Tetramethoxychalcone, a Chalcone Derivative, Suppresses Proliferation, Blocks Cell Cycle Progression, and Induces Apoptosis of Human Ovarian Cancer Cells

Zihao Qi1,2,*, Mingming Liu1,2,*, Yang Liu1,2,3, Meiqin Zhang2,3,*, Gong Yang1,2,4,*

1 Cancer Institute, Fudan University Shanghai Cancer Center, Shanghai, China, 2 Department of Oncology, Shanghai Medical College, Fudan University, Shanghai, China, 3 Department of Gynecological Oncology, Fudan University Shanghai Cancer Center, Shanghai, China, 4 Central Laboratory, the Fifth People’s Hospital of Shanghai, Fudan University, Shanghai, China

Abstract

In the present study, we investigated the in vitro antitumor functions of a synthetic chalcone derivative 4,3’,4’,5’-tetramethoxychalcone (TMOC) in ovarian cancer cells. We found that TMOC inhibited the proliferation and colony formation of cisplatin sensitive cell line A2780 and resistant cell line A2780/CDDP, as well as ovarian cancer cell line SKOV3 in a time- and dose-dependent manner. Treatment of A2780 cells with TMOC resulted in G0/G1 cell cycle arrest through the down-regulation of cyclin D1 and CDK4, and the up-regulation of p16, p21 and p27 proteins. We demonstrated that TMOC might induce cell apoptosis through suppressing Bcl-2 and Bcl-xL, but enhancing the expression of Bax and the cleavage of PARP-1. Treatment of TMOC also reduced the invasion and migration of A2780 cells. Finally, we found that TMOC inhibited the constitutive activation of STAT3 signaling pathway and induced the expression of the tumor suppressor PTEN regardless of the p53 status in cell lines. These data suggest that TMOC may be developed as a potential chemotherapeutic agent to effectively treat certain cancers including ovarian cancer.

Citation: Qi Z, Liu M, Liu Y, Zhang M, Yang G (2014) Tetramethoxychalcone, a Chalcone Derivative, Suppresses Proliferation, Blocks Cell Cycle Progression, and Induces Apoptosis of Human Ovarian Cancer Cells. PLoS ONE 9(9): e106206. doi:10.1371/journal.pone.0106206

Editor: Chih-Pin Chuu, National Health Research Institutes, Taiwan

Received April 16, 2014; Accepted August 3, 2014; Published September 2, 2014

Copyright: © 2014 Qi et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are included within the paper.

Funding: This study was supported by grants from the National Natural Science Foundation of China (No. 81071839 for M. Liu, and Nos. 91129721 and 81372797 for G. Yang), by China Postdoctoral Science Foundation (No. 2013MS31126 for M. Liu, by the Shanghai Pujiang Program (11PJ1402200) from the Shanghai Municipal Government of China for G. Yang, and by the Doctoral Fund of Ministry of Education of China (2012007110079) for G. Yang. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* Email: piaozmq@hotmail.com (MZ); yanggong@fudan.edu.cn (GY)

These authors contributed equally to this work.

Introduction

Ovarian cancer is the leading cause of death from gynecologic malignancies in women. Due to the lack of sensitive and specific methods for early detection, nearly 60–70% of ovarian cancer patients are diagnosed at advanced stages [1,2]. Despite advances in treatment of ovarian cancer, predominantly involving cytoreductive surgery followed by platinum-based chemotherapy, the survival rate of ovarian cancer patients remains very low [3]. Clinical problems including acquired resistance to conventional chemotherapies as well as the metastatic and invasive capabilities of the disease have severely impaired the treatment success [4,5]. Therefore, the continued development of novel therapeutic agents for ovarian cancer, especially for the platinum resistant cells, is still urgent.

Naturally occurring products from various plants are always important in the discovery of new therapeutic agents [6,7]. For instance, chalcone derivatives (molecules containing 1,3-diphenyl-2-propen-1-one groups), one of the major classes of natural products with widespread distribution in spices, tea, beer, fruits and vegetables, display various interesting biological activities including anti-inflammatory, antimicrobial, antioxidant, and anticancer properties [8–10]. Specifically, as a structure mimics of combretastatin A-4(CA-4), 3’,4’,5’-trimethoxychalcone was reported to exhibit antimitotic properties caused by the inhibition of tubulin polymerization [11–14].

In our efforts to discover cytotoxic agents against ovarian cancer cells, a series of CA-4 related compounds were synthesized and evaluated for their anti-proliferative activities in human epithelial ovarian cancer cell line A2780 in vitro (data not shown). Among these compounds, 4,3’,4’,5’-tetramethoxychalcone (TMOC, Fig. 1A) possessed the highest inhibitory potency against A2780 cells. However, subsequent in vitro assays showed that TMOC did not interrupt the tubulin polymerization (Fig. 1B), indicating that the anti-cancer mechanism of TMOC still remains to be elucidated.

