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Gossypin inhibits gastric cancer growth by direct targeting AURKA and RSK2

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Abstract

Gossypin is a flavone extracted from *Hibiscus vitifolius* that has been reported to exhibit anti-inflammatory, antioxidant, and anticancer activities. However, the anticancer properties of gossypin and its molecular mechanism of action against gastric cancer have not been fully investigated. In the present study, we report that gossypin is an AURKA and RSK2 inhibitor that suppresses gastric cancer growth. Gossypin attenuated anchorage-dependent and -independent gastric cancer cell growth as well as cell migration. Based on the results of *in vitro* screening and cell-based assays, gossypin directly binds to and inhibits AURKA and RSK2 activities and their downstream signaling proteins. Gossypin decreased S phase and increased G2/M phase cell cycle arrest by reducing the expression of cyclin A2 and cyclin B1 and the phosphorylation of the CDC protein. Additionally, gossypin also induced intrinsic apoptosis by activating caspases and PARP and increasing the expression of cytochrome c. Our results demonstrate that gossypin is an AURKA and RSK2 inhibitor that could be useful for treating gastric cancer.

Keywords

gossypin; AURKA; RSK2; gastric cancer; apoptosis

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AUTHOR CONTRIBUTION

L.W. and X.W. performed the *in vitro* experiments and assisted with the cell based experiments; X.Z and F.M. assisted with the cell based assays; H.C performed the computer modeling; K.L. and A.M.B. supervised the data analysis and manuscript editing; Z.D. and D.J.K. supervised the overall experimental design, provided the idea and prepared the manuscript

Supplemental material

Supplemental Figure (1) and associated figure legend are provided in Supplemental Material and are available online with the paper.

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INTRODUCTION

Gastric cancer (GC) is the fourth most common type of cancer and the second leading cause of cancer-related mortality in the world (Jemal et al., 2011). Over the last few years, the incidence of gastric cancer has remarkably decreased due to the development of new screening techniques. However, the 5-year survival rate remains low, as most patients with GC are diagnosed at advanced or metastatic stages (Van Cutsem et al., 2016; Matsuoka and Yashiro, 2018). Thus, challenges remain to identify targeted therapies or combinations of these therapies with chemotherapy in the field of gastric cancer research (Song et al., 2016).

Natural botanicals have been reported to possess anticancer activity and emphasize the importance of discovering the important source of potential medicines (Gullett et al., 2010). Natural compounds are widely used in the field of cancer therapy presumably due to high efficacy, low toxicity, and few side effects (Taylor and Jabbarzadeh, 2017). Previous studies indicated that flavonoids, coumarins, alkaloids, and polysaccharides extracted from plants have low toxicity along with good therapeutic effects against tumor growth (Ishibashi and Ohtsuki, 2008). A natural flavone originally isolated from *Hibiscus vitifolius*, gossypin (3,5,8,3',4'-pentahydroxy-7-O-glucosyl flavone), has been reported to inhibit proliferation of colon cancer and leukemia cells (Babu et al., 2003). Additionally, gossypin exerts additional anticancer activities, including induction of apoptosis and reduction of cell invasion through the mediation of MEKs, cyclin D1, CHK1, PARP, or TAK1 proteins (Babu et al., 2003; Bhaskaran et al., 2013; Shi et al., 2012). Gossypin significantly reduced the growth of A375 melanoma xenograft tumors *in vivo* (Bhaskaran et al., 2013).

The three human homologues of the Aurora kinases (A, B and C) are essential for proper execution of various mitotic events and are important for maintaining genomic integrity (Katayama et al., 2003). Mitotic errors can cause a variety of chromosome irregularities and enhance tumorigenesis (Li and Li, 2006). Aurora kinase A (AURKA) is mainly localized at the mitotic spindle poles during mitosis, where it regulates the functions of centrosomes, and is essential for mitotic progression (Marumoto et al., 2005). AURKA is aberrantly expressed in various cancer cells and is located in chromosomal region 20q13 where it is amplified especially in gastric cancer (Sakakura et al., 2001). AURKA phosphorylates downstream signaling molecules such as histone H3 (Crosio et al., 2002), p53 (Katayama et al., 2004), and PLK1 (Macurek et al., 2008). AURKA inhibitors have been reported to block AURKA mitotic functions by inducing apoptosis of cancer cells (Keen and Taylor, 2004). Several AURKA inhibitors, including MLN8237, hesperadin, VX-680, VE465 and barasertib, exhibit preclinical activity against various solid tumors. Clinical trials are ongoing to verify the effects of these inhibitors (Mahadevan et al., 2003; Hamada et al., 2003; Ikezoe, 2008) in order to demonstrate the therapeutic potential of inhibiting AURKA.

