RESEARCH ARTICLE

Addition of Gallic Acid Overcomes Resistance to Cisplatin in Ovarian Cancer Cell Lines

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Abstract

Objective: Ovarian cancer is one of the leading causes of cancer-related mortality in women, and is often associated with drug resistance. Therefore, finding effective drugs, including naturally derived compounds, is urgently needed. Herein, we aimed to test the anti-cancer potential of gallic acid monohydrate (GA) and its congeners on cisplatin-sensitive (A2780S), and resistant (A2780CP) ovarian cancer and normal ovarian (HOSE6-3) cell lines. **Methods:** Cytotoxicity was assessed by AlamarBlue and CCK08 assays by exposing cells to different concentrations of cisplatin (0-21µg/mL), GA and its congeners (0-100µg/mL), and a combination of GA and cisplatin. Apoptosis was estimated by Hoechst stain and monitoring the relative RNA expression of the apoptotic effector caspase-3 using qRT-PCR. **Results:** GA decreased cell viability in a concentration-dependent manner in all cell lines, with an IC_{50} of 19.39µg/mL (A2780S), 35.59 µg/mL (A2780CP), and 49.32µg/mL (HOSE6-3). GA displayed higher cytotoxicity than its congeners. An apoptotic rate estimation of approximately 20% and 30% was obtained in A2780S and A2780CP. While the cytotoxicity observed with cisplatin and GA was comparable, combining the two enhanced the cytotoxicity significantly, especially in the A2780CP cell line ($p<0.05$). **Conclusion:** These data suggest that GA may help overcome the resistance. Hence, the cytotoxic effects of GA, especially on chemo-resistant ovarian cancer cells merit further investigation.

Keywords: Ovarian cancer- gallic acid- cisplatin- cytotoxicity- chemo-resistance

Introduction

Ovarian cancer is the 8th most common cause of death from cancer among women worldwide and the 2nd most common cause of death from gynecological cancer (Bray et al., 2018). Epithelial ovarian cancer (EOC) accounts for 90% of all ovarian cancers, and high-grade EOC accounts for the majority (Kurman, 2014; Matulonis et al., 2016). The vast majority of patients with high-grade EOC are diagnosed with stage III/IV disease (Kurman, 2014). Debulking surgery and a combination of cisplatin and paclitaxel, with or without anti-angiogenic therapy has been the standard of care. However, the vast majority of patients experience a relapse of disease (Bristow et al., 2002; McGuire and Markman, 2003; du Bois et al., 2009; Markman, 2010; Burger et al., 2011; Perren et al., 2011). More recently, a small subset of patients with mutations in BRCA1 or BRCA2 gene or those with homologous recombination deficiency have been shown to respond to inhibitors of the poly (ADP-ribose) polymerase (PARP) enzyme, leading to a prolongation of disease-free survival (Konstantinopoulos et al., 2010; Ledermann et al., 2012; Oza et al., 2018). However, subsequently the disease becomes resistant to chemotherapy, including cisplatin. Despite the advances in treatment, the 5-year survival for stage III and IV disease remains 15-25% (Peres et

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al., 2018). Therefore, new strategies and compounds are urgently needed to achieve better control and to overcome the resistance to frontline chemotherapy.

Gallic acid (GA, 3,4,5-trihydroxybenzoic acid) is a polyphenolic compound commonly found in many plants (Kahkeshani et al., 2019). Gallic acid has been reported to have anticancer activity again lung cancer (You and Park, 2010), breast cancer (Wang et al., 2014), prostate cancer (Kaur et al., 2009), leukaemia (Madlener et al., 2007), and cervical cancer (You et al., 2010). Gallic acid was also reported to induce apoptosis in stomach cancer, colon cancer, adipocytes, and have an antiangiogenic effect in glioma cells (Yoshioka et al., 2000; Hsu et al., 2007; He et al., 2016; Sourani et al., 2016). Moreover, GA derivatives seem to have cytotoxic activities as well. For example, 3,4-Dihydroxybenzoic acid (Protocatechuic acid) was shown to exhibit an anti-proliferative effect on human gastric adenocarcinoma cells (Lin et al., 2007), while 4-Hydroxybenzoic acid derivatives were found to increase protein acetylation levels, arrest cell cycle progression, and subsequently trigger leukaemia cells to programmed cell death without affecting the normal cells (Seidel et al., 2014).

