10-Gingerol Inhibits Ovarian Cancer Cell Growth by Inducing G2 Arrest

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Introduction
Ginger root has a tradition of use as a remedy for numerous ailments that include nausea, loss of appetite, cramps, diarrhea, heartburn, migraines, colds, influenza, and arthritis.1 Ginger also contains a number of bioactive compounds with anticancer activities.2 Gingerols, which are a series of pungent phenolic homologs that differ in unbranched alkyl chain length, are important biologically active constituents of the rhizomes of ginger.3 Numerous laboratory studies attest to the anticancer activities of 6-gingerol, which has been the focus of most research.4 For example, 6-gingerol induces G1 phase cell cycle arrest and death of colorectal cancer cells, inhibits hepatocarcinoma cell motility and invasion, and the activity of metastasis-promoting matrix metalloproteinase-9.5,6 Anticancer activities have also been attributed to 10-gingerol (Figure 1), which we have recently shown to be is a potent inhibitor of breast cancer cell growth, acting via blockade of cellular proliferation and induction of programmed cell death.7 In a mouse model of triple-negative breast cancer, orthotopic tumor growth and metastasis to multiple organs is also inhibited by 10-gingerol.8 However, information regarding the biological activity of 10-gingerol in ovarian cancer cells is limited. Ovarian cancer is the leading cause of death in gynecologic cancer patients, and is in urgent need of new treatments because of its aggressive nature, high rate of recurrence, and proclivity to develop resistance to chemotherapeutic drugs.9,10 In this study, we assessed the impact of 10-gingerol on the growth of HEY, OVCAR3, and SKOV-3 ovarian cancer cell lines, as well as the mechanism of action of 10-gingerol in HEY cells.

Materials and Methods

Materials
10-Gingerol (purity >98%) was from Chengdu Biopurify Phytochemicals Ltd. (Chengdu, Sichuan, China). RNase A was from Qiagen (Hilden, Germany). Annexin V-FLUOS was from Roche Applied Sciences (Laval, QC). Oregon Green 488 dye was from Invitrogen (Burlington, ON). Triton X-100, aprotinin, dimethyl sulfoxide

Figure 1. Chemical structure of 10-gingerol.
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...where MCF cells were placed into 25 cm² flasks and treated with medium alone, vehicle (DMSO), or 10-gingerol for 72 h. Before culture, a sample of cells was fixed with 1% [w/v] paraformaldehyde to establish baseline fluorescence. Fixed cells were stored at 4°C. At the end of culture cells were fixed and a minimum of 1 × 10⁴ counts per sample were analyzed with a FACSCaliber flow cytometer and Cell Quest™ software (version 3.3; BD Biosciences, Mississauga, ON). The formula, MCF_{baseline} = \left(2^n\right) (MCF_{sample}), where MCF_{baseline} is the baseline mean channel fluorescence (MCF) was used to calculate the number of cell divisions (n).

**Cytotoxicity assay**
Approximately 3 × 10⁴ HEY cells were placed into 25 cm² flasks and treated with medium alone, vehicle (DMSO), or 10-gingerol for 24 h. Cell were then stained with Annexin V-FLUOS, as recommended by the supplier, at 1 µg/mL in flow cytometry buffer (10 mM HEPES, 140 mM NaCl, 5 mM CaCl₂). Then, cells were washed and resuspended in flow cytometry buffer. The percentage of healthy and dead (apoptotic or necrotic) cells was determined by flow cytometry.

**Cell cycle analysis**
HEY cells were synchronized in G0 by serum-starvation for 24 h. Cells were then placed in complete DMEM and added to 6-well plates at 2.5 × 10⁶ cells/well. After 72 h treatment with vehicle (DMSO) or 10-gingerol, cells were placed in ice-cold PBS, followed by the drop-wise addition of ice-cold 70% ethanol. After storage at -20°C for at least 24 h, cells were stained with PI at 0.02 mg/ml in flow cytometry buffer (10 mM HEPES, 140 mM NaCl, 5 mM CaCl₂). The percentage of healthy cells was determined by flow cytometry software (Verity Software House, Topsham, ME) was used to determine the percentage of cells in different phases of the cell cycle.