Mitogen-activated protein kinase (MAPK) signaling pathways play a central role in transducing extracellular signals to intracellular target proteins involved in cell growth, proliferation, cell motility, and apoptosis (Cargnello and Roux, 2011). RSK is downstream effectors of the MAPK signaling cascade that regulate diverse cellular processes such as cell cycle, motility, apoptosis, and proliferation in various cancers (Pearson et al., 2001). RSK

phosphorylate downstream signaling proteins such as CREB (De Cesare et al., 1998), c-Jun (Arul and Cho, 2013), ATF1 (Liu et al., 2011), and histone H3 (Strelkov and Davie, 2002). RSKs enhance the proliferation of prostate cancer cells and inhibiting RSK suppresses the growth of breast cancer cells (Clark et al., 2005; Smith et al., 2005). Additionally, the RSK1 and/or RSK2 proteins are highly expressed in many types of cancers and promote tumor growth and survival (Anjum and Blenis, 2008; Casavieri et al., 2017). Therefore, RSKs are important therapeutic targets in various cancers. In this study, we evaluated the anticancer activity of gossypin in gastric cancer cells through its targeting of AURKA and RSK2. Gossypin could be a lead anticancer compound and deserves further investigation for prevention and treatment of gastric cancer.

MATERIALS AND METHODS

Cell lines

HGC27 human gastric cancer cells were purchased from the Chinese Academy of Sciences Typical Culture Collection (Shanghai, China). AGS human gastric cancer cells and JB6 mouse epidermal cells were purchased from American Type Culture Collection (ATCC). All cells were cytogenetically tested and authenticated before being frozen. Each vial of frozen cells was thawed and maintained in culture for a maximum of 8 weeks. Enough frozen vials were available for each cell line to ensure that all cell-based experiments were conducted on cells that had been tested and in culture for 8 weeks or less. HGC27 cells were cultured in Minimum Essential Medium with Earle's Balanced Salts (MEM/EBSS) supplemented with 1% non-essential amino acid (NEAA), 10% FBS (Biological Industries, Cromwell, CT, USA) and 1% antibiotic-antimycotic. AGS cells were cultured in Roswell Park Memorial Institute medium 1640 (RPMI1640) supplemented with 10% FBS and 1% antibiotic-antimycotic. JB6 cells were cultured in minimal Eagle's medium (MEM) supplemented with 5% FBS and 1% antibiotic-antimycotic.

Reagents and antibodies

Gossypin (purity: > 90% by HPLC) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and CNBr-activated Sepharose 4B beads were from GE Healthcare (Piscataway, NJ, USA). The phosphorylation Aurora B (T232) antibody was obtained from Sigma-Aldrich and the total Aurora B antibody was from Abcam (Cambridge, MA, USA). The antibody to detect β -actin was from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and all the other antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Active AURKA, active RSK2, MBP (AURKA substrate), and ATF1 (RSK2 substrate) human recombinant proteins for kinase assays were purchased from SignalChem (Richmond, BC, Canada).

Cell proliferation assay

HGC27 (2.0×10^3 cells per well) or AGS (0.7×10^3 cells per well) cells were seeded in 96-well plates and incubated for 24 h. Cells were treated with different concentrations of gossypin for 72 h. Twenty μ l of the MTT solution (Solarbio, Beijing, China) were added to each well and incubated for 2 h at 37°C in a 5% CO₂ incubator. The cell culture medium was removed and then 200 μ l of DMSO were added to each well and crystal formation was

observed. Absorbance was measured at 570 nm using the Thermo Multiskan plate-reader (Thermo Fisher Scientific, Waltham, MA, USA).