In our previous study, the anticancer activity of 8 marine natural products and 32 extracts of marine organisms from Oman waters were studied (Dobretsov et al., 2016). Among the tested pure compounds, GA isolated from leaves of a mangrove tree Avicennia marina showed potent anticancer activity against the MCF-7 breast cancer cell line (Dobretsov et al., 2016). The inhibitory concentrations of GA against the MCF-7 cell line were >100 µg/mL. At the same time, GA at this concentration did not have quantifiable activity on the human fibroblast cells.

The primary objective of the present study, was to assess the anti-cancer potential (cytotoxic activity) of GA monohydrate (7) and its congeners (3,4-dihydroxybenzoic acid (1), 3,5-dihydroxybenzoic acid (2), 4-hydroxybenzoic acid (3), 3-hydroxybenzoic acid (4), 3-fluoro-4-hydroxybenzoic acid (5), 4-fluoro-3-hydroxybenzoic acid (6) (Figure 1) on cisplatin-sensitive (A2780S), cisplatin-resistant (A2780CP) ovarian cancer cell lines, and immortalized epithelial ovarian cell line (HOSE6-3). Also, the synergistic cytotoxic action of cisplatin and GA was examined on the cancer cell lines by exposing cells to both the agents simultaneously.

Materials and Methods

Reagents

GA monohydrate, $C_6H_2(OH)_3COOH$-H_2O, M.W.= 188.14 g/mol, was purchased from Cica reagent, Kanto Chemicals, Japan. A stock solution of GA monohydrate (188.14 g/mol) was prepared in absolute ethanol (EMD Millipore) at 100 µg/mL and stored at 4°C. GA congeners (1-6, Figure 1) were purchased from AcrosOrganics, UK, as solid compounds and their solutions were prepared in absolute ethanol so that they were equivalent to 188.14 g/mol of GA monohydrate. Congeners are structurally related compounds, and congeners 1-6 and GA monohydrate (compound 7) are all derived from

benzoic acid. The purity of compounds 1-7 was confirmed through TLC, and the structures were confirmed by NMR spectroscopy. 1H NMR was recorded on Jeol 400 MHz spectrometer. Chemical shifts (δ) are expressed in ppm. DMSO-d6 was used as a solvent, and tetramethylsilane (TMS) was used as the internal standard. Cisplatin was purchased from Mylan, USA as a 50 mg/mL solution.

1H NMR data of Gallic acid monohydrate (GA) and its congeners (1-6) are shown in Table S1 [see supplementary file].

Cell culture and treatment

Ovarian cancer cells sensitive (A2780S) and resistant to cisplatin (A2780CP) were provided by Prof. Benjamin K. Tsang from the University of Ottawa, Canada, and were maintained in Dulbecco's Modified Eagle's Medium, sodium pyruvate (Gibco). The A2780S cell line had been derived from a patient with a metastatic endometrioid type of ovarian carcinoma who had not been exposed to treatment. The cell line has epithelial morphology, and cells grow as a monolayer in tissue culture flasks. A2780CP was developed after repeated passages to cisplatin. Human ovarian surface epithelial cells (HOSE6-3) was provided by Prof. GSW Tsaw, from the University of Hong Kong, and was maintained in Minimum Essential Medium (MEM) (Gibco). All culturing media were supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Gibco) in a humidified incubator (5% CO_2) at 37°C.