Materials and Methods

Materials

TMOC was synthesized according to the previous report and was determined by spectra including 1H-NMR, 13C-NMR, and high resolution mass spectrum (HRMS) which were great agreed with the literatures [14,15]. The purity of TMOC was more than 98% which was analyzed by HPLC.
RPMI-1640 medium and fetal bovine serum (FBS) were purchased from Thermo Scientific (South Logan, UT, USA). MTT, propidium indide (PI), 4,6-diamidino-2-phenylindole (DAPI), and the antibody to β-Actin were purchased from Sigma-Aldrich (St Louis, MO, USA). Gentian violet was purchased from Solarbio (Beijing, China). The Annexin V-FITC/PI apoptosis detection kit, invasion chambers, matrigel, and the antibody to p21 were purchased from BD Biosciences (Franklin Lakes, NY, USA). Cell lysis buffer and BCA protein assay kit were purchased from Beyotime (Shanghai, China). PVDF membrane and chemiluminescent reagents were from Millipore (Billerica, MA, USA). Antibodies to cyclin D1, CDK4, p16, p21, Bcl-xL, Bax, STAT3, p53, PTEN, c-myc were from Santa Cruz Biotechnology. Antibodies to phospho-Src (Tyr416), Src, phospho-STAT3 (Tyr705) and cleaved-PARP-1 were purchased from Cell Signaling Technology.

Cell culture and transfection

The human epithelial ovarian cancer cell lines A2780 and SKOV3 were purchased from ATCC. The cisplatin resistant ovarian cancer cell line A2780/CDDP was kindly provided by Prof. Ling-Ya, Pan [16]. Immortalized but pre-neoplastic human ovarian epithelial cells T29 was derived from ovarian surface epithelial cell lines IOSE-29 as described previously [17]. Cells were routinely cultured with RPMI-1640 supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin in a humidified incubator at 37°C in an atmosphere of 5% CO2. For transfection studies, cells were transiently transfected with STAT3-CA (constitutive active mutant, A661C and N663C) [18,19], STAT3-DN (dominant negative mutant, Y705F) [20] or the control vector using Fugene HD (promega). The STAT3 constructs were gifts from Dr. Hesham M. Amin (MD Anderson Cancer Center, Houston, Texas, USA).

In vitro anti-proliferation assay

The in vitro anti-proliferative activity of TMOC was measured by the MTT reagent, as described in the literature [21]. Briefly, 5×10^3 cells in 100 µL of medium per well were plated in 96-well plates. After incubated for 24 h, the cells were treated with different concentration of TMOC or DMSO (as negative control) for 24 h, 48 h or 72 h. Then, the medium with the compound or DMSO was replaced with 200 µL of fresh medium containing 10% MTT (5 mg/mL in PBS) in each well and incubated at 37°C for 4 h. Last, the MTT-containing medium was discarded and 150 µL of DMSO per well was added to dissolve the formazan crystals newly formed. Absorbance of each well was determined with a microplate reader (Synergy H4, Bio-Tek) at a 590 nm wavelength. The inhibition rates of proliferation were calculated with the following equation:

\[
\text{Inhibition ratio} = \frac{(OD_{\text{DMSO}} - OD_{\text{drug}})}{(OD_{\text{DMSO}} - OD_{\text{blank}})} \times 100%
\]

Colony formation assay

5×10^2 cells per well were seeded in six-well plates at a single cell density. 48 h later, the cells were treated with different concentrations of TMOC or DMSO (as negative control) for 48 h. Then the medium was replaced with fresh medium to allow cell growth for one week. The cells were fixed with methyl alcohol for 15 min and stained with gentian violet for 30 min. Colonies consisting of more than 50 cells were counted.

Cell cycle analysis

Cell cycle status was detected by flow cytometry according to a previously published method [22], and were analyzed by Multicycle AV (for windows, version 320) software. Briefly, cells were first treated with various concentrations of TMOC or DMSO for 24 h, and then harvested, washed twice with 1× PBS, and resuspended in 200 µL of 1× PBS. The cells were fixed in 4 mL of ice-cold 75% ethanol at −20°C overnight and stained with 500 µL PI (50 µg/mL, Sigma) containing 0.1% RNase (1 mg/mL, Sigma) for 15 min in dark condition at room temperature. The cells were then analyzed by flow cytometry (Cytomics FC 500 MPL, Beckman Coulter). The results were indicated as mean values from three independent determinations.

