Effects of trans-(±)-kusunokinin on chemosensitive and chemoresistant ovarian cancer cells

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Abstract. Ovarian cancer ranks eighth in cancer incidence and mortality among women worldwide. Cisplatin-based chemotherapy is commonly used for patients with ovarian cancer. However, the clinical efficacy of cisplatin is limited due to the occurrence of adverse side effects and development of cancer chemoresistance during treatment. Trans-(±)-kusunokinin has been previously reported to inhibit cell proliferation and induce cell apoptosis in various cancer cell types, including breast, colon and cholangiocarcinoma. However, the potential effects of (±)-kusunokinin on ovarian cancer remains unknown. In the present study, chemosensitive ovarian cancer cell line A2780 and chemoresistant ovarian cancer cell lines A2780cis, SKOV-3 and OVCAR-3 were treated with trans-(±)-kusunokinin to investigate its potential effects. MTT, colony formation, apoptosis and multi-caspase assays were used to determine cytotoxicity, the ability of single cells to form colonies, induction of apoptosis and multi-caspase activity, respectively. Moreover, western blot analysis was performed to determine the proteins level of topoisomerase II, cyclin D1, CDK1, Bax and p53-upregulated modulator of apoptosis (PUMA). The results demonstrated that trans-(±)-kusunokinin exhibited the strongest cytotoxicity against A2780cis cells with an IC_{50} value of 3.4 μM whilst also reducing the colony formation of A2780 and A2780cis cells. Trans-(±)-kusunokinin also induced the cells to undergo apoptosis and increased multi-caspase activity in A2780 and A2780cis cells. This compound significantly downregulated topoisomerase II, cyclin D1 and CDK1 expression, but upregulated Bax and PUMA expression in both A2780 and A2780cis cells. In conclusion, trans-(±)-kusunokinin suppressed ovarian cancer cells through the inhibition of colony formation, cell proliferation and the induction of apoptosis. This pure compound could be a potential targeted therapy for ovarian cancer treatment in the future. However, studies in an animal model and clinical trial need to be performed to support the efficacy and safety of this new treatment.

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Introduction

Cancer is the second leading cause of mortality globally. In 2020, ovarian cancer was the 8th most commonly diagnosed cancer in the world (1). There are five subtypes of epithelial ovarian cancer: High-grade serous carcinoma (HGSC; 75%); clear cell carcinoma (CC; 6%); endometroid carcinoma (EC; 10%), low-grade serouscarcinoma (3%); and mucinous carcinoma (MC; 6%) (2). Risk factors for ovarian cancer include older age at menopause, hormone replacement therapy and genetic alterations (3). Genomic variations that have been previously associated with ovarian cancer include mutations in BRCA1/2 and TP53, high copy number of KRAS, BRAF, cyclin E1, phosphatidylinositol 3-kinase (PI3K) catalytic subunit, β-catenin and HER2, in addition to the loss of the copy number of PTEN (4,5).

The first-line chemotherapeutic treatment method for ovarian cancer is platinum-based chemotherapy, including cisplatin, carboplatin and oxaliplatin (6). Cisplatin is a platinum drug that interacts directly with DNA, resulting in the formation of DNA adducts leading to cell death (7). Although the drug response rate for ovarian cancer is 60-80%, the majority of patients eventually become resistant to platinum-based drugs and suffer from relapses (8). Platinum-based drugs can cause adverse side effects, including anaphylaxis, cytopenia, hepatotoxicity, ototoxicity and cardiotoxicity (9). Therefore, novel therapeutic agents for ovarian cancer treatment that are more effective and with minimal cytotoxicity are in urgent demand. Over the past decade, targeted therapy is becoming an important form of ovarian cancer treatment strategy due to its direct effects on cancer (10). Furthermore, it causes less damage to normal non-cancerous cells (11). There are various types of targeted therapy for ovarian cancer treatment, including VEGF (bevacizumab), EGFR (cetuximab), HER2 (trastuzumab), mTOR (temsirolimus) (12) and poly-ADP ribose polymerase (PARP1; rucaparib) (13) inhibitors.

Lignans are secondary plant metabolites that have been reported to confer a number of biologically active properties, such as anti-inflammatory, antimicrobial, antiviral and anticancer effects (14). Lignan-based compounds etoposide and teniposide have been applied to treat leukemia, testicular and lung cancer (15). In particular, lignan compounds isolated from plants have also been found to exert anticancer effects on ovarian cancer cells. Magnolol has been documented to inhibit cell proliferation, migration and invasion in HER2-overexpressing ovarian cancer cells (16). In addition, daurinol and deoxyschizandrin has been reported to induce cell cycle arrest by inhibiting topoisomerase IIα (17) and cyclin E expression (18), respectively. Arctigenin has also been demonstrated to inhibit STAT3 phosphorylation and induce caspase-3-dependent apoptosis (19).

Kusunokinin is a dibenzylbutyrolactone lignan that can be found in a wide variety of plants, such as *Haplophyllum vulcanicum* (20), *Wikstroemia sikokiana* (21), *Aristolochia malmeana* (22), *Aristolochia cymbifera* (23), *Wikstroemia indica* (24), *Piper cernuum* (25) and *Piper nigrum* (26). Other lignan compounds, including (-)-cubebin, (-)-hinokinin and (-)-arctigenin, can also be found with (-)-kusunokinin (22,25). (-)-Cubebin, (-)-hinokinin and (-)-arctigenin have been found to confer anticancer effects against colon (HT-29) (27), breast (MCF-7 and SKBR-3) (28) and ovarian (OVCAR-3 and SKOV-3) cancers (19). The anticancer effects of trans-(-)-kusunokinin isolated from *P. nigrum* have been previously evaluated *in vitro* and *in vivo*. Trans-(-)-kusunokinin can exert cytotoxic effects on breast, colorectal and lung cancer cells. Furthermore, it can reduce breast tumor growth in rats (29). This compound can also induce breast cancer cell apoptosis by decreasing topoisomerase II expression whilst increasing that of Bax, caspase-8, -7 and -3 (26). In addition, trans-(-)-kusunokinin can inhibit N-nitrosomethylurea-induced rat mammary tumor growth by suppressing c-Src, PI3K, AKT and ERK1/2 signaling and the expression of proliferative proteins c-Myc, E2F transcription factor-1, CDK1 and cyclin B1. Trans-(-)-kusunokinin has also been found to decrease the expression of migratory proteins MMP-2, MMP-9 and E-cadherin (29). Furthermore, synthetic trans-(±)-kusunokinin was demonstrated to induce cytotoxicity against breast cancer and cholangiocarcinoma cells whilst increasing multi-caspase activity (30). Trans-(-)-kusunokinin can also interact with colony stimulating factor 1 receptor (CSF1R) and HER, proteins associated with cancer cell proliferation (31,32) and aldo-keto reductase family 1 member B1 (AKR1B1), a protein associated with migration (33). Synthetic racemic trans-(±)-kusunokinin, which consists of trans-(-)-kusunokinin and trans-(+)-kusunokinin (Fig. 1A), can reduce CSF1R protein expression and subsequently inhibit AKT and STAT3 activity and the expression of downstream molecules cyclin D1 and CDK1, leading to cell cycle arrest at the G_2/M phase in MCF-7 cells (30,31). Additionally, this effective compound has been demonstrated to decrease Ras, ERK and cyclin B1 expression in breast cancer cells (32).