Cell viability assay

AlamarBlue (Invitrogen, USA) and Cell Counting Kit-8 (CCK8 from Sigma-Aldrich) were used to determine the IC_{50} of GA in A2780S, A2780CP and HOSE6-3 cell lines. Briefly, cells were cultured in a 96-well plate (Corning) at a density of 15,000 cells/well for 24h. The growth media was subsequently discarded and replaced with serum-free media and the cells were exposed to different concentrations of GA (0 µg/mL, 3.125 µg/mL(16.6µM) , 6.25 µg/mL(33.2 µM) , 12.5 µg/mL (66.4 µM) , 15.63 µg/mL(83 µM), 18.75 µg/mL(99.6 µM), 25 µg/mL(132.8 µM) , 28.125 µg/mL(149.4 µM), 31.25 µg/mL(166 µM), 34.375 µg/mL(182.7 µM), 37.50 µg/mL(199.3 µM), 40.625 µg/mL(215.7 µM), and 46.75 µg/mL(248.5 µM), 50 µg/mL(265.7 µM) and 100 µg/mL(531.5 µM) for 24h. A few wells were treated with an equal volume of ethanol used to prepare the different concentrations for the negative control. The corresponding absorbance was used in the calculation of the cell viability. On the third day, the detection dye, AlamarBlue or CCK8 solution, was added. The plate was incubated for few hours (3hours with AlamarBlue and 1hour with CCK8), then read in a plate reader Multiskan spectrum (ThermoFisher Scientific, USA) 570/600 nm for AlamarBlue and 450/650 nm for CCK8. For consistency and statistical analysis, the viability of the control cells (untreated cells) was set as 100%, and experiments were repeated for three times.

Combination Index Calculation

To assess whether GA and cisplatin had a cytotoxic synergistic effect on A2780S and A2780CP cell lines, we

used ComboSyn software (https://www.combosyn.com) to calculate the combination index (CI). Response to different doses of cisplatin and GA were used to generate a detailed report for each cell line (see supplementary file).

Apoptosis Analysis

Apoptosis was determined, as described previously (Al-Bahlani et al., 2017a; Al-Bahlani et al., 2017b). Briefly, A2780S and A2780CP cell lines were cultured in a 24-well plate (Corning) at a density of 150,000 cells/well and incubated overnight. Growth media was refreshed and different concentrations of GA (0 μg/mL, 3.125 μg/mL, 6.25 μg/mL, 12.5 μg/mL, 25 μg/mL, 50 μg/mL and 100 μg/mL) were added to cells and incubated for 24h. On the third day, cells were harvested, washed with Dulbecco's phosphate-buffered saline, with no calcium and no magnesium (DPBS) (GIBCO, Thermo Fisher), stained with Hoechst 33258dye (Sigma-Aldrich) in 10% formalin (1:50), and then preserved in the dark at 4°C until visualization (within 1-2weeks). Apoptotic cells were identified based on their morphology (smaller size and fragmented DNA) under a fluorescent microscope with a DAPI filter. We estimated the percentage of cells undergoing apoptosis by microscopic examination of different random fields at 40X and 100X magnification. We examined at least three different areas for each experiment, and the number of apoptotic cells was counted to estimate the percentage of survival. For consistency and accuracy, experiments were repeated three times.

RNA extraction and qRT-PCR analysis

RNA was extracted from cell lines using the PureLink RNA mini kit (Invitrogen). Briefly, cells were trypsinized in 0.25% trypsin-EDTA (Gibco), pelleted by centrifugation, and incubated in lysis buffer. 70% Ethanol was added to provide the appropriate binding of RNA to the silica membrane once the lysate was transferred into the RNeasy spin column. Columns were washed with washing buffer, and the RNA was eluted using RNase free water. 1μg of RNA was then reverse transcribed to make cDNA, using the high capacity cDNA reverse transcription kit following the manufacturer's instructions (Applied Biosystems). Ten ng of cDNA and TaqMan pre-optimized probes were used to assess the gene expression of the caspase3 gene using qRT-PCR run on 7500 fast real-time PCR machine (Applied Biosystems, Austin, TX). All obtained data were normalized to GAPDH expression, and the relative expression was obtained using the $2^{-\Delta\Delta Ct}$ method. The experiment was repeated two times (in triplicate) separately.