Anchorage-independent cell growth

Cells (8×10^3 cells per well) suspended in complete growth medium supplemented with 10% FBS were added to 0.3% agar with or without different concentrations of gossypin in a top layer over a base layer of 0.6% agar with or without different concentrations of gossypin. The cultures were maintained at 37°C in a 5% CO₂ incubator for 2 weeks and then colonies were counted under a microscope using the Image-Pro Plus software (v.6) program (Media Cybernetics, Rockville, MD, USA).

Migration assay

Transwell chambers (Corning, Bedford, MA, USA) were coated outside with Matrigel and dried for 30 min at room temperature under sterile conditions. The lower compartment was filled with 600 µl complete medium with or without gossypin. Samples containing 5×10^4 cells in 200 µl serum-free medium were added to the upper compartment with or without gossypin. The chambers were incubated for 48 h at 37°C in a 5% CO₂ atmosphere. After incubation, cells that had migrated into the lower compartment or attached to the lower side of the membrane were carefully collected.

Immunofluorescence

Cells (3.0×10^4 cells per well) were seeded into 4-well chamber slides (Thermo Fisher Scientific, Waltham, MA, USA) for 24 h and treated with gossypin for 24 h. Cells were fixed with 4% paraformaldehyde at room temperature for 20 min and permeabilized with 0.3% Triton X-100 at room temperature for 15 min followed by incubation with an antibody to detect phosphorylated histone H3 at serine 10 (Cell Signaling Technology, 1:200) for 24 h. Cells were incubated with an FITC-labeled secondary antibody (Thermo Fisher Scientific) for 1 h at room temperature. The nucleus was counterstained with DAPI (Solarbio, Beijing, China). Cells were mounted on microscope slides and fluorescence were visualized by confocal microscope (Nikon, Tokyo, Japan).

In vitro kinase assay

The kinase assays were performed according to the instructions provided by Upstate Biotechnology (Billerica, MA, USA). The active recombinant AURKA (300 ng) or RSK2 (200 ng) protein was mixed with different concentrations of gossypin in reaction buffer (Cell Signaling Technology) and incubated at room temperature for 15 min. The MBP (AURKA substrate) or ATF1 (RSK2 substrate) and ATP were added and the mixtures were incubated at 30°C for 30 min. The reaction was terminated by adding 10 µl protein loading buffer and the mixture was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). AURKA or RSK2 activity was evaluated using a serine and threonine phosphorylation antibody.

Computational modeling of AURKA and RSK2 with gossypin

To confirm whether gossypin can bind with AURKA or RSK2, we performed *in silico* docking using the Schrödinger Suite 2017 software programs. AURKA and RSK2 crystal structures were first downloaded from the Protein Data Bank (PDB) and then prepared under the standard procedures of the Protein Preparation Wizard program (Schrödinger Suite 2017). Hydrogen atoms were added consistent with a pH of 7 and all water molecules were removed. The ATP-binding site-based receptor grid of AURKA and RSK2 were generated for docking, respectively. Gossypin was prepared for docking by default parameters using the LigPrep program. Then, the docking of gossypin with AURKA and RSK2 was accomplished with default parameters under the extra precision (XP) mode using the program Glide, respectively. Herein, we could get the best-docked representative structures.

Pull-down assay using CNBr-Sepharose 4B beads

HGC27 cell lysates (500 µg) or a recombinant human AURKA (200 ng) or RSK2 (200 ng) protein was incubated with gossypin-Sepharose 4B beads (Sepharose 4B beads as a control) in reaction buffer (50 mM Tris pH 7.5, 5 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.01% NP40, 2 µg/mL bovine serum albumin). After incubation with gentle rocking overnight at 4°C, the beads were washed 5 times with buffer (50 mM Tris pH 7.5, 5 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.01% NP40) and then binding was visualized by Western blotting.