Cell apoptosis analysis

To detect apoptosis, cells were incubated with the different concentrations of TMOC or DMSO (as negative control) for 24 h. The cells were harvested, washed twice with cold 1×PBS, and resuspended in 200 µL binding buffer at the density of 1×10^5 cells/mL. The cells were then stained with 5 µL Annexin-V and
Anti-Tumor Effects of a Chalcone Compound TMOC

A

Inhibition ratio (%) vs. TMOC concentration (μM)

A2780

24 h, 48 h, 72 h

A2780 CDDP

24 h, 48 h, 72 h

SKOV3

24 h, 48 h, 72 h

T29

24 h, 48 h, 72 h

B

Concentration and effect on cell culture

A2780

0 μM, 0.3125 μM, 0.625 μM, 1.25 μM, 2.5 μM

A2780 CDDP

0 μM, 0.625 μM, 1.25 μM, 2.5 μM, 5 μM

SKOV3

0 μM, 0.625 μM, 1.25 μM, 2.5 μM, 5 μM

C

Number of colonies vs. TMOC concentration (μM)

A2780

Number of colonies

A2780 CDDP

Number of colonies

SKOV3

Number of colonies
PI, for 15 min in dark condition at room temperature and subjected to analysis by flow cytometry. The early apoptosis was evaluated based on the percentage of cells with Annexin V+/PI−, while the late apoptosis was that of cells with Annexin V+/PI+.

The results were indicated as mean values from three independent determinations.

**DAPI and PI staining**

Nuclear morphological and membrane integral changes of apoptosis were determined by DAPI and PI staining respectively, as described previously [23,24]. 3 × 10⁵ cells were seeded in 6-well plates and cultured for 48 h, followed by treatment with diluent or with desired concentrations of TMOC for 24 h. For DAPI staining, cells were washed with PBS for three times, fixed with methanol, and permeabilized with 0.1% Triton X-100, followed by staining with DAPI (1:2000 dilution, in 1x PBS) at 37°C for 15 min in dark condition at room temperature and pictures of each wounded monolayer were taken at 0, 36 and 72 h. After staining, the cells were washed with PBS to remove unbound dye (PI) and observed using fluorescent microscopy (Olympus). Fluorescent images were recorded using a cooled CCD camera.

**Wound healing assay**

To detect cell motility, cells were seeded in 6-well plates and grown to 90% confluence. A single scratch wound was created on monolayer cells by using a sterile micropipette tip. Subsequently, medium with cellular debris was removed, and fresh serum-free medium (without FBS supplementation) containing different concentrations of TMOC or DMSO was added. Cells were incubated at 37°C for 24 h. (C) Colony formation rate after treatment with TMOC for 24 h. doi:10.1371/journal.pone.0106206.g002

**Statistical analysis**

The data were calculated using Graph Pad Prism and expressed as mean ± S.E. The values of IC₅₀ were fitted using a nonlinear regression model with a sigmoidal dose response. Comparisons between control and treated groups were determined by paired t test or one-way ANOVA followed by Tukey’s multiple comparison tests. Results were considered statistically significant at the p<0.05 level.

**Results**

**TMOC suppresses cell growth**

We first determined the anti-proliferative effects of TMOC on human ovarian carcinoma cells, including A2780 (p53 wild-type), A2780/CDDP (cisplatin resistant subline of A2780, p53 mutant) and SKOV3 (p53 null) cells, as well as pre-neoplastic ovarian epithelial T29 cells. Cells were treated with various concentrations (ranging from 0.3125 to 40 μM) of TMOC for 24, 48, and 72 hours. As shown in Fig 2A, treatment of A2780 cells with TMOC resulted in a corresponding decrease of cell proliferation and viability in a dose- and time-dependent manner. Similar effects were obtained on treatment of A2780/CDDP and SKOV3 cells (Fig 2A). In contrast, the sensitivity of T29 cells to TMOC was much low, as the concentration of TMOC effective on T29 cells was detected at 40 μM in 72 h. The IC₅₀ values were calculated and listed in Table 1. Thus, these data suggest that TMOC does have a cytotoxic effect on ovarian tumor cells regardless p53 status, but processes less cytotoxicity in pre-neoplastic ovarian epithelial cells. Moreover, we also tested the anti-proliferative activities of chalcone (1,3-Diphenyl-2-propen-1-one), which is the mother compound of TMOC (Table 1).

**Transwell invasion assay**

Cell invasion was assayed using chambers pre-coated with matrigel [25]. Briefly, 5 × 10⁴ cells in 300 μL serum-free RPMI-1640 were seeded into the upper chamber. The chamber was placed into a 24-well plate and the lower wells contained RPMI-1640 medium with 10% FBS and different concentrations of TMOC or DMSO (as a negative control). After 24 h of incubation, the cells on the upper surface of chamber were carefully swabbed with a cotton swab. The cells migrated through the chamber were fixed with methanol, stained with crystal violet and subsequently counted from 5 different areas from each well under inverted microscope. At least three independent experiments were performed.