CSF1R, AKR1B1 and HER2 are overexpressed in ovarian cancer but not in the normal ovarian surface epithelium (34-36). In particular, CSF1R and HER2 expression were previously found to be upregulated upon the induction of cisplatin-resistance in ovarian cancer cells compared with that in cisplatin-sensitive cells (34,36). Since trans-(±)-kusunokinin can bind CSF1R, AKR1B1 and HER2, which causes the inhibition of breast cancer cell proliferation and induction on programmed cell death, the present study investigated the potential effects of this compound on chemosensitive and chemoresistant ovarian cancer cells.

Materials and methods

Cell culture. SKOV-3 and OVCAR-3 cells were obtained from the American Type Culture Collection. A2780 and A2780cis cells were purchased from the European Collection of Authenticated Cell Cultures and Addexbio Technologies, respectively. A2780 are defined as chemosensitive (cisplatin-sensitive) cells and as a type of EC in the epithelial ovarian cancer subtype (37). A2780cis, SKOV-3 and OVCAR-3 are chemoresistant cells and are classified as EC, CC and HGSC in the epithelial ovarian cancer subtype, respectively (37,38). All cells were maintained in RPMI-1640 medium (Thermo Fisher Scientific, Inc.) with different supplements. Medium for A2780, A2780cis and SKOV-3 cells was supplemented with 10% FBS (Thermo Fisher Scientific, Inc.), 1% penicillin/streptomycin (Thermo Fisher Scientific, Inc.) and 1% L-glutamine (Thermo Fisher Scientific, Inc.). Medium for OVCAR-3 cells was supplemented with 20% FBS, 1% penicillin/streptomycin and 1% L-glutamine. For A2780cis cells, 1 μM cisplatin (Sigma-Aldrich; Merck KGaA) was added into RPMI-1640 complete medium every two passages to maintain cisplatin resistance. All cells were maintained at 37˚C in a 5% CO_2 humidified incubator.

Cytotoxicity assay. A2780, A2780cis, SKOV-3 and OVCAR-3 cells were seeded at $1x10^4$, $2.5x10^4$, $2x10^4$ and $3.7x10^4$ cells/well, respectively, into a 96-well plate and incubated for 24 h at 37˚C with 5% CO_2. Next, cells were treated with the various concentrations of racemic trans-(±)-kusunokinin (0-26 μM) [synthesis procedure as previously described (31)], cisplatin (0-80 μM) (Sigma-Aldrich; Merck KGaA) and doxorubicin (0-5 μM; Sigma-Aldrich; Merck KGaA) for 72 h at 37˚C. Cell viability was then assessed using MTT tetrazolium salt (Invitrogen; Thermo Fisher Scientific, Inc.). Absorbance of the wells were then detected at wavelengths of 570 and 650 nm using a Varioskan™ LUX Multimode Microplate Reader (Thermo Fisher Scientific, Inc.). The cell viability rate was expressed as a percentage of untreated control (100% of cell viability), which corresponded to cells treated with only 0.5% DMSO. The percentage of cell survival was calculated as previously described (39). The half maximal inhibitory concentration (IC_{50}) values were calculated by linear approximation regression of the percentage of cell survival vs. the compound concentration.

Colony formation assay. A2780 and A2780cis cells were seeded at $1x10^3$ and $2x10^3$ cells/well, respectively, into a 3.5-cm culture dish and incubated for 24 h at 37˚C with 5% CO_2. Next, the cells were treated with 1X or 2X IC_{50} concentration of trans-(±)-kusunokinin and cisplatin for 72 h at 37˚C in a 5% CO_2. Medium was then removed and cells were washed with 1X PBS. Fresh complete medium was added into the plates for further culture. After 5 days of incubation at 37˚C, colonies were stained with 0.5% crystal violet

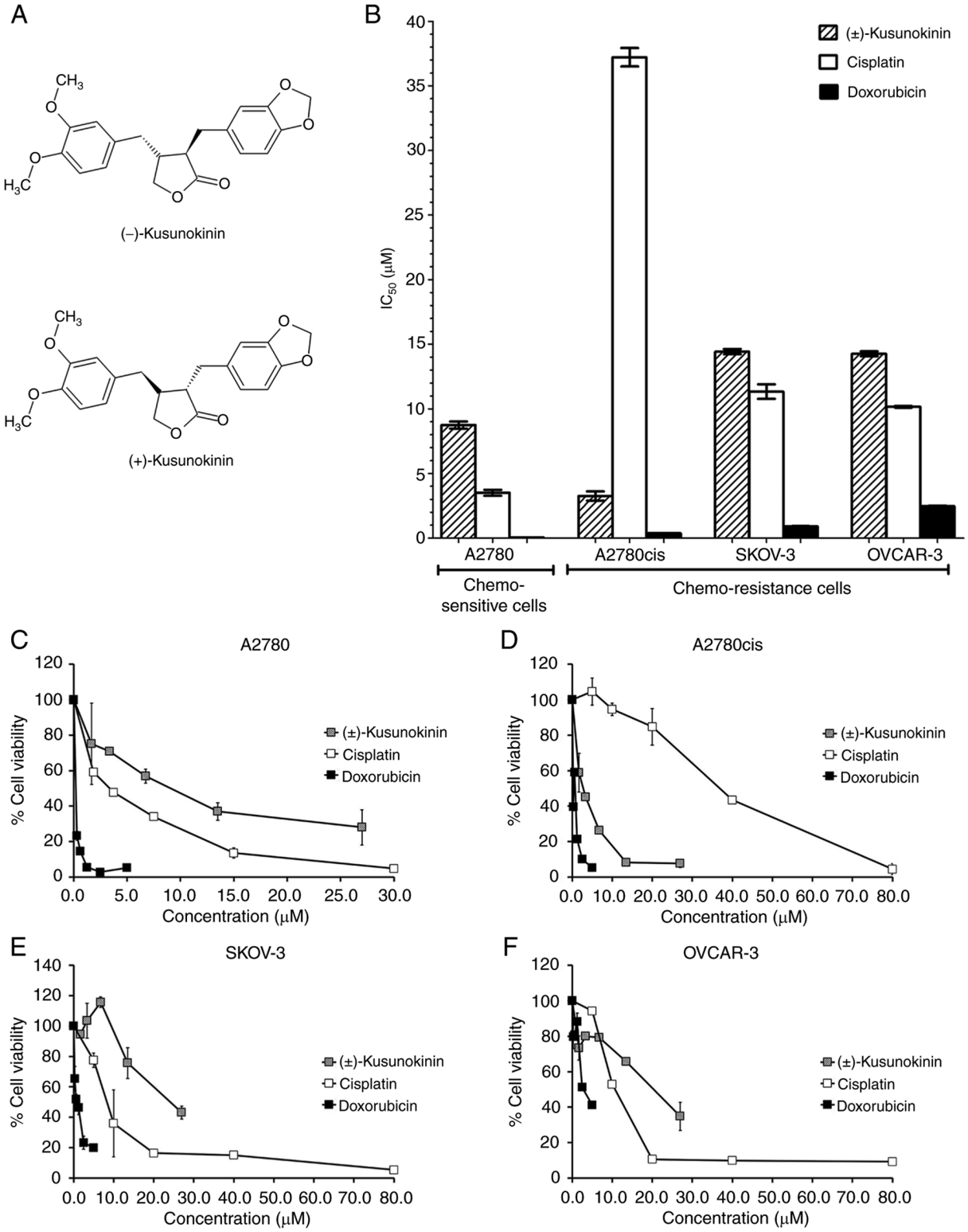

Figure 1. Cytotoxicity of synthetic trans-(±)-kusunokininon ovarian cancer cells. MTT assay was used to detect cytotoxicity of ovarian cancer cells treated with various concentrations of cisplatin, doxorubicin and (A) racemic trans-(±)-kusunokinin for 72 h. (B) The IC_{50} value and percentage of cell viability for (C) A2780, (D) A2780cis, (E) SKOV-3 and (F) OVCAR-3 cells are calculated and quantified. All data represent the mean ± SD. n=3. A2780cis, cisplatin-resistant A2780 cells.

solution in 25% methanol (Sigma-Aldrich; Merck KGaA) for 10 min at room temperature and counted under a light inverted microscope (Olympus Corporation). A colony was defined as a cluster of ≥50 cells (40). The percentage of colonies was calculated using the following formula: Colonies (%)=(Number of colonies in treatment group/Number of colonies in control) x100.