Cell cycle analysis

Cells were seeded into 60-mm culture dishes (6.5×10^4 cells per dish) and incubated for 24 h. Cells were synchronized by serum starvation for 24 h and treated with gossypin for 48 h in 10% serum-supplemented medium. Cells were harvested by trypsinization and washed with cold phosphate buffered saline (PBS) and then fixed in 1 mL 70% cold ethanol. After rehydration, cells were disrupted in 0.6% Triton X-100 and digested with 100 µg/ml RNase for 1 h. Cells were subsequently stained with 20 µg/ml propidium iodide (Clontech, Palo Alto, CA, USA) for 15 min and then cell cycle was analyzed by flow cytometry.

Apoptosis assay

Cells were plated into 60-mm dishes (6.5×10^4 cells per dish) and incubated for 24 h. Cells were treated with different doses of gossypin for 72 h and collected by trypsinization and then stained with Annexin V (BioLegend, San Diego, CA, USA) and propidium iodide. Cell staining was accomplished following the product instructions (Clontech, Palo Alto, CA, USA) and apoptosis was analyzed by flow cytometry.

Statistical analysis

All quantitative results are expressed as mean values $\pm$ S.D. or $\pm$ S.E. Significant differences were determined using the Student's *t* test or one-way analysis of variance (ANOVA). A *p* value of < 0.05 was considered to be statistically significant. The statistical package for social science (SPSS) for Windows (IBM, Inc. Armonk, NY, USA) was used to calculate the *p*-value to determine statistical significance.

RESULTS

Gossypin inhibits gastric cancer cell growth

Gossypin is a 3,5,8,3',4'-pentahydroxy-7-O-glucosyl flavone and its structure is shown in Figure 1A. We first determined whether gossypin could affect growth of gastric cancer cells. Cells were treated with gossypin at various doses and growth was analyzed by MTT or soft agar assay. Results showed that growth of gastric cancer cells was significantly inhibited by gossypin treatment in a dose dependent manner (Fig. 1B, C). Additionally, we investigated the effect of gossypin on cell migration and data indicated that gossypin significantly suppressed migration (Fig. 1D).

Gossypin is an AURKA and RSK2 inhibitor

We used *in vitro* kinase assays to screen for potential molecular targets of gossypin. Results showed that gossypin strongly suppressed the activity of AURKA and RSK2 compared to other kinases (Supplemental Fig. 1). Additionally, to determine the effect of gossypin on various epidermal growth factor (EGF)-induced signaling molecules, we used JB6 epidermal skin cells. Following serum starvation for 48 h, JB6 cells were treated with gossypin for 3 h before treatment with EGF for 30 min. Results showed that phosphorylation of histone H3 at serine 10 and ATF1 at serine 63 were strongly suppressed by gossypin treatment, whereas phosphorylation of AURKA, RSK2, and other molecules were not affected (Fig. 2A). To confirm the molecular targets of gossypin, HGC27 gastric cancer cells were treated with gossypin for 1 h and then the expression level of various signaling molecules was analyzed by Western blotting. Results indicated that gossypin strongly suppressed phosphorylation of histone H3 at serine 10, c-Jun at serine 63, and ATF1 at serine 63 (Fig. 2B). Additionally, we used an immunofluorescence assay to determine whether gossypin could affect the phosphorylation of histone H3. Results showed that gossypin strongly suppressed the phosphorylation of histone H3 in AGS gastric cancer cells (Fig. 2C). We next performed *in vitro* kinase assays with an active recombinant AURKA or RSK2 protein to examine whether gossypin affected AURKA or RSK2 activity. Results indicated that gossypin strongly inhibited the activity of AURKA and RSK2 in a dose-dependent manner (Fig. 2D, E). These results demonstrated that gossypin is an AURKA and RSK2 inhibitor.

Gossypin directly binds with AURKA and RSK2

To determine whether gossypin interacts with the AURKA or RSK2 protein, we created a computational molecular docking model following protocols in the Schrodinger Suite 2017. The results of computational docking studies suggested that gossypin formed several contacts with the ATP pocket of AURKA as well as the ATP pocket of N- and C-terminals of RSK2 (Fig. 3A, B). This finding indicated that gossypin might be a potential direct inhibitor of AURKA and RSK2. Images were generated with the UCSF Chimera program (Pettersen et al., 2004). To validate the binding between gossypin and AURKA or RSK2, we performed *in vitro* pull-down assays using gossypin-conjugated Sepharose 4B beads (or Sepharose 4B as a negative control) with HGC27 gastric cancer cell lysates or a recombinant AURKA or RSK2 protein. Results showed that gossypin directly binds with the AURKA or RSK2 protein (Fig. 3C, D).