**Western blot analysis**

Cells were treated with different concentrations of TMOC or DMSO (as a negative control) for 24 h, and then were harvested, washed with cold 1×PBS twice, lysed with cell lysis buffer for 30 min on ice, and centrifuged at 12,000 rpm for 15 min at 4°C. The concentration of total protein was determined by BCA protein assay kit. Equal amounts (30 μg per load) of protein samples were subjected to SDS-PAGE electrophoresis and transferred on to polyvinylidene fluoride (PVDF) membranes which was then blocked in 10% non-fat milk, and reacted with primary antibodies. After incubation with the secondary antibodies conjugated with horseradish peroxidase (HRP), the protein bands were developed with the chemiluminescent reagents.

**Table 1. The IC₅₀ values and water solubility of TMOC and chalcone.**

|            | TMOC  | chalcone |
|------------|-------|----------|
| IC₅₀ (μM)  |       |          |
| A2780      | 24 h  | 3.5      |
|            | 48 h  | 1.3      |
|            | 72 h  | 0.6      |
| A2780/CDDP | 24 h  | 8.5      |
|            | 48 h  | 4.3      |
|            | 72 h  | 0.9      |
| SKOV3      | 24 h  | 5.2      |
|            | 48 h  | 3.6      |
|            | 72 h  | 1.1      |
| T29        | 24 h  | >40      |
|            | 48 h  | 30.1     |
|            | 72 h  | 7.4      |
| Water solubility (μg/mL) | 4 | <1 |

doi:10.1371/journal.pone.0106206.t001
Figure 3. The effect of TOMC on cell cycle progression. (A) Cell cycle distribution after treatment with different concentrations of TMOC for 24 h. (B) Quantitative analysis of TMOC-treated cells. (C) Regulation of cell cycle associated proteins in A2780 cells. The experiments were repeated three times, and a representative experiment is shown. * \( p < 0.05 \), ** \( p < 0.01 \) compared to control.

\[ \text{doi:10.1371/journal.pone.0106206.g003} \]
Figure 4. Apoptosis induced by TMOC. (A) DAPI nuclei staining was used to visualize the apoptosis induced by TMOC. Arrows represent the apoptotic cells. Scale bar = 10 μm. (B) PI uptake was analyzed under a fluorescent microscope. Scale bar = 50 μm. (C) Representative flow cytometry profiles of apoptosis and (D) quantitative results obtained using Annexin V/PI staining. (E) Western blots of apoptotic related proteins. The assay was repeated three times, and a representative result is shown. * p<0.05, ** p<0.01 compared to control.

doi:10.1371/journal.pone.0106206.g004
Anti-Tumor Effects of a Chalcone Compound TMOC

A

0 h  36 h  72 h
0 μM
1 μM
2 μM

B

Relative distance (%)

0  100  150
0  36  72

Hours after wounding

C

0 μM  2.5 μM  5 μM

D

Number of invaded cells

0  100  200  300  400  500
0  2.5  5

TMOC concentration (μM)
Compared with TMOC, chalcone exhibited much weak effect on the inhibition of both neoplastic and pre-neoplastic cell growth, and present poor water solubility, which may indicate that the four methoxy groups are essential for the anti-cancer activities and physiochemical properties.

In the following experiments, we determined the effect of TMOC on the colony formation ability of the ovarian cancer cell lines. Colony formation assay is an in vitro cell survival assay based on the ability of a single cell to proliferate indefinitely, thereby retaining its reproductive ability to form a colony consisting of at least 50 cells. Colony formation assay is the method of choice to determine cell reproductive death after treatment with cytotoxic agents, and now widely used to determine the cytotoxicity induced by various chemotherapeutic agents [26]. In this study, as shown in Fig. 2B, treatment of A2780, A2780/CDDP, and SKOV3 cells with TMOC at concentrations of 0.3125, 0.625, 1.25 and 2.5 μM for 48 hours dose-dependently inhibited colony formation, when compared to treatment with diluent (DMSO). The numbers of colonies formed by cells treated with TMOC or diluent were summarized in Fig 2C, which confirmed the inhibited effect of TMOC on the growth of ovarian cancer cells.

**TMOC induces G0/G1 phase cell cycle arrest**

Chemical anti-tumor agents can inhibit cell proliferation through induction of cell cycle arrest. Therefore, to understand how the cell growth was inhibited by TMOC, the cell cycle progression was analyzed by quantitating DNA content using flow cytometry. We treated A2780 cells with TMOC for 24 h and examined the DNA content by propidium iodide (PI) staining (Fig. 3A). As illustrated in Fig. 3B, compared with control cells treated with the diluent, when A2780 cells were treated with high concentrations of TMOC at 5, 10 and 20 μM, the percentage of cells in G0/G1 phase was elevated from 42.5% to 64.1%, and the percentage of cells in S and G2/M phase was decreased concomitantly. Because cell cycle progression is regulated by cyclin/cyclin-dependent kinase (CDK) complexes, the uncontrolled expressions of cyclins and/or CDKs may lead to cell cycle dysregulation and tumorigenesis [27]. Therefore, we examined whether TMOC affected cyclins or CDKs expressions. As shown in Fig. 3C, treatment of cells with TMOC down-regulated the expressions of cyclin D1 and CDK4, but up-regulated the expressions of p16, p21\(^{Cip1}\) and p27\(^{Kip}\) in a dose-dependent manner. Taken together, the altered expressions of the cell cyclins and CDK inhibitors might be associated with cell cycle arrest caused by TMOC.