**Western blot analysis**
HEY cells were placed into 6-well plates at 2.5 × 10⁶ cells/well and treated with vehicle (DMSO) or 10-gingerol for 48 h. Cells were then collected and placed in ice-cold PBS, followed by the drop-wise addition of ice-cold 70% ethanol. After storage at -20°C for at least 24 h, cells were stained with PI at 0.02 mg/ml in PBS containing 0.1% [v/v] Triton X-100 and 0.2 mg/ml DNase-free RNase A. Flow cytometry and ModFit LT 3.0 software (Verity Software House, Topsham, ME) was used to determine the percentage of cells in different phases of the cell cycle.
onto 10% Tris-HCl polyacrylamide gels containing 375 mM Tris-HCl [pH 8.8], 0.1% SDS [w/v], 0.1% ammonium persulfate [w/v], and 0.15% tetramethylethylenediamine [v/v]. Gels were run for 1 h at 200 V in running buffer (20 mM Tris-HCl [pH 8.3], 200 mM glycine, and 0.1% SDS [v/v]). An iBlot (Invitrogen) was used to transfer protein onto a nitrocellulose membrane, which was then blocked for 1 h in 5% skim milk powder [w/v] dissolved in TTBS (20 mM Tris-HCl [pH 7.6], 200 mM NaCl, and 0.05% Tween 20 [v/v]). Membranes were incubated overnight at 4°C with primary antibodies, at the manufacturer’s recommended concentrations, in TTBS containing 5% skim milk powder, then washed and incubated at room temperature for 1 h with the appropriate HRP-conjugated secondary antibody (1:10000) in TTBS containing 5% skim milk powder. Blots were washed and protein bands were visualized with enhanced chemiluminescence reagent (GE Healthcare Canada, Mississauga ON) using the Chemidoc Touch (BioRad, Mississauga, ON).

**Statistics**
Microsoft Excel and GraphPad Prism (version 7) were used for data analysis. Analysis of variance (ANOVA) and the Tukey-Kramer comparisons test was used to assess statistical significance (P < 0.05).

**Results and Discussion**

**Inhibitory effect of 10-gingerol on the growth of ovarian cancer cells**
The impact of 10-gingerol on the growth of 3 different ovarian cancer cell lines was assessed using MTT assays. We observed a time- and dose-dependent inhibition of the growth of HEY ovarian cancer cells; 34 ± 6% growth inhibition (P < 0.05 vs. vehicle control) at 24 h by 100 µM 10-gingerol, 71 ± 14% growth inhibition (P < 0.05 vs. vehicle control) at 72 h by 200 µM 10-gingerol (Figure 2A). Visual examination of HEY cell cultures showed an approximate 50% reduction in cell number, relative to vehicle-treated cultures, after 24 h exposure to 100 µM 10-gingerol (Figure 2B). This was consistent with the results from MTT assays. A growth-inhibitory effect of 10-gingerol was also observed in OVCAR3 (33 ± 5% growth inhibition, P < 0.05 vs. vehicle control) and SKOV-3 (38 ± 7% growth inhibition, P < 0.05 vs. vehicle control) ovarian cancer cell cultures after 72 h exposure to 200 µM 10-gingerol (Figure 2C). Subsequent investigations used HEY cells because this ovarian cancer cell line was most sensitive to growth inhibition by 10-gingerol. Decreased ovarian cancer cell growth in the presence of 10-gingerol was consistent with an earlier report that ginger extract, which contains gingerols plus other bioactive compounds, suppresses the growth of A2780, SKOV-3 and CaOV3 ovarian cancer cell lines, as assessed by sulforhodamine B assays. Importantly, the same study implies a selective effect on malignant cells since ginger extracts do not impact the growth of normal human surface ovarian epithelial cells.

**Cytostatic effect of 10-gingerol on ovarian cancer cells**
Since MTT assays do not differentiate between cytostatic and cytotoxic effects, we stained HEY cells with Oregon Green 488 dye or Annexin V-FLUOS and PI in order to determine the effect of 10-gingerol on cell proliferation and cell viability, respectively, by flow cytometry. Figure 3A shows that exposure of HEY cells to 100 or 200 µM 10-gingerol for 72 h resulted in fewer rounds of cell division (30% and 28% reduction in rounds of cell division, respectively; P < 0.05 vs. vehicle control). A similar inhibitory effect on the proliferation of triple-negative breast cancer cells was seen when these cells were treated with 10-gingerol. In contrast, Figure 3B shows that there was no loss of viability when HEY cells cultured for 24 h in the presence of 200 µM 10-gingerol (4 ± 1%...
apoptotic plus necrotic cells in vehicle-treated culture vs. 5 ± 1% apoptotic plus necrotic cells in 10-gingerol-treated cultures, \( P > 0.05 \). This finding was in sharp contrast to the apoptotic effect of 10-gingerol on triple-negative mammary carcinoma cells after only 24 h treatment.\(^7\) The effect of 10-gingerol therefore appears to differ between ovarian and breast cancer cells.