Western blotting

Treated cells were harvested, and the pellet was washed in cold PBS (Dibco, US). Cells were lyzed in RIPA lysis buffer (Santa Cruz), supplemented with protease and phosphatase inhibitor cocktail (Thermo Scientific) in a concentration of 100:1. The obtained lysate was centrifuged at 4°C to remove any remaining cell debris, and the supernatant containing the proteins was subjected to Bradford assay for quantification. 50 μg of proteins were resolved on 10% SDS-PAGE for 90 minutes at 90V, then transferred to a PVDF membrane (Thermo Scientific) for 1 hour at 70V. The PVDF membrane was then blocked in 5% non-fat dry milk (BioRad) dissolved in 1X TBST for 30 minutes at room temperature. The blocked membrane was then exposed to primary antibody (1:1000 of β-actin mouse monoclonal antibody (Santa Cruz) and caspase3 rabbit monoclonal antibody (Abcam) at 4°C overnight. The membrane was then washed in 1X TBST three times for 5 mines each, followed by exposing the membrane to HRP-conjugated secondary antibody (donkey anti-mouse IgG-HRP from Santa Cruz) (1:5,000 dilution) and anti-rabbit IgG-HRP (Cell Signaling). Membranes were then washed three times each for 5 min in 1X TBST before the revelation using the ECL kit (Thermo Scientific). The chemiluminescence signal was captured using the BioRad chemidoc touch imaging system, and proteins were quantified using ImageJ software. The experiment was repeated three times separately (Figures S1-S6).

Statistical analysis

All experiments were performed as triplicate and repeated at least three times independently. The results are reported as the mean ± standard deviation (SD). Means were compared using one-way ANOVA followed by a Tukey or Dunnett 2-sided post-hoc tests. Prior to analysis, the normality of the data and homogeneity of the variables were tested. The difference between means was considered significant at (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Results

Cell viability assays
Determination of IC_{50} in Cisplatin

We used AlamarBlue and CCK8 reagents to assess cisplatin's cytotoxicity in A2780S, A2780CP, and HOSE6-3 cell lines exposed to 9 different doses ranging from 0 to 21 μg/mL. A dose-dependent effect of cisplatin on the three cell lines is shown in Figure 2A. The difference in the viability of the cisplatin-treated cells, compared to the control, was statistically significant at doses more than 7.5 μg/mL for A2780S and HOSE6-3 cell lines. However, for A2780CP cells, significance was obtained only at 21μg/mL, which confirmed the resistance of A2780CP cells to cisplatin. The recorded IC_{50} values were 8.8 μg/mL (262μM), 9.4 μg/mL (103μM), and 23.1 μg/mL (189 μM) for HOSE6-3, A2780S, and A2780CP cell lines, respectively, as shown in Figure 2A.

Determination of IC_{50} for GA

The inhibitory effects of GA monohydrate against A2780S, A2780CP, and HOSE6-3 cell lines were determined using AlamarBlue and CCK8 cell viability assays. Figure 2B shows a dose-dependent cell viability inhibitory effect of GA against the three cell lines. The IC_{50} of GA was determined to be 19.39 μg/mL (103 μM), 35.59 μg/mL (189 μM), and 49.32 μg/mL (262 μM) for A2780S, A2780CP, and HOSE6-3 cell lines, respectively. Figure 2B shows the significant inhibition of cell growth at a concentration of 15.63 μg/mL in A2780S (p<8.3 E-06), 34.375 μg/mL (p<0.006) in A2780CP, but only weak inhibitory effect in HOSE6-3 (p<4.2 E-05). We
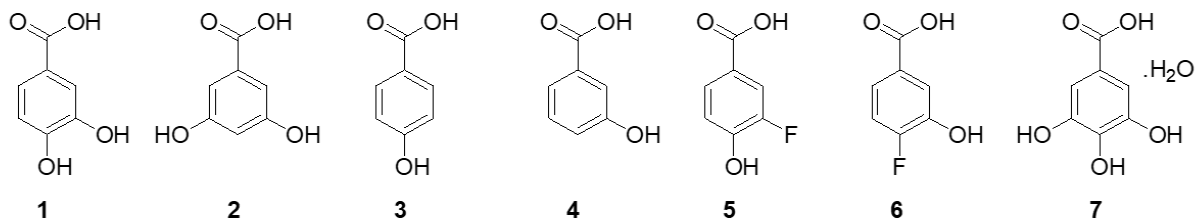
Figure 1. Gallic Acid Monohydrate (7) and Its Congeners: 1: 3,4-Dihydroxybenzoic acid, 2: 3,5-Dihydroxybenzoic acid, 3: 4-Hydroxybenzoic acid, 4: 3-Hydroxybenzoic acid, 5: 3-Fluoro-4-hydroxybenzoic acid, 6: 4-Fluoro-3-hydroxybenzoic acid.