Apoptosis assay and multi-caspase activation assay. To investigate the effects of trans-(±)-kusunokinin on apoptosis

and caspase activity in ovarian cancer cells, A2780 and A2780cis cells were seeded into a 12-well plate at cell densities of $1.5x10^5$ and $2x10^5$ cells per well, respectively. A2780 and A2780cis cells were treated with the IC_{50} concentration of trans-(±)-kusunokinin and incubated at 37˚C. Next, treated cells were harvested at 0, 24, 48 and 72 h.

For apoptosis assay, $2.5x10^4$ treated cells were stained with 50 μl Muse® Annexin V & Dead Cell reagent (Muse® Annexin V & Dead Cell kit, cat. no. MCH100105; EMD Millipore) and incubated at room temperature for 20 min. Apoptotic cells were analyzed using Muse® Cell Analyzer (EMD Millipore) and results were analyzed by Muse 1.8 analysis software (30). The low-left quadrant represented the live cells that were not stained with Annexin V or 7-aminoactinomycin D (7AAD). The low-right quadrant represented cells in the early stages of apoptosis, which were stained with only Annexin V. By contrast, the upper-right quadrant represented cells at the late stages of apoptosis, which were stained with both Annexin V and 7AAD. The upper-left quadrant represented dead cells (possibly necrotic), where cells were not stained with Annexin V but were stained with 7AAD.

For multi-measuring caspase activity, $2.5x10^4$ treated cells were incubated in 50 μl Muse® MultiCaspase working solution at 37˚C for 30 min for multi-measuring caspase activity. Subsequently, 50 μl caspase 7AAD working solution (Muse® MultiCaspase kit; cat. no. MCH100109; EMD Millipore) was added and the cells were incubated at room temperature for 5 min. Multi-caspase activity were analyzed using Muse® Cell Analyzer (EMD Millipore) and results were analyzed by Muse 1.8 analysis software (30). In this assay, VAD peptide, an effective pan-caspase (caspase-1, -3, -4, -5, -6, -7, -8, and -9) inhibitor, bind to the active sites of the caspases, leading to an increase in fluorescent intensity in the caspase-positive cell population. 7AAD was used to detect double-stranded DNA damaged/dead cells. The lower-left, lower-right, upper-right and upper-left quadrants represented live cells exhibiting caspase activity, late stages of caspase activity or are dead following caspase activiation and necrosis cells, respectively.

Western blot analysis. A2780 and A2780cis cells were treated with the IC_{50} concentration of trans-(±)-kusunokinin and harvested at 0, 24, 48 and 72 h after incubation at 37˚C. Cell pellets were suspended in RIPA buffer (Pierce; Thermo Fisher Scientific, Inc.). Protein concentration of whole cell lysates was determined using the Bio-Rad Bradford Protein assay (Bio-Rad Laboratories, Inc.). In total, 50 μg total protein were then separated by 12% SDS-PAGE and transferred onto nitrocellulose membranes (EMD Millipore). Next, membranes were blocked in 5% non-fat dry milk dissolve in 1X Tris-buffered saline, 0.5% Tween 20 (TBST) for 1 h at room temperature. Afterwards, the membranes were incubated with primary antibodies against topoisomerase IIα (1:1,000; rabbit monoclonal antibody; cat. no. 12286; Cell Signaling Technology, Inc.), cyclin D1 (1:500; rabbit polyclonal antibody; cat. no. 2922; Cell Signaling Technology, Inc.), CDK1 (1:500; mouse monoclonal antibody; cat. no. sc-53219; Santa Cruz Biotechnology, Inc.), Bax (1:500; rabbit polyclonal antibody; cat. no. 2772; Cell Signaling Technology, Inc.), p53-upregulated modulator of apoptosis (PUMA; 1:500; rabbit polyclonal antibody; cat. no. 4976; Cell Signaling Technology, Inc.), CSF1R (1:500; mouse monoclonal antibody; cat. no. sc-46662; Santa Cruz Technology, Inc.) and GAPDH (internal control; 1:5,000; mouse monoclonal antibody, cat. no. CB1001; EMD Millipore) for 3 h at room temperature. For AKR1B1, the membrane was incubated with rabbit polyclonal antibody (cat. no. AV48180; Sigma-Aldrich; Merck KGaA) at 4˚C overnight. After incubation with topoisomerase IIα, cyclin D1, CDK1, Bax, PUMA, CSF1R and AKR1B1 antibodies, the membranes were then incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit (cat. no. 7074; Cell Signaling Technology, Inc.) or horse anti-mouse IgG antibodies (cat. no. 7076; Cell Signaling Technology, Inc.) at dilution 1:2,500 for 1 h at room temperature. HRP-conjugated horse anti-mouse IgG antibody at dilution 1:10,000 was used to detect GAPDH and incubated for 1 h at room temperature. Immunoreactive bands were detected with SuperSignal™ West Dura Extended Duration substrate kit (Thermo Fisher Scientific, Inc.). The intensity of band was analyzed using the ImageJ software (version 1.5.3; National Institutes of Health).

Statistical analysis. All data were expressed as the mean ± standard deviation. Data from the two groups was assessed by the unpaired Student's t-test, while one-way analysis of variance followed by Bonferroni's post hoc test (GraphPad Prism8; GraphPad Software, Inc.) was used to analyze the data in multiple groups. $P<0.05$ was considered to indicate a statistically significant difference. All results were determined in ≥3 independent experiments.

Results

Effects of trans-(±)-kusunokinin on the viability of ovarian cancer cells. To evaluate the effects of trans-(±)-kusunokinin on chemosensitive (A2780) and chemoresistant (A2780cis, SKOV-3 and OVCAR-3) ovarian cancer cell lines, the cytotoxicity of trans-(±)-kusunokinin was detected using MTT assay. Chemotherapeutic drugs cisplatin and doxorubicin were used as a positive control. The IC_{50} values of trans-(±)-kusunokinin, cisplatin and doxorubicin of the ovarian cancer cell lines are shown in Fig. 1B. Trans-(±)-kusunokinin exhibited a stronger cytotoxic effect on A2780cis cells compared with that by cisplatin. In addition, trans-(±)-kusunokinin exerted the strongest levels of cytotoxicity against A2780cis (IC_{50}, 3.25±0.62 μM) and A2780 (IC_{50}, 8.75±0.47 μM) cells, which were used as representatives of chemoresistant and chemosensitive ovarian cancer cells, respectively. Trans-(±)-kusunokinin showed a cytotoxic effect on SKOV-3 and OVCAR-3 cells with IC_{50} values of 14.43±0.34 and 14.26±0.32 μM, respectively. Nevertheless, trans-(±)-kusunokinin did not show cytotoxicity stronger than doxorubicin in A2780, A2780cis, SKOV3 and OVCAR-3 cells. Trans-(±)-kusunokinin also markedly decreased cell viability in all cell lines tested in a dose-dependent manner (Fig. 1C-F).