**TMOC induces cellular apoptosis**

DAPI nuclei staining was used to visualize the apoptosis induced by TMOC. As shown in Fig. 4A, the nuclear apoptosis of TMOC-treated A2780 cells were characterized by condensed and fragmented chromatin stained with strong blue fluorescent dots, whereas control cells were stained with uniform blue [24]. In addition, the apoptosis was also indicated by staining with PI, a membrane impairment nuclear dye, as shown in Fig. 4B. These results indicated that TMOC could induce cellular apoptosis. To further determine the number and stage of apoptotic cells, Annexin-V/PI double staining was applied to quantify the number of apoptotic cells treated with TMOC [22]. As illustrated in Fig. 4C and 4D, the total proportions of cells stained with Annexin V+/PI\(^{-}\) (the right lower quadrant representing early apoptosis) and Annexin V+/PI\(^{+}\) (the right upper quadrant representing late apoptosis and necrosis) cells were increased from 1.2% to 35.5% after treatment of A2780 cells with TMOC at 5, 10 and 20 μM for 24 h. These data further confirmed that TMOC could strongly induce cellular apoptosis of ovarian cancer cells in a dose-dependent manner.

Next, we investigated the signaling pathway involved in TMOC induced apoptosis by Western blot analysis. We showed that (Fig. 4E), TMOC up-regulated the expression of the pro-apoptotic protein Bax, but down-regulated the expressions of the anti-apoptotic proteins Bcl-2 and Bcl-xL in a dose dependent manner. Moreover, compared with control cells, exposure to TMOC induced the cleavage of poly(ADP-ribose) polymerase-1 (PARP-1), a marker of cells undergoing apoptosis [28]. These results suggested that TMOC might induce cellular apoptosis through these proteins.

**TMOC suppresses cell migration and invasion**

Metastatic spreading, one of the features in ovarian cancer cells, is an important factor to low the survival rate of patients [29], while anti-cancer agents can not only inhibit cancer cell growth, but may also halt cell metastases. In this study, to determine whether TMOC suppresses invasion and migration in ovarian cancer cells, we performed wound healing and transwell invasion assays. In wound healing assay, after a single wound was scratched on monolayer of A2780 cells, the medium was changed with serum-free medium containing TMOC or diluent (as control) in order to avoid the influence of cell proliferation. As shown in Fig. 5A and 5B, the migration of cells was significantly decreased by TMOC. The suppression of migration was not due to apoptosis or growth arrest as the concentration of TMOC at 0.5 μM or 1 μM only induced a relative low toxicity in A2780 cells. The results obtained from the transwell assay, showed that TMOC inhibited the invasion and migration of A2780 cells in a dose-dependent manner (Fig. 5C and 5D).

**Signaling pathways involved in TMOC-mediated anti-cancer effects**

Signal transducer and activator of transcription-3 (STAT3), an oncogenic transcription factor, is often constitutively active in human cancer cells [18]. Recent studies have shown that the activation of STAT3 plays a pivotal role in the survival, hyper-proliferation, and metastatic progression of ovarian cancer [30,31]. Once activated, the phosphorylated STAT3 may up-regulate the expression of genes such as apoptosis inhibitors (Bcl-xL, Bcl-2), cell cycle regulators (cyclin D1) and oncogenic transcription factors (c-myc) in tumorigenesis [32,33]. Therefore, we employed western blot to test whether the suppression of STAT3 signaling pathway was involved in the TMOC-mediated anti-cancer effects. Indeed, as shown in Fig. 6A, with the increase of TMOC concentration, the phosphorylation of STAT3 as well as the expression of c-myc, a known downstream target of STAT3 [33,34], was dose-dependently suppressed in A2780, A2780/CDDP and SKOV3 cells, whereas no effect on the expression levels of total STAT3 was observed.
To further confirm the role of STAT3 in TMOC-induced cytotoxicity, we transfected A2780 and A2780/CDDP cells with STAT3 constitutively active (S3-CA) plasmid, STAT3 dominant negative (S3-DN) plasmid or control vector, respectively, and then determined the anti-proliferative effect of TMOC on the transfected cells. As shown in Fig. 6C, introduction of S3-CA significantly rescued the anti-proliferative effect of TMOC. In contrast, introduction of S3-DN sensitized cancer cells to TMOC treatment. Collectively, these data suggest that TMOC may elicit its anti-cancer activity through inhibition of STAT3 signaling pathway.