Gossypin induces G2/M phase cell cycle arrest

To determine the effect of gossypin on cell cycle in HGC27 and AGS gastric cancer cells, we performed flow cytometry analysis. Cells were synchronized by serum starvation for 24 h and cell cycle was released with serum with or without gossypin for 48 h. The results indicated that gossypin reduces S phase and induces G2 phase cell cycle arrest (Fig. 4A, B). Additionally, we investigated whether gossypin affects the expression of G2 phase marker proteins. After serum starvation for 24 h, cells were treated with different doses of gossypin for 48 h and cell cycle marker proteins were analyzed by Western blotting. Results showed that gossypin reduced the expression of cyclin A2, cyclin B1, and phosphorylated CDC2 proteins (Fig. 4C).

Gossypin promotes apoptosis of gastric cancer cells

To investigate whether gossypin increases gastric cancer cell death, we examined cell viability. HGC27 gastric cancer cells were treated with gossypin for 72 h and then suspended cells (dead) and attached cells (live) were counted. The number of suspended cells was significantly increased by treatment with gossypin (Fig 5A, *left panel*) and the number of adherent cells was significantly decreased (Fig 5A, *right panel*) in the gossypin-treated group. Additionally, we examined whether gossypin induces apoptosis of gastric cancer cells. Cells were treated with gossypin for 72 h and the expression of annexin V was analyzed. Results indicated that that early and late apoptosis in gossypin-treated cells were strongly increased compared to control cells (Fig. 5B, C). To investigate gossypin-induced apoptotic signaling, pro-apoptotic or anti-apoptotic marker protein expression was analyzed by Western blotting. Results showed that the expression of pro-apoptotic marker proteins (cleaved caspase 3, 7 and 9, cleaved PARP and cytochrome c) was strongly increased by gossypin and the expression of anti-apoptotic marker protein (Bcl-xL) was decreased (Fig. 5D). These results suggested that gossypin induces signaling of intrinsic cellular apoptosis.

DISCUSSION

The anti-cancer properties of natural products have been suggested to play an important role in the prevention of cancers (Li et al., 2015). Gossypin is traditionally used for the treatment of diabetes (Venkatesan and Sorimuthu Pillai, 2012) and exerts anti-inflammatory (Rajagopal et al., 2018), antioxidant (Katary and Salahuddin, 2017), and anticancer activities (Babu et al., 2003). However, the mechanistic targets of gossypin have not been clearly elucidated. In this study, we found that gossypin induced anticancer activities, including inhibition of cell growth and cell migration in gastric cancer (Fig. 1B-D) and gossypin directly suppresses AURKA and RSK2 protein activities (Supplemental Fig. 1 and Fig. 2D, E). AURKA is frequently overexpressed in many human cancers including gastric cancer and is heavily involved in cancer development (Marumoto et al., 2005). Additionally, AURKA promotes chemoresistance to anticancer agents such as paclitaxel and docetaxel in various cancers (Tanaka et al., 2007; Long et al., 2012). Recently, Alisertib (MLN8237), as a selective AURKA inhibitor, was reported to induce apoptosis in human tongue squamous cell carcinoma cells *in vitro* and *in vivo* (Niu et al., 2017). Another AURKA inhibitor, TC-A2317, reportedly disrupts mitotic progression and inhibits proliferation of various cancer cell types (Min et al., 2016). Additionally, the MAPK signaling pathway is frequently