Trans-(±)-kusunokinin treatment inhibits colony formation. In the present study, the effects of trans-(±)-kusunokinin on the capacity of the single-cell proliferation by A2780 and A2780cis cells were next investigated. The result showed that both IC_{50} and 2X IC_{50} concentrations of trans-(±)-kusunokinin significantly inhibited colony formation by A2780 cells compared with that in the corresponding control group

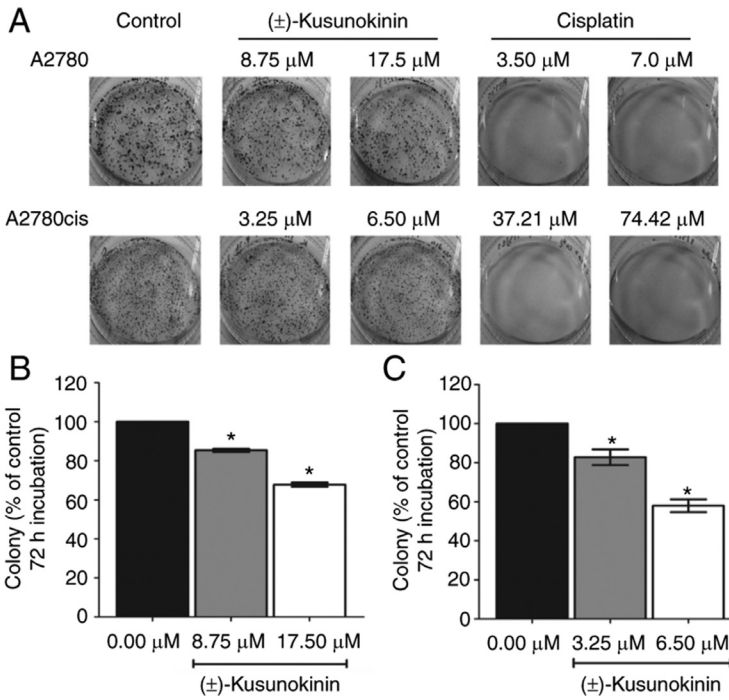

Figure 2. Inhibitory effects of trans-(±)-kusunokinin on colony formation in ovarian cancer cell lines. (A) A2780 and A2780cis cells were treated with 1X IC_{50} and 2X IC_{50} of trans-(±)-kusunokinin and cisplatin. The percentage of (B) A2780 and (C) A2780cis colony formation were then quantified. All data represent the mean ± SD. n=3. *P<0.05 vs. 0.00 μM. A2780cis, cisplatin-resistant A2780 cells.

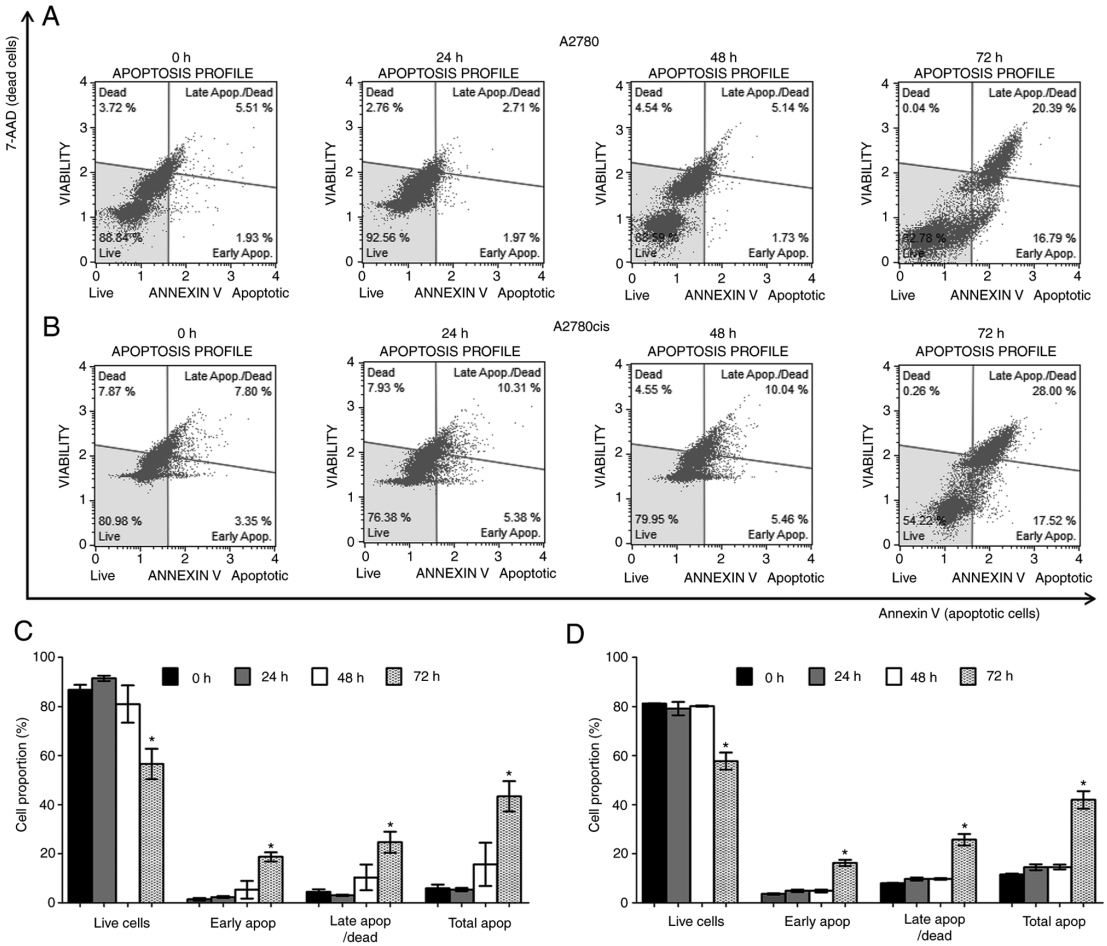

Figure 3. Trans-(±)-kusunokinin promotes apoptosis. (A) A2780 and (B) A2780cis cells were treated with 8.75 and 3.25 μM trans-(±)-kusunokinin, respectively, for 24, 48 and 72 h. The percentage of live cells, early apoptotic, late apoptotic/dead and total apoptotic (C) A2780 and (D) A2780cis cells compared with that of non-treated cells at 0 h are quantified. All data represent the mean ± SD. n=3. *P<0.05 vs. non-treated cells (0 h). A2780cis, cisplatin-resistant A2780 cells.