Several studies have demonstrated that the constitutive activation of STAT3 is often triggered by the non-receptor tyrosine kinase c-Src and negatively regulated by the tumor suppressors p53 and PTEN [35, 36]. Thereby, we also examined whether TMOC could suppress the activation of c-Src kinase and modulate the expression of p53 and PTEN in the three ovarian cancer cell lines. As shown in Fig. 6D, we found that TMOC dose-dependently inhibited the phosphorylation of c-Src kinase, whereas the total level of c-Src remained unchanged. Meanwhile, TMOC up-regulated the expression levels of PTEN in all of the three cell lines, but only up-regulated p53 in A2780 cells.

**Discussion**

In an attempt to develop novel chemotherapeutic agents with less harmful side-effects to treat cancer, natural products and their synthetic analogs have received high attention [37]. For instance, many studies have shown that several chalcone compounds, both derived from nature and synthetic versions, exhibit cytotoxic and antitumor activities [38]. In this study, we intended to study the anti-cancer activity and the underlying mechanism of TMOC, a synthetic chalcone compound, in ovarian cancer cell lines. We showed that TMOC significantly inhibited the proliferation and colony formation of A2780, A2780/CDDP, and SKOV3 cells (Fig. 2), which suggests that TMOC may be an effective chemotherapeutic agent against both cisplatin sensitive and resistant ovarian cancer cells. Importantly, we found that TMOC exhibited less toxicity to pre-neoplastic human ovarian epithelial cells than to neoplastic cells under the same concentration.

We found that TMOC induced G0/G1 cell cycle arrest through the down-regulation of cyclin D1 and CDK4, and the up-regulation of p16, p21 and p27 proteins (Fig. 3C). The cyclin D1/CDK4 complex is responsible for cell cycle progression in early G1 phase and is frequently overexpressed in various human carcinomas including ovarian cancer [39–41]. The p16 protein is a specific inhibitor of CDK-cyclin D complex, preventing the phosphorylation of Rb and cell cycle reentry at G0/G1 phase [39].

We observed p21 and p27, which belong to Cip/Kip family, negatively regulate the cell cycle progression through inhibition of CDK-cyclin complexes [42]. Apoptosis is normally a balanced system tightly regulated by anti-apoptotic and pro-apoptotic effectors, including proteins of the Bcl-2 family. The anti-apoptotic proteins Bcl-2 and Bcl-xL promote cell survival whereas the pro-apoptotic protein Bax induces the programmed cell death. The ratio of Bax/Bcl-2 is critical for the induction of apoptosis and determines whether cells will undergo apoptosis [43]. In the present study, TMOC treatment resulted in the increase of Bax but led to the decrease of Bcl-2 and Bcl-xL (Fig. 4E). The increase of the Bax/Bcl-2 ratio from 0.07 to 1.90 may be responsible for the concomitant apoptosis due to the disruption of mitochondrial membrane potential and the inactivation of key cellular proteins such as PARP-1.

Accumulating studies provide strong evidence that the STAT3 activation has been linked with a variety of tumors including multiple myeloma, ovarian cancer, breast cancer, prostate cancer, and so on [44]. Thus, the suppression of STAT3 signaling pathway has emerged as an effective way for cancer therapy. In our present investigation, the results from Western blots showed that the phosphorylation of STAT3 and its upstream protein tyrosine kinases c-Src (Fig. 6A and 6D) was inhibited by TMOC, which was also supported by the down-regulation of transcriptionally regulated targets such as cyclin D1, Bcl-xL and c-myc [33]. Besides, we identified that TMOC could up-regulate the expression of PTEN in all of the three cell lines but only up-regulated p53 in A2780 cells.

In conclusion, we provide strong evidence that TMOC inhibits cell growth and motility, and induces cell cycle arrest and apoptosis of human ovarian cancer cells through repressing STAT3 and c-Src activation. However, further study may be needed to validate the potential application of TMOC in cancer treatment.

**Author Contributions**

Conceived and designed the experiments: GY MZ. Performed the experiments: QZ ML YL. Analyzed the data: GY ZQ ML. Contributed reagents/materials/analysis tools: GY. Contributed to the writing of the manuscript: GY ML.

