.canlet.2014.08.041 (2014).
- Venero, J. L., Burguillos, M. A., Brundin, P. & Joseph, B. The executioners sing a new song: killer caspases activate microglia. *Cell Death Differ* **18**, 1679–1691, doi:10.1038/cdd.2011.107 (2011).
- Corbiere, C., Liagre, B., Terro, F. & Beneytout, J. L. Induction of antiproliferative effect by diosgenin through activation of p53, release of apoptosis-inducing factor (AIF) and modulation of caspase-3 activity in different human cancer cells. *Cell Research* **14**, 188–196, doi:10.1038/sj.cr.7290219 (2004).
- Chen, S. Y. *et al.* zVAD-induced autophagic cell death requires c-Src-dependent ERK and JNK activation and reactive oxygen species generation. *Autophagy* **7**, 217–228 (2011).
- Guerra, M. T. *et al.* Mitochondrial Calcium Regulates Rat Liver Regeneration Through the Modulation of Apoptosis. *Hepatology* **54**, 296–306, doi:10.1002/hep.24367 (2011).

20. Wang, X. *et al.* Deletion of MCL-1 causes lethal cardiac failure and mitochondrial dysfunction. *Genes Dev* **27**, 1351–1364, doi:10.1101/gad.215855.113 (2013).
21. Zi, X. & Simoneau, A. R. Flavokawain A, a novel chalcone from kava extract, induces apoptosis in bladder cancer cells by involvement of Bax protein-dependent and mitochondria-dependent apoptotic pathway and suppresses tumor growth in mice. *Cancer Res* **65**, 3479–3486, doi:10.1158/0008-5472.CAN-04-3803 (2005).
22. George, N. M., Evans, J. J. D. & Luo, X. A three-helix homo-oligomerization domain containing BH3 and BH1 is responsible for the apoptotic activity of Bax. *Gene Dev* **21**, 1937–1948, doi:10.1101/gad.1553607 (2007).
23. Mellier, G., Huang, S., Shenoy, K. & Pervaiz, S. TRAILing death in cancer. *Molecular aspects of medicine* **31**, 93–112, doi:10.1016/j.mam.2009.12.002 (2010).
24. Yee, C., Yang, W. & Hekimi, S. The intrinsic apoptosis pathway mediates the pro-longevity response to mitochondrial ROS in *C. elegans*. *Cell* **157**, 897–909, doi:10.1016/j.cell.2014.02.055 (2014).
25. Levenon, A. L. Activation of stress signaling pathways by oxidized and nitrated lipids. *Free radical biology & medicine* **75** Suppl 1, S8, doi:10.1016/j.freeradbiomed.2014.10.846 (2014).
26. Lin, S. H. & Shih, Y. W. Antitumor effects of the flavone chalcone: inhibition of invasion and migration through the FAK/JNK signaling pathway in human gastric adenocarcinoma AGS cells. *Mol Cell Biochem* **391**, 47–58, doi:10.1007/s11010-014-1986-6 (2014).
27. Wang, R. A. *et al.* Apoptosis drives cancer cells proliferate and metastasize. *J Cell Mol Med* **17**, 205–211, doi:10.1111/j.1582-4934.2012.01663.x (2013).
28. Dong, Y. Z., Morris-Natschke, S. L. & Lee, K. H. Biosynthesis, total syntheses, and antitumor activity of tanshinones and their analogs as potential therapeutic agents. *Nat Prod Rep* **28**, 529–542, doi:10.1039/c0np00035c (2011).
29. Walsh, C. T. The chemical versatility of natural-product assembly lines. *Accounts Chem Res* **41**, 4–10, doi:10.1021/ar7000414 (2008).
30. Lee, Y. H. *et al.* A new synthetic chalcone derivative, 2-hydroxy-3',5,5'-trimethoxychalcone (DK-139), suppresses the Toll-like receptor 4-mediated inflammatory response through inhibition of the Akt/NF-kappa B pathway in BV2 microglial cells. *Exp Mol Med* **44**, 369–377, doi:10.3858/em.2012.44.6.042 (2012).