activated in gastric cancer by multiple types of genomic amplifications or mutations (Deng et al., 2012). RSKs are downstream effectors of MAPK signaling and enhance tumor growth (Anjum and Blenis, 2008; Casalvieri et al., 2017) and RSK inhibitors have been developed for cancer therapeutics (Deng et al., 2017; Neise et al., 2013). Therefore, dual targeting of AURKA and RSK2 could assist in the application of a molecular targeted therapy against gastric cancer. In the present study, gossypin induced cytotoxicity in gastric cancer cells by concurrently inhibiting both RSK2 and AURKA (Fig. 2D, E). Additionally, its activity was clearly confirmed by the attenuated phosphorylation of histone H3, c-Jun, or ATF1, which are direct substrates of AURKA or RSK2 (Fig. 2A, B). Binding of gossypin with these kinases was confirmed by docking studies and pull-down experiments, which clearly supported the interaction (Fig. 3).

Using computer docking and NMR studies, gossypin was reported to directly bind to the CDK2 protein (Kim et al., 2008), but evidence was not provided as to whether gossypin treatment affected its phosphorylation or activity in gastric cancer cells. Our results of cell cycle protein expression indicated that phosphorylation of CDK2 was little changed by gossypin treatment (data not shown). Therefore, the effect of gossypin on CDK2 protein activity should be evaluated in gastric cancer cells. Gossypin inhibits transforming growth factor β -activated kinase 1 (TAK1)-mediated NF- κ B activation and enhances apoptosis (Kunnumakkara et al., 2007). However, the mechanism explaining how gossypin could inhibit NF- κ B signaling to induce apoptosis is not clear. Additionally, high doses of gossypin (150 μ M) inhibited the activity of CDK4 in melanoma cells (Bhaskaran et al., 2013). Based on the results of our *in vitro* kinase assay, gossypin (20 μ M) strongly inhibited the activity of AURKA (Fig. 2D) and RSK2 (Fig. 2E), suggesting that AURKA and RSK2 are the major targets of gossypin in gastric cancer cells.

The RSK proteins have been found to inhibit apoptosis by protecting mitochondrial integrity (Smith et al., 2005; Xian et al., 2009; Tan et al., 1999) and mediating the activity of pro- or anti-apoptotic proteins, including Bcl-2, Bcl-xL, Bad, and caspases (Doehn et al., 2009). Additionally, BI-D1870 (an RSK2 inhibitor) or knockdown of the *RSK2* gene inhibits myeloma cell proliferation and induces apoptosis (Shimura et al., 2012). Therefore, we investigated whether gossypin could induce apoptosis in gastric cancer cells. Our results indicated that gossypin induced apoptosis of gastric cancer cells through the inhibition of the AURKA and RSK2 proteins (Fig. 5A-C). Inhibition of these proteins resulted in an induction of the cleavage of caspases (9, 7 and 3) and PARP and induced cytochrome c expression and reduced Bcl-xL expression (Fig. 5D). In conclusion, our findings demonstrate that gossypin is a potent AURKA and RSK2 inhibitor that could be useful for preventing or treating gastric cancers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations:

AURKA	aurora kinase A
RSK2	ribosomal protein S6 kinase 2
ATF1	activating transcription factor 1
AKT	v-akt murine thymoma viral oncogene homolog
CDC2	cell division cycle protein 2

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Figure 1. Gossypin suppresses growth and migration of gastric cancer cells.

(A) Chemical structure of gossypin. (B) Effect of gossypin on anchorage-dependent growth of gastric cancer cells. Cells were treated with gossypin at various concentrations and then incubated for 72 h. Cell growth were measured at an absorbance of 570 nm. (C) Effect of gossypin on anchorage-independent growth of gastric cancer cells. Cells were treated with gossypin and incubated for 2 weeks. Colonies were counted using a microscope and the Image-Pro PLUS (v.6) computer software program. (D) Effect of gossypin on cell migration in gastric cancer cells. Cells were treated with gossypin and incubated for 48 h. Migrated cells were counted. For B-D, data are shown as means $\pm$ S.D. of triplicate values from 3

independent experiments and the asterisk (*) indicates a significant ($p < 0.05$) inhibitory effect of gossypin.

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Figure 2. Gossypin is a potent AURKA and RSK2 inhibitor.