selected the IC_{50} value for each cell line for subsequent experiments. We also exposed the three cell lines to the IC_{50} of GA at different time points (6, 12, 24, and 48 hours). At 24 hours of exposure, all three cell lines showed a significant decrease in the viability ($p<2.1E-08$ for A2780S, $p<4.5E-05$ for A2780CP, and $p<0.003$ for HOSE6-3), and hence we chose the exposure time of 24 hours for subsequent experiments.

Cytotoxicity of GA and its congeners (1-6)

We then studied the cytotoxic effect of GA monohydrate and different congeners (1-6) using both AlmarBlue and CCK8 assays on the A2780S and A2780CP cell lines

to determine the type of benzoic acid with the highest cytotoxicity. At 24 hours, GA monohydrate had the highest cytotoxic effect on A2780S ($p<0.034$ using CCK8 and $p<2.9E-07$ using AlamarBlue), and A2780CP cells ($p<0.004$ using CCK8 and $p<1.7E-04$ using AlamarBlue). In contrast, there was no significant change in cell death of A2780S and A2780CP when exposed to all other congeners (1-6) of GA (Figure 3). Therefore, the GA monohydrate was chosen for further analysis.

Synergistic effect of cisplatin and GA cytotoxicity

To determine whether a combination of cisplatin and GA would induce more cytotoxicity, the A2780S, and
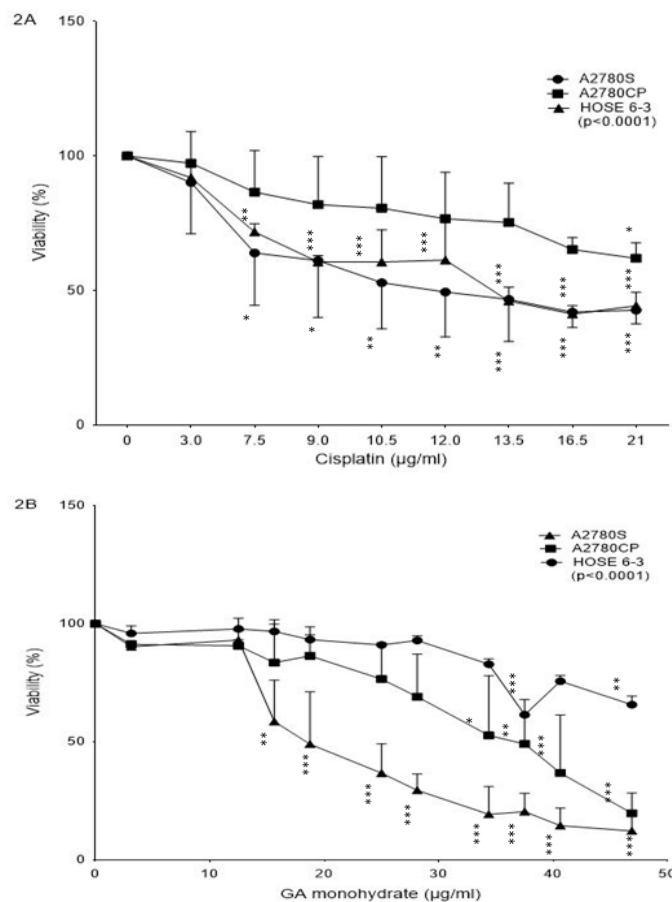