**G2 arrest in ovarian cancer cells mediated by 10-gingerol**

We next performed cell cycle analysis and assessed cell cycle protein levels in HEY cells cultured with or without 10-gingerol. Figure 4A shows that following 72 h exposure to 200 µM 10-gingerol, the percentage of G2 phase cells was increased (20 ± 4 % in 10-gingerol-treated cultures vs. 4 ± 1% in vehicle-treated cultures, \( P < 0.05 \)), while the proportion of cells in G1 was decreased (60 ± 5 % in 10-gingerol-treated cultures vs. 86 ± 1% in vehicle-treated cultures, \( P < 0.05 \)). There are conflicting reports of S phase and G1 phase arrest in breast cancer cells following exposure to 10-gingerol.\(^7,12\) However, to our knowledge, this is the first report of 10-gingerol-induced G2 growth arrest. Western blot analysis (Figure 4B) revealed decreased expression of cyclin A, B1, and D3 proteins in HEY cells after 48 h treatment with 10-gingerol. Cyclin A is involved in G2 to M phase transition, as well as cell cycle progression through S phase, cyclin B1 regulates mitosis, and D type cyclins are required for G1 entry.\(^13\) Reduced expression of cyclin A accounts for the accumulation of ovarian cancer cells. (A) Oregon Green 488-stained HEY cells were cultured for 72 h in the absence or presence of the indicated concentrations of 10-gingerol, and flow cytometric analysis was used to determine the mean number of divisions ± SEM in 5 independent experiments; asterisk denotes \( p < 0.05 \) compared to the vehicle control. (B) HEY cells were cultured for 24 h in the absence or presence of the indicated concentrations of 10-gingerol, then stained with Annexin V-FLUCOS and PI for determination of the proportion of viable cells and cells in early apoptosis or late apoptosis/necrosis by flow cytometry. Data are shown as the mean percent cell death ± SEM.

Figure 3. Anti-proliferative effect of 10-gingerol on ovarian cancer cells. (A) HEY cells were cultured for 72 h in the absence or presence of the indicated concentrations of 10-gingerol, and flow cytometric analysis was used to determine the mean number of divisions ± SEM in 5 independent experiments; asterisk denotes \( p < 0.05 \) compared to the vehicle control. (B) HEY cells were cultured for 24 h in the absence or presence of the indicated concentrations of 10-gingerol, then stained with Annexin V-FLUCOS and PI for determination of the proportion of viable cells and cells in early apoptosis or late apoptosis/necrosis by flow cytometry. Data are shown as the mean percent cell death ± SEM.

Figure 4. G2 arrest and reduced cyclin A, B1 and D3 expression in 10-gingerol-treated ovarian cancer cells. (A) HEY cells were cultured for 72 h in the absence or presence of 10-gingerol, then stained with PI for flow cytometric analysis of DNA content. Flow cytometry histograms are representative of 3 independent experiments. Data shown are the mean ± SEM; asterisk denotes \( p < 0.05 \) compared to the vehicle control. (B) HEY cells were cultured for 48 h in the absence or presence of the indicated concentrations of 10-gingerol, then cell lysates were prepared and analyzed by western blotting for cyclin A, B1 and D3 expression, as well as ß-actin expression to confirm equal loading of protein. Blots shown are representative of 3 independent experiments.
of 10-gingerol-treated HEY cells in the G2 phase. The decreased proportion of 10-gingerol-treated HEY cells in G1 phase is likely due to the combined effects of decreased cyclin B1 and D3 expression.

**Conclusion**

We demonstrate here, for the first time, that the natural phenolic compound, 10-gingerol, suppresses ovarian cancer cell growth. The cytostatic effect of 10-gingerol was the result of G2 phase cell cycle arrest. The reduced growth of ovarian cancer cells in the presence of 10-gingerol supports further investigation for the possible use of 10-gingerol in the management of ovarian cancer; however, oral administration of ginger extract (up to 2 g daily), while well tolerated by healthy humans, yields only 1.5 µM 10-gingerol in plasma, which is well below the amount needed to suppress ovarian cancer cell growth. Any future clinical application will therefore require the development of effective delivery modalities such as intravenous injection of 10-gingerol-loaded nanoparticles, which has already been shown to enhance the therapeutic effect of curcumin.

**Ethical Issues**

Not applicable.

**Conflict of Interest**

The authors declare that they have no conflict of interests.

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**References**