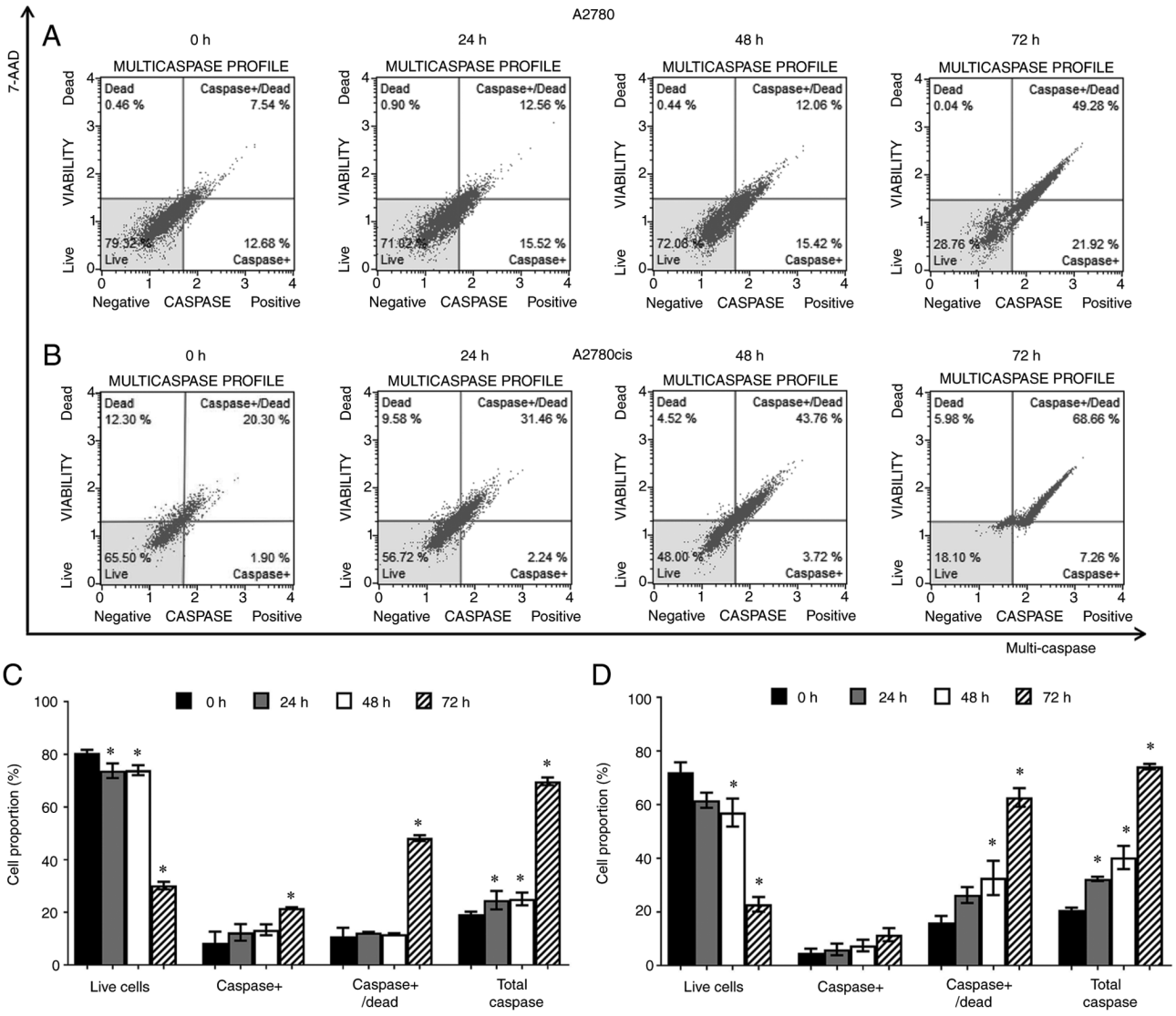

Figure 4. Trans-(±)-kusunokinin stimulates multi-caspase activity. (A) A2780 and (B) A2780cis cells were treated with 8.75 and 3.25 μM of trans-(±)-kusunokinin, respectively, and harvested at 24, 48 and 72 h. The percentage of live cells, caspase+, caspase+/dead, dead and total caspase (caspase+ and caspase+/dead) (C) A2780 and (D) A2780cis cells compared with that of non-treated cells at 0 h were quantified. All data represent the mean ± SD. n=3. *P<0.05 vs. non-treated cells (0 h). A2780cis, cisplatin-resistant A2780 cells.

(Fig. 2A and B). In addition, IC_{50} and 2X IC_{50} concentrations of trans-(±)-kusunokinin significantly inhibited colony formation by A2780cis cells compared with that in the corresponding control group (Fig. 2A and C). However, treatment with both IC_{50} and 2X IC_{50} concentrations of cisplatin almost completely inhibited the formation of colonies by A2780 and A2780cis cells (Fig. 2A).

Trans-(±)-kusunokinin induces apoptosis on both chemoresistant and chemosensitive ovarian cancer cells. To investigate if the inhibition of cell proliferation of trans-(±)-kusunokinin was associated with apoptosis, A2780 and A2780cis cells were incubated with the IC_{50} concentration of (±)-kusunokinin at 8.75 and 3.25 μM, respectively. The proportion of apoptotic cells was determined using Annexin V-FITC staining assay. Double-stranded DNA of damaged or dead cells was quantified using 7AAD, a fluorescent intercalator. Results showed that trans-(±)-kusunokinin significantly decreased the proportion of live cells at 72 h compared with that in non-treated cells (0 h) for both A2780 (Fig. 3A and C) and A2780cis cells (Fig. 3B and D). In addition, trans-(±)-kusunokinin significantly increased the number of early apoptotic, late apoptotic/dead and total apoptotic A2780 and A2780cis cells at 72 h compared with that of non-treated cells (0 h; Fig. 3C and D). In total, >10% of apoptotic cells were found on non-treated A2780cis cells at 0, 24, 48 and 72 h. However, trans-(±)-kusunokinin significantly decreased the number of live cells and increased the number of early apoptotic, late apoptotic/dead and total apoptotic cells at 72 h compared with non-treated cells (72 h) (Fig. S1).

Trans-(±)-kusunokinin enhances multi-caspases activity. To verify the effects of trans-(±)-kusunokinin on the apoptotic mechanism, a multi-caspase activity assay (analyzing caspases -1, -3, -4, -5, -6, -7, -8 and -9) was performed. A2780 and A2780cis cells were incubated with 8.75 and 3.25 μM of trans-(±)-kusunokinin, respectively, for 24, 48 and 72 h. The results showed that compared with that in the 0 h group,