(A) Effect of gossypin on EGF-induced activation of various signaling proteins in JB6 cells. Serum-starved (0.1% FBS; 48 h) JB6 cells were treated with various doses of gossypin for 2 h followed by treatment with EGF for 30 min. (B) Effect of gossypin on various signaling proteins in HGC27 gastric cancer cells. Cells were treated with gossypin and then signaling pathway proteins were detected by Western blotting. (C) Gossypin inhibits phosphorylation of histone H3 in AGS gastric cancer cells. Cells were treated with gossypin for 24 h and detected by immunofluorescence assay. Red color indicates phosphorylated histone H3 at serine 10 and blue color shows DAPI. (D-E) Gossypin suppresses AURKA and RSK2

kinase activity in a dose-dependent manner. The effect of gossypin on AURKA or RSK2 activity was assessed by an *in vitro* kinase assay using AURKA (active, 200 ng) and MBP (AURKA substrate, 400 ng) or RSK2 (active, 200 ng) and ATF1 (RSK2 substrate, 300 ng) proteins. The effect of gossypin on kinase activities was determined by Western blotting using an antibody to detect phosphorylated serine and threonine. Band density was measured using the Image J (NIH) software program. For all data, similar results were obtained from 3 independent experiments.

Figure 3. Gossypin directly binds to RSK2.

(A) Modeling of gossypin binding with AURKA at the ATP binding pocket (*left panel*); and Ligand Interaction Diagram (LID) of the binding (*upper right panel*). (B) Modeling of gossypin binding with the N-terminal domain (NTD) at the ATP binding pocket of RSK2 (*upper left panel*) and LID of the binding (*upper right panel*). Modeling of gossypin binding with the C-terminal domain (CTD) at the ATP binding pocket of RSK2 (*lower left panel*) and LID of the binding (*lower right panel*). The AURKA, NTD RSK2 and CTD RSK2 structures are shown as ribbon representation and gossypin is shown as stick representation. LID legend is shown below. Gossypin directly binds to AURKA or RSK2 in (C) gastric cancer cell lysates or (D) as recombinant proteins. The recombinant proteins or cell lysates

were incubated with gossypin-conjugated Sepharose 4B beads or with Sepharose 4B beads alone. The pulled down proteins were analyzed by Western blotting. For C and D, similar results were obtained from 3 independent experiments.

Figure 4. Gossypin induces G2/M phase arrest.

(A-B) Effect of gossypin on cell cycle in HGC27 or AGS gastric cancer cells. Cells were synchronized by serum starvation for 24 h and treated with gossypin for 48 h in 10% serum-supplemented medium and then stained with propidium iodide (PI). Cell cycle was analyzed by Fluorescence Activated Cell Sorting (FACS). For B, data is shown as mean values $\pm$ S.D. ($n = 3$) and the asterisk (*) indicates a significant ($p < 0.05$) difference compared to untreated control cells. (C) Effect of gossypin on cell cycle marker proteins. Cells were synchronized by serum starvation for 24 h and treated with gossypin for 48 h in 10% serum-supplemented medium and analyzed by Western blotting. Band density was measured using the Image J (NIH) software program. For C, similar results were obtained from 3 independent experiments.

Figure 5. Gossypin increases apoptosis of gastric cancer cells.

(A) Gossypin induces cell death. Cells were seeded in a 6-well plate and treated with gossypin at the indicated doses for 72 h. The number of suspended or attached cells was determined using a hemacytometer. (B-C) Gossypin induces apoptosis. Cells were seeded with gossypin in medium supplemented with 10% FBS and then incubated for 72 h. Cells were stained with annexin V and propidium iodide (PI) and apoptosis was determined by Fluorescence Activated Cell Sorting (FACS). For A-C, data are shown as mean values $\pm$ S.D. ($n = 3$) and the asterisk (*) indicates a significant ($p < 0.05$) difference compared to untreated control cells. (D) Gossypin strongly induces expression of apoptotic marker proteins. Cells were treated with gossypin for 72 h and the levels of cleaved caspases, cleaved PARP, cytochrome c and BcL-xL proteins were determined by Western blotting using β -actin as the loading control. Band density was measured using the Image J (NIH) software program. Similar results were obtained from 3 independent experiments.