**References**

1. Bowtell DD (2010) The genesis and evolution of high-grade serous ovarian cancer. Nature Reviews Cancer 10: 803–809.
2. Siegel R, Naishadham D, Jemal A (2013) Cancer statistics, 2013. CA: a cancer journal for clinicians 63: 11–30.
3. Pujade-Lauraine E, Wagger U, Asavall-Lundquist E, Gembki V, Heywood M, et al. (2010) Pegylated liposomal doxorubicin and carboplatin compared with paclitaxel and carboplatin for patients with platinum-sensitive ovarian cancer in late relapse. Journal of Clinical Oncology 28: 3323–3329.
4. Lengyel E (2015) Ovarian cancer development and metastasis. American journal of pathology 177: 1053–1064.
5. Kipp E, Tan DS, Kaye SB (2015) Meeting the challenge of ascites in ovarian cancer: new avenues for therapy and research. Nature Reviews Cancer 13: 273–282.
6. Harvey AL (2008) Natural products in drug discovery. Drug discovery today 13: 894–901.
7. Demain AL, Vaishnav P (2011) Natural products for cancer chemotherapy. Microbial biotechnology 4: 687–698.
8. Nowakowska Z. (2007) A review of anti-inflammatory and anti-inflammatory chalcones. European Journal of Medicinal Chemistry 42: 125–137.
9. Orlikova B, Tasdemir D, Golaš F, Dicato M, Diederich M (2011) Dietary chalcones with chemopreventive and chemotherapeutic potential. Genes and nutrition 6: 125–147.
10. Yadav VR, Prasad S, Sung B, Aggarwal BB (2011) The role of chalcones in suppression of NF-κB-mediated inflammation and cancer. International immunopharmacology 11: 295–309.
11. Ducati S, Mackenzie G, Greedy R, Armitage S, Chabert JFD, et al. (2011) Combretastatin-like chalcones as inhibitors of microtubule polymerization. Part 2: Structure-based discovery of alpha-aryl chalcones. Bioorganic & medicinal chemistry 19: 7711–7722.
12. Ducati S, Reunisson D, Wu M, Kendall A, Chabert JFD, et al. (2009) Combretastatin-like chalcones as inhibitors of microtubule polymerization. Part 1: synthesis and biological evaluation of antivacular activity. Bioorganic & medicinal chemistry 17: 7696–7710.
13. Dyrauger C, Wickstrom M, Friden-Saxin M, Friberg A, Dahlen K, et al. (2011) Inhibitors and promoters of tubulin polymerization: Synthesis and biological evaluation of chalcones and related dienones as potential anticancer agents. Bioorganic & medicinal chemistry 19: 2659–2665.
14. Sahin IB, Alih WF, Chiardulli LD, Cordeiro MN, Canevarolo RR, et al. (2013) Cytotoxicity 3, 4, 5-trimethoxychalcones as mitotic arresters and cell migration inhibitors. European journal of medicinal chemistry 63: 501–510.
15. Edwards ML, Steenberk DM, Sunkara PS (1998) Chalcones: a new class of antimiotic agents. Journal of medicinal chemistry 53: 1948–1954.
16. Yan XD, Yin J, Yao HY, Mao N, Yang YL, et al. (2010) Increased Expression of Annexin A1 Is a Mechanism of Platinum Resistance in Ovarian Cancer. Cancer research 70: 1616–1621.
17. Liu J, Yang G, Thompson-Lanza JA, Glassman A, Hayes K, et al. (2004) A genetically defined model for human ovarian cancer. Cancer Res 64: 1655–1663.
18. Bromberg JF, Wrezowczyk MH, Deegan G, Zhao Y, Pesnell RG, et al. (1999) Stat3 as an oncoprobe. Cell 98: 295–305.
19. Pernet V, Joly S, Jordi N, Dalkara D, Guzik-Kornacka A, et al. (2013) Misguidance and modulation of axonal regeneration by Stat3 and Rho/ROCK pathway. British Journal of Dermatology 166: 564–574.
20. Karpfen A, Paillard V, Sanders M (1996) Dominant negative stat3 mutant inhibits interleukin-6-induced Jak-STAT signal transduction. J Biol Chem 271: 5961–5964.
21. van Meeloo J, Kaspers GJ, Gloor J (2011) Cell sensitivity assays: the MTT assay. Cancer Cell Culture: Springer. pp.237–245.
22. Wang Z, Hou J, Lu L, Qi Z, Sun J, et al. (2013) Small Ribosomal Protein Subunit S7 Suppresses Ovarian Tumorigenesis through Regulation of the PI3K/AKT and MAPK Pathways. PLoS one 8: e79117.
23. Xi G, Hu X, Wu B, Jiang H, Young CY, et al. (2011) Autophagy inhibition promotes paclitaxel-induced apoptosis in cancer cells. Cancer letters 307: 141–149.
24. Ma Ls, Jiang C, Gu M, Lu R, Lin SX, et al. (2013) Fhuopisin C induces oncosis of human breast adenocarcinoma cells. Acta Pharmacologica Sinica 34: 1093–1100.