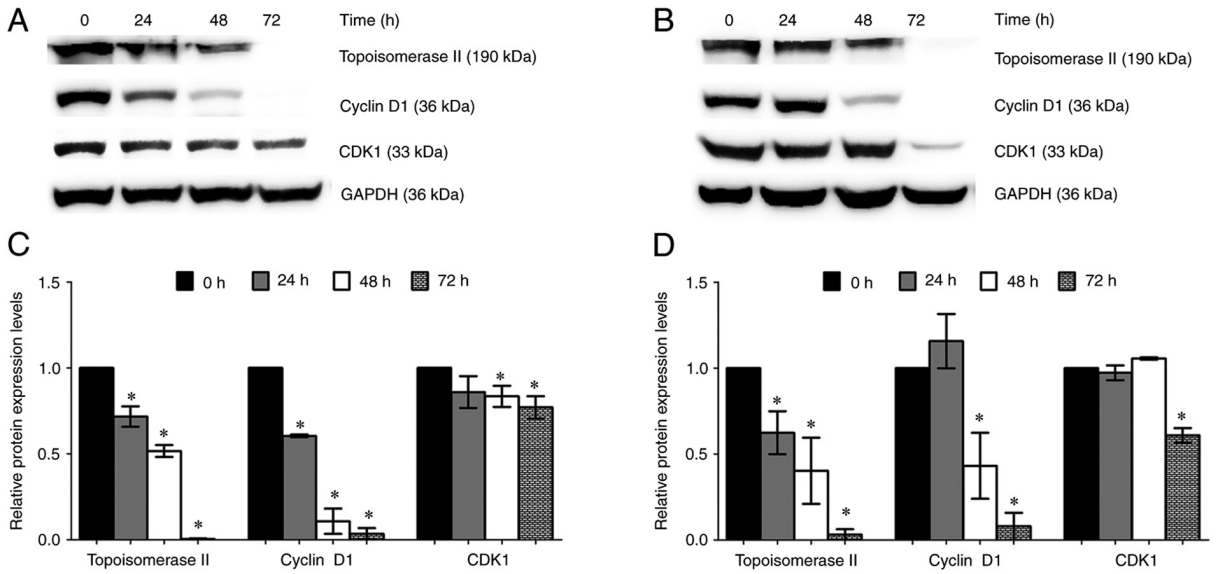

Figure 5. Effect of trans-(±)-kusunokinin on cell proliferation proteins. (A) A2780 and (B) A2780cis cells were treated with 8.75 and 3.25 μM of trans-(±)-kusunokinin, respectively, and incubated for 24, 48 and 72 h. Topoisomerase II (190 kDa), cyclin D1 (36 kDa), CDK1 (33 kDa) and GAPDH (36 kDa) protein expression were detected using western blot analysis. Protein levels of (C) A2780 and (D) A2780cis were quantified by normalization with GAPDH band intensity. All data represent the mean ± SD. n=3. *P<0.05 vs. control group (0 h). A2780cis, cisplatin-resistant A2780 cells.

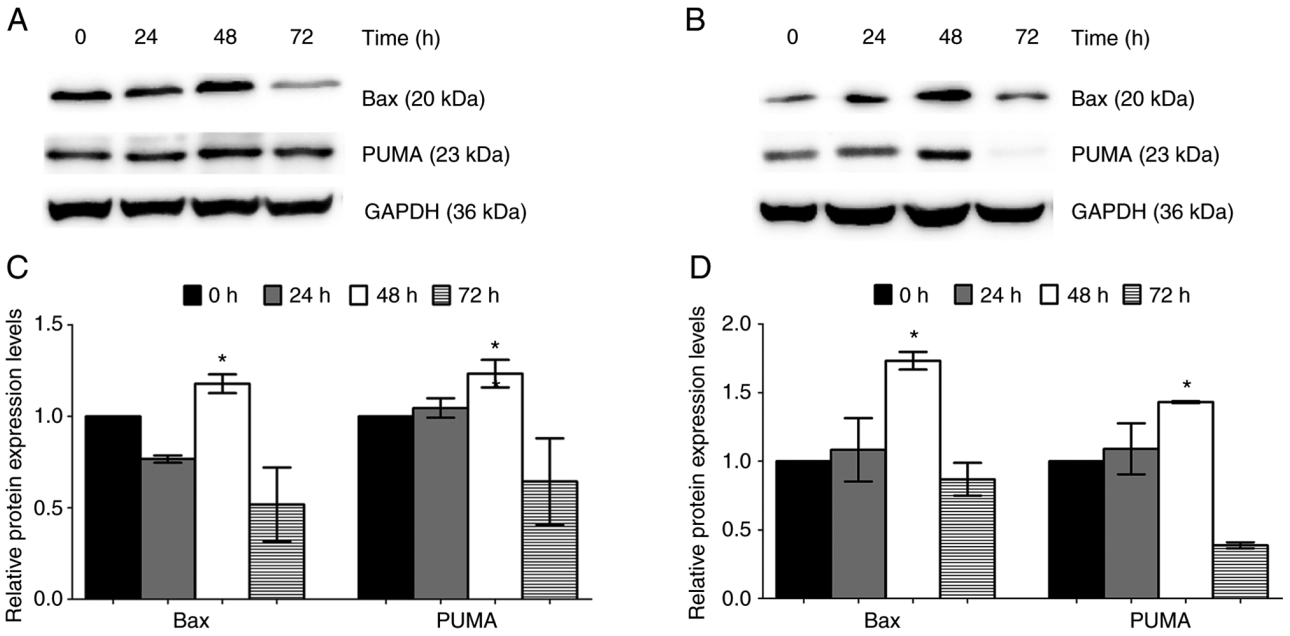

Figure 6. Effect of trans-(±)-kusunokinin on apoptotic proteins. (A) A2780 and (B) A2780cis cells were treated with 8.75 and 3.25 μM of trans-(±)-kusunokinin, respectively, and incubated for 24, 48 and 72 h. Bax (20 kDa), PUMA (18 kDa) and GAPDH (36 kDa) proteins were detected using western blot analysis. Protein expression of (C) A2780 and (D) A2780cis was quantified following normalization to GAPDH. All data represent the mean ± SD. n=3. *P<0.05 vs. control group (0 h). A2780cis, cisplatin-resistant A2780 cells; PUMA, p53-upregulated modulator of apoptosis.

trans-(±)-kusunokinin significantly decreased the percentage of live A2780 (Fig. 4A and C) and A2780cis (Fig. 4B and D) cells in a time-dependent manner, but especially at 72 h. In addition, trans-(±)-kusunokinin increased the percentage of caspase+, caspase+/dead and total caspase A2780 and A2780cis cells in a time-dependent manner compared with that in the control 0 h group, especially at 72 h (Fig. 4C and D). In total, >10% of caspase+/dead and total caspase cells were found in the non-treated A2780 and A2780cis groups at 0, 24, 48 and 72 h. Nevertheless, trans-(±)-kusunokinin significantly decreased the percentage of live cells and increased the percentage of caspase+/dead and total caspase at 72 h compared with non-treated cells (72 h). Moreover, the percentage of caspase+, caspase+/dead and total caspase A2780cis cells were also increased in a time-dependent manner compared with non-treated cells at 24 to 72 h (Fig. S2).

Trans-(±)-kusunokinin suppresses the expression of proteins associated with cell proliferation. CSF1R and AKT, proliferation proteins, were found at significantly