31. Zhang, S. *et al.* A new brominated chalcone derivative suppresses the growth of gastric cancer cells *in vitro* and *in vivo* involving ROS mediated up-regulation of DR5 and 4 expression and apoptosis. *Toxicology and applied pharmacology* **309**, 77–86, doi:10.1016/j.taap.2016.08.023 (2016).
32. Perier, C. *et al.* Apoptosis-Inducing Factor Deficiency Sensitizes Dopaminergic Neurons to Parkinsonian Neurotoxins. *Ann Neurol* **68**, 184–192, doi:10.1002/ana.22034 (2010).
33. Kalghatgi, S. *et al.* Bactericidal Antibiotics Induce Mitochondrial Dysfunction and Oxidative Damage in Mammalian Cells. *Sci Transl Med* **5**, doi:10.1126/scitranslmed.3006055 (2013).
34. Szczepanek, K., Chen, Q., Larner, A. C. & Lesniewski, E. J. Cytoprotection by the modulation of mitochondrial electron transport chain: the emerging role of mitochondrial STAT3. *Mitochondrion* **12**, 180–189, doi:10.1016/j.mito.2011.08.011 (2012).
35. Giorgio, M., Trinei, M., Migliaccio, E. & Pelicci, P. G. Hydrogen peroxide: a metabolic by-product or a common mediator of ageing signals? *Nature reviews. Molecular cell biology* **8**, 722–728, doi:10.1038/nrm2240 (2007).
36. Herrera, B. *et al.* Activation of caspases occurs downstream from radical oxygen species production, Bcl-x(L) down-regulation, and early cytochrome C release in apoptosis induced by transforming growth factor beta in rat fetal hepatocytes. *Hepatology* **34**, 548–556, doi:10.1053/jhep.2001.27447 (2001).
37. Marsden, V. S. *et al.* Apoptosis initiated by Bcl-2-regulated caspase activation independently of the cytochrome c/Apaf-1/caspase-9 apoptosome. *Nature* **419**, 634–637, doi:10.1038/nature01101 (2002).
38. Moorjani, N. *et al.* Activation of apoptotic caspase cascade during the transition to pressure overload-induced heart failure. *J Am Coll Cardiol* **48**, 1451–1458, doi:10.1016/j.jacc.2006.05.065 (2006).
39. Gray, D. C., Mahrus, S. & Wells, J. A. Activation of Specific Apoptotic Caspases with an Engineered Small-Molecule-Activated Protease. *Cell* **142**, 637–646, doi:10.1016/j.cell.2010.07.014 (2010).
40. Wang, X. D. The expanding role of mitochondria in apoptosis. *Gene Dev* **15**, 2922–2933 (2001).
41. Jin, C. Y. *et al.* Genistein sensitizes human hepatocellular carcinoma cells to TRAIL-mediated apoptosis by enhancing Bid cleavage. *Anti-cancer drugs* **20**, 713–722, doi:10.1097/CAD.0b013e32832e8998 (2009).
42. Koyani, C. N. *et al.* Activation of the MAPK/Akt/Nrf2-Egr1/HO-1-GCLC axis protects MG-63 osteosarcoma cells against 15d-PGJ2-mediated cell death. *Biochemical pharmacology* **104**, 29–41, doi:10.1016/j.bcp.2016.01.011 (2016).

Acknowledgements

This work was supported by National Natural Science Foundation of China (Project no. 81273393 for Yanbing Zhang and Project no. U1404821 for Cheng-Yun Jin); The Scientific Innovation Talent Award from Department of Education of Henan Province (no. 15HASTIT036 for Cheng-Yun Jin).

Author Contributions

C.Y.J. designed the study and revised the article, S.Y.Z., D.J.F., Y.B.Z. and H.M.L. were responsible for the chalcone derivatives synthesis. T.Y.L. contributed most of the experiments *in vitro* and X.Y.W. was in charge of instruments assay. H.Q.D. screened and cultured the DR5^{-/-} MGC803 cell line. H.D.X., Y.C.L., L.L.L. made the research work *in vivo*. L.Z. cooperated to write the manuscript. All authors reviewed the manuscript.

Additional Information

Competing Interests: The authors declare that they have no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>.

© The Author(s) 2017