25. He S, Yang Y, Liu X, Huang W, Zhang X, et al. (2012) Compound Astragalus and Salvia miltiorrhiza extract inhibits cell proliferation, invasion and collagen synthesis in keloid fibroblasts by mediating transforming growth factor-β/Smad pathway. British Journal of Dermatology 166: 564–574.
26. Franken NA, Rodenmond HM, Staj J, Haveman J, Van Bee C (2006) Clonogenic assay of cells in vitro. Nature protocols 1: 2315–2319.
27. Vermeulen K, Van Bockstaele DR, Berneman ZN (2003) The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. Cell proliferation 36: 131–149.
28. D’Amours D, Sallmann FR, Dixit VM, Pouier GG (2001) Gain-of function of poly (ADP-ribose) polymerase-1 upon cleavage by apoptotic proteases: implications for apoptosis. Journal of cell science 114: 3771–3778.
29. Nauza H, Monell DJ (2005) Ovarian cancer metastatic: integrating insights from disparate model organisms. Nature Reviews Cancer 5: 355–366.
30. Silver DL, Naora H, Liu J, Cheng W, Montell DJ (2004) Activated signal transducer and activator of transcription (STAT) 3 localization in focal adhesions and function in ovarian cancer cell motility. Cancer Research 64: 5550–5558.
31. Yu H, Ballard D, Jove R (2009) STAT3s in cancer inflammation and immunity: a leading role for STAT3. Nature Reviews Cancer 9: 798–809.
32. Shan KS, Sano S, Kajiguchi K, Anders J, Komazawa N, et al. (2004) Disruption of Stat3 reveals a critical role in both the initiation and the promotion stages of epithelial carcinogenesis. Journal of Clinical Investigation 114: 720–728.
33. Yu H, Kooyarski M, Ballard D (2007) Crosstalk between cancer and immune cells: role of STAT3 in the tumour microenvironment. Nature Reviews Immunology 7: 41–51.
34. Kuzni NS, Nakajima K, Ichiba M, Fukada T, Narimatsu M, et al. (1999) STAT3 is required for the gp130-mediated full activation of the e-cmy gene. J Exp Med 189: 63–73.
35. Ram PT, Kengar R (2001) G protein coupled receptor signaling through the Src and Stat3 pathway: role in proliferation and transformation. Oncogene 20: 1601–1606.
36. Mukhopadhyay UK, Mooney P, Jia L, Eves R, Rapti L, et al. (2010) Doubles game: Src-Stat3 versus p53-PTEN in cellular migration and invasion. Molecular and cellular biology 30: 4980–4995.
37. Fulda S (2010) Modulation of apoptosis by natural products for cancer therapy. Planta medica 76: 1073–1079.
38. Regev A, Katsar A, B-chiparous-Litina D (2013) Chalcones in Cancer: Understanding their Role in terms of QSAR. II part. Mini Reviews in Medicinal Chemistry 13: 952–970.
39. Kunime T, Tsuchida H, Kawabata M, Insue T, Umesaki N, et al. (1999) The p16-cyclin D1/CDK4-pRb pathway and clinical outcome in epithelial ovarian cancer. Clinical cancer research 5: 4152–4157.
40. Wolter FA, Akoğlu B, Claussnitzer A, Stein J (2001) Downregulation of the cyclin D1/CDK4 complex occurs during resveratrol-induced cell cycle arrest in colon cancer cell lines. Journal of nutrition 131: 2197–2203.
41. Bali A, O’Brien PM, Edwards LS, Sutherland RL, Hacker NF, et al. (2004) Cyclin D1, p53, and p21Waf1/Cip1 expression is predictive of poor clinical outcome in serous epithelial ovarian cancer. Clinical cancer research 10: 5168–5177.
42. Sherr CJ, Roberts JM (1999) CDK inhibitors: positive and negative regulators of G1-phase progression. Genes & development 13: 1501–1512.
43. Korsmeyer SJ, Shutter JR, Vesey DJ, Merry DE, Oltvai ZN (1993) Bcl-2/Bax: a game: Src-Stat3 versus p53-PTEN in cellular migration and invasion. Molecular and cellular biology 30: 4980–4995.
44. Siveen KS, Sikka S, Surana R, Dai X, Zhang J, et al. (2014) Targeting the STAT3 signaling pathway in cancer: role of synthetic and natural inhibitors. Biochim Biophys Acta 1845: 136–154.
45. Naora H, Montell DJ (2005) Ovarian cancer metastatic: integrating insights from disparate model organisms. Nature Reviews Cancer 5: 355–366.
46. Silver DL, Naora H, Liu J, Cheng W, Montell DJ (2004) Activated signal transducer and activator of transcription (STAT) 3 localization in focal adhesions and function in ovarian cancer cell motility. Cancer Research 64: 5550–5558.