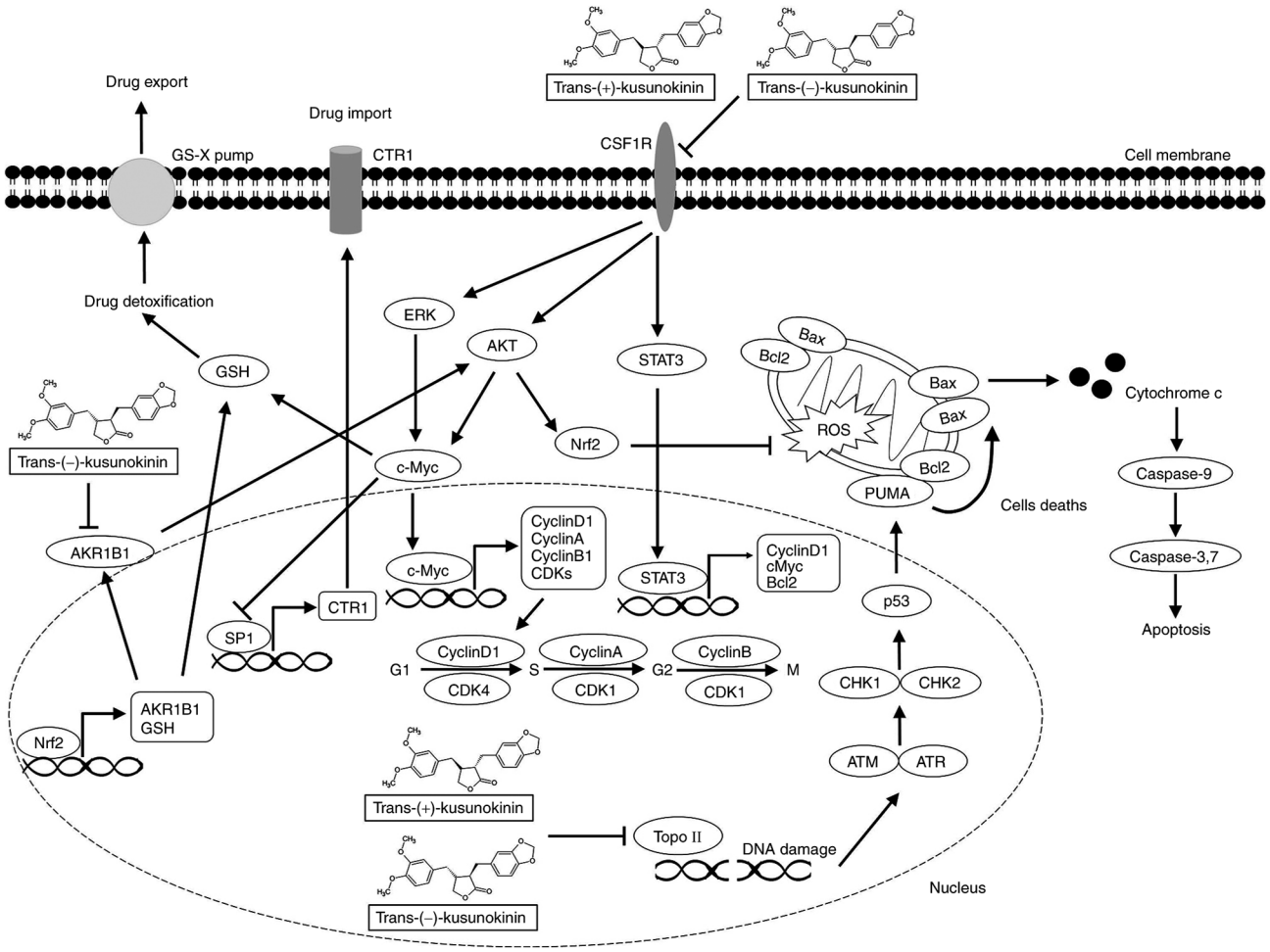

Figure 7. Schematic representation of the possible molecular mechanisms underlying the anticancer activity of trans-(±)-kusunokinin on ovarian cancer. Trans-(±)-kusunokinin inhibited CSF1R, topoisomerase II and AKR1B1, and could lead to the suppression of cell proliferation and drug resistance in cisplatin-resistance ovarian cancer cells (A2780cis).

higher levels in A2780cis cells compared with A2780 cells (Fig. S3). Due to the action of trans-(±)-kusunokinin on the inhibition of colony formation on ovarian cancer cells, the expression of cyclin D1, CDK1 and topoisomerase II, proteins associated with cell proliferation (41,42), were determined using western blot analysis. Chemosensitive (A2780) and chemoresistant (A2780cis) ovarian cancer cells were treated with 8.75 and 3.25 μM trans-(±)-kusunokinin, respectively, for 24, 48 and 72 h. The results showed that topoisomerase II and cyclin D1 expression was significantly decreased in A2780 cells at 24, 48 and 72 h compared with that in cells in the 0 h control group (Fig. 5A and C). In addition, trans-(±)-kusunokinin significantly decreased topoisomerase II and cyclin D1 expression at 48 and 72 h in A2780cis cells compared with that in cells in the 0 h control group (Fig. 5B and D). CDK1 expression was significantly suppressed by trans-(±)-kusunokinin treatment at 72 h in both ovarian cancer cell lines tested (Fig. 5).

Trans-(±)-kusunokinin increases the expression of apoptotic proteins. To verify the observations of the induction of apoptosis by trans-(±)-kusunokinin on both chemosensitive (A2780) and chemoresistant (A2780cis) ovarian cancer cells, proteins associated with the intrinsic apoptosis pathway Bax and PUMA were investigated. After treatment with the IC_{50} concentration of trans-(±)-kusunokinin, Bax and PUMA expression was significantly increased at 48 h after the treatment of A2780 and A2780cis cells compared with that in the 0 h control group. However, Bax and PUMA were downregulated at 72 h in both A2780 and A2780cis cells (Fig. 6).

Discussion

Trans-(-)-kusunokinin can be extracted from black pepper (*P. nigrum*) and has been reported to inhibit breast (MCF-7, MDA-MB-468 and MDA-MB-231), colon (HT-29 and SW-620) and lung (A-549) cancer cells (26,29). In addition, the synthetically derived trans-(±)-kusunokinin has also been found to inhibit breast cancer (MCF-7, MDA-MB-468 and MDA-MB-231), colon cancer (HT-29) and cholangiocarcinoma (KKU-M213 and KKU-K100) cells (30). In the present study, the potential effect of synthetic trans-(±)-kusunokinin on cisplatin-sensitive (A2780) and cisplatin-resistant (A2780cis, SKOV-3 and OVAR-3) ovarian cancer cells was investigated. It was first found that trans-(±)-kusunokinin exerted particularly potent cytotoxic effects against A2780cis, even to a higher extent compared with A2780 cells. Ovarian cancer cells were previously demonstrated to exhibit higher expression levels

of CSF1R, AKR1B1 and HER2 compared with those in the normal ovarian surface epithelium (34-36). High expression levels of CSF1R and HER2 promote cisplatin-resistance in ovarian cancer cells (34,36). EC exhibits a number of genetic features, including the overexpression of K-ras, HER2 and β-catenin genes and dysfunctions in PTEN and p53 gene expression (43). Proposed targets for EC therapy include mTOR, AKT, PI3K, MEK, HER2, VEGF, receptor tyrosine kinases, CSF1R and PARP (44). Trans-(±)-kusunokinin acts on a variety of proteins that have been previously linked to the genetic features of EC, including CSF1R, AKT, PI3K, RAS and HER2 (29,31-33).

For colony formation, A2780 and A2780cis cells were incubated with the trans-(±)-kusunokinin for 72 h. Ideally, a colony should be defined to be >50 cells. Cells incubated with the IC_{50} concentrations of trans-(±)-kusunokinin retained their cell division abilities by ~80% in A2780 and A2780cis cells. However, 2X IC_{50} concentrations of trans-(±)-kusunokinin inhibited colony formation in both cell lines. In addition, cells incubated with cisplatin were unable to divide (3-5 cells/colony). Therefore, they could not be counted, since the staining was too weak due to insufficient cells/colonies. This suggests that trans-(±)-kusunokinin bound to their target proteins in a reversible manner, such that after this drug was removed, the cells returned to their proliferative states through this process was not as efficient. In addition, the cells did not show 50% inhibition of colony formation because the cells likely recovered after the withdrawal of trans-(±)-kusunokinin. By constrast, cisplatin binds the purine bases of DNA irreversibly, which causes DNA damage, such that even after the drug was removed, the cells could not recover. Therefore, few cells in the cisplatin treatment group remained alive. Hence, in conclusion the ovarian cancer cells treated with trans-(±)-kusunokinin may have recovered after trans-(±)-kusunokinin removal, which resumed colony formation activities in both cell lines.

Trans-(±)-kusunokinin was found to inhibit A2780 and A2780cis cell proliferation through the induction of apoptosis and multi-caspase activity. However, >10% apoptosis and dead A2780cis cells were observed even at 0 h (non-treated cell group). This effect could be due to cell death during experimental protocol, which has been previously reported (45,46). Therefore, additional assays on the apoptosis of non-treated (0 h) cells at 24, 48 and 72 h were performed to verify the effects of trans-(±)-kusunokinin on apoptosis at 72 h (Fig. S1). It was observed that >10% caspase+/dead A2780 and A2780cis cells were also seen at 0 h (non-treated cell group). This effect may be due to apoptosis occurring during the experimental process. These results were previously reported (47-49). Therefore, measurements of multi-caspase activity in non-treated cells at 24, 48 and 72 h were performed, which served as an internal control to assess the function of trans-(±)-kusunokinin on multi-caspase activity at 72 h (Fig. S2).

In total there are five mechanisms that contribute to cisplatin resistance: Decreased drug import; increased drug export; increased drug inactivation by detoxification enzymes; increased DNA damage repair; and inactivated cell death signaling (50). Trans-(±)-kusunokinin may be involved in all key drug resistance mechanisms through the suppression of CSF1R, AKT, ERK, c-Myc, STAT3 and Bcl-2 signaling, which was previously reported (29-30). Overexpression of copper transporter 1 (CTR1) has been found to increase cisplatin uptake. Therefore, low expression levels of CTR1 promote ovarian cancer resistance to platinum-based drugs (51). Specificity protein (Sp1) is a transcription factor that can upregulate the expression of CTR1 (52). By contrast, Sp1 can also be suppressed by c-Myc (53). c-Myc is involved in the response to oxidative stress. This protein can activate glutathione-directed survival pathways, which are involved in cellular detoxification, redox balance and stress response in tumor cells (54,55). In addition, nuclear factor-erythroid 2 related factor 2 (Nrf2) has been found to regulate the expression of AKR1B1 (56). The AKR1B1 enzyme serves an important role in drug detoxification and can regulate the development and progression of breast and ovarian cancers (50,56). Suppression of CSF1R expression by trans-(±)-kusunokinin leads to the reduction of AKT, ERK and STAT3 signaling, followed by the reduced expression of Bcl-2, which is associated with the activation of cell death (55,57,58).

Trans-(±)-kusunokinin may also reverse the mechanism of doxorubicin resistance through the suppression of AKT in MCF-7 cells (31). Doxorubicin-resistant cells tend to overexpress Nrf2, which then suppresses ROS production in cells to negate the effects of doxorubicin (59). One potential upstream signaling component of Nrf2 was previously found to be AKT (60). Therefore, we hypothesize that the reduction of CSF1R and AKT may lead to reduced Nrf2 expression.

In the present study, trans-(±)-kusunokinin inhibited topoisomerase II, cyclin D1 and CDK1 expression, consistent with a previous finding (30,31). Both natural trans-(-)-kusunokinin and synthetic trans-(±)-kusunokinin were previously found to downregulate topoisomerase II, CDK1 and cyclin D1 in breast cancer cells (MCF-7) (26,29-31). These results support the findings from the present study that trans-(±)-kusunokinin inhibited ovarian cancer cell proliferation through suppressing the expression of proteins involved in cell proliferation. Lignan-based compounds, such as daurinol and (-)-hinokinin, has been found to decrease topoisomerase II and cyclin D1 expression in ovarian (SNU-840) and breast (MCF-7 and SKBR-3) cancer cells, respectively (17,28). During cell proliferation, cyclin D1 and CDKs are downstream proteins in the CSF1R pathway (61) and are also transcribed by c-Myc (62). CSF1R translocates into the nucleus whilst complexed with CSF1 and bind to the promoter region of cyclin D1, c-Myc and c-Jun (61). Specifically, CSF1R and c-Myc were previously found to be overexpressed in cisplatin-resistant cells (SKOV-3/CR, CaoV-3/CR, A2780CP20 and A2780cis), where they served an important role in the cisplatin resistance mechanism (34,63). Additionally, activation of the STAT3 signaling pathway is another potential mechanism in the chemoresistance of ovarian cancer cells (64). STAT3 is expressed at higher levels in A2780cis cells compared with A2780 cells (65) and can regulate the expression of cell cycle (c-Myc and cyclin D1), anti-apoptosis (Bcl-xL, Bcl-2 and survivin), angiogenesis (VEGF and IL-8) and migration (MMP-2 and MMP-9) proteins (66). Taken together, the action of trans-(±)-kusunokinin found in the present study may have occurred through the suppression of signaling proteins, especially CSF1R.

The induction of DNA damage by cisplatin induces apoptosis by activating the intrinsic pathway on ovarian cancer. p53 is a tumor suppressor protein that responds to DNA damage

and induces apoptotic proteins such as Bax, Bak and PUMA in intrinsic pathways at the mitochondria (67). In addition, inhibition of topoisomerase II causes transient breaks in the double-strand DNA (68). Consequently, ataxia telangiectasia mutated and ataxia telangiectasia and rad3-related (a checkpoint protein) can detect the double-stranded breaks in the DNA and activate checkpoint kinases (CHK)1 and CHK2, in addition to p53 (69). p53 triggers the transcription of various apoptotic genes, such as PUMA and phorbol-12-myristate-13-acetate-induced protein 1. PUMA activates the pro-apoptotic Bcl-2 family member of proteins, including Bax and Bak (70). These two proteins then trigger cytochrome *c* release from the mitochondria, followed by the induction of caspases-9 and -3 (71). Topoisomerase II is another important target for anticancer drugs, including etoposide, doxorubicin, daunorubicin and mitoxantrone (72). The present study verified the action of trans-(±)-kusunokinin on the induction of apoptosis, multi-caspase activity and expression of apoptotic proteins. Topoisomerase II was found to be decreased at 24 h, followed by increased PUMA and Bax expression at 48 h (Fig. 6). Bax then induced mitochondrial dysfunction and caspase activity at 72 h. Consistent with findings from a previous study, natural trans-(-)-kusunokinin downregulated topoisomerase II expression whilst upregulating p53 expression at 24 h. The downstream proteins of p53, including p21, Bax, cytochrome *c*, cleaved caspases-7 and -8, were sequentially activated (26). Synthetic trans-(±)-kusunokinin was also found to induce apoptosis and multi-caspase activity in breast cancer cells (30). However, the protein levels of PUMA and Bax were decreased at 72 h, which could be due to the half-life of the proteins. A previous study showed that Bax expression was increased at 48 h but was decreased at 72 and 96 h (26). Other natural compounds isolated from cotton seed (AT101) increased PUMA expression at 12 h but then decreased at 24 h in A2780cis cells (73).

A2780cis cells have high expression levels of c-Myc and cyclin D1 expression along with high levels of ERK and AKT activation, which serve a role in mediating the cisplatin resistance mechanism (63,74-76). It was found that CSF1R and AKR1B1 expression was significantly higher in A2780cis cells compared with that in A2780 cells (Fig. S3). These results support the hypothesis on the action of trans-(±)-kusunokinin on cisplatin-resistant cells. Therefore, the activity of trans-(±)-kusunokinin in the suppression of cisplatin-resistance in ovarian cancer cells could be due to its action and binding activity on the CSF1R and AKR1B1 proteins (Fig. 7). However, the combinatorial treatment of trans-(±)-kusunokinin with chemotherapeutic drugs should be evaluated in future studies to confirm the mechanism underlying drug resistance of ovarian cancer cells. Furthermore, it is necessary to investigate the underlying molecular mechanisms in the combination treatment.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author's contributions

PG contributed to the study conception and design. NMA performed the majority of the experiments, as well as the statistical analysis, and drafted the initial manuscript. TR performed the apoptosis and multi-caspase assays. TT performed the western blot analysis. PG was responsible for data analysis and revised the final version of the manuscript. PG and NMA confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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