Figure 2. The Effect of Cisplatin (2A) and Gallic Acid (2B) on A2780S, A2780CP and HOSE6-3. Cells were exposed to different concentrations of cisplatin (0-21µg/mL) and GA monohydrate (0-100µg/mL) for 24 hours. Data represent the means ± SD from three independent experiments. All data were analyzed by one-way ANOVA followed by Dunnett 2-sided post-hoc test. *P < 0.05, **P < 0.01, ***P < 0.001.
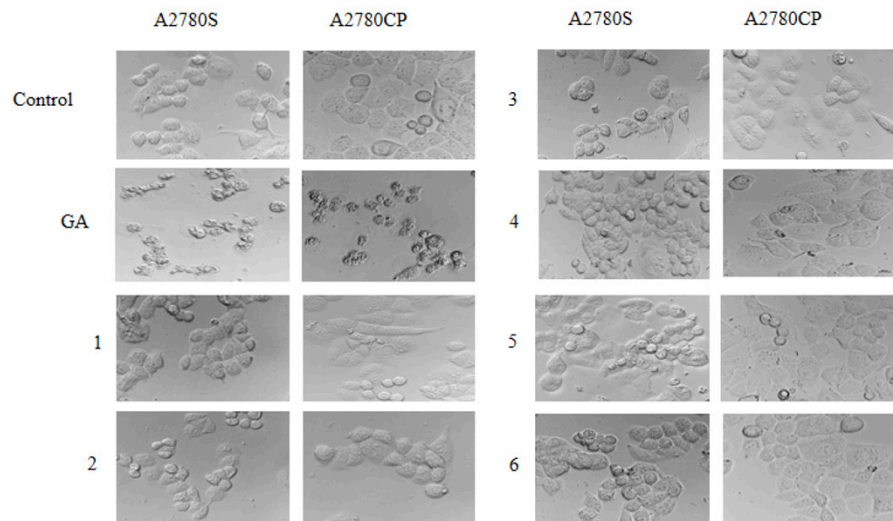
Figure 3. The Anticancer Activity of GA and Its Congeners (1-6) on A2780S and A2780CP. All compounds were added at 103 µM (equivalent to gallic acid monohydrate IC_{50} of 19.4 µg/mL) in A2780S and at 189 µM (equivalent to gallic acid monohydrate IC50 of 35.6 µg/mL) in A2780CP. Images were taken under transmitted light.

A2780CP cells were exposed for 24 hours to cisplatin, GA, or a combination of cisplatin and GA using the IC_{50} values. There was no significant difference in the cell viability with either cisplatin or GA alone. However, when cisplatin and GA were applied together, the viability decreased in both cell lines ($p<0.055$ in A2780S and $p<0.042$ in A2780CP), with a much greater cytotoxicity being observed in A2780CP cells (Figure 4).

Moreover, the combination index revealed a synergistic effect of GA and cisplatin on A2780S cells at the IC_{50} doses (CI=0.3785 at IC_{50}). Similarly, a synergistic effect was obtained for GA and cisplatin on the A2780CP cell line (CI=6.5x10^{-6} at IC_{50}). The combination of GA and cisplatin

had a synergistic effect at IC_{75}, IC_{90}, and IC_{95} values on both cell lines (see supplementary file for the combination index calculation).

Apoptosis analysis

Apoptosis was determined by exposing A2780S and A2780CP cells to different concentrations (0, 3.125, 6.25, 12.5, 25, 50, and 100 µg/mL) of GA for 24 hours and subsequently stained with Hoechst dye. Apoptosis was confirmed by morphological changes, including the fragmentation of nuclei and the smaller sized cells. The obtained apoptotic cell number seems to increase in a dose-dependent manner (Figure 5). The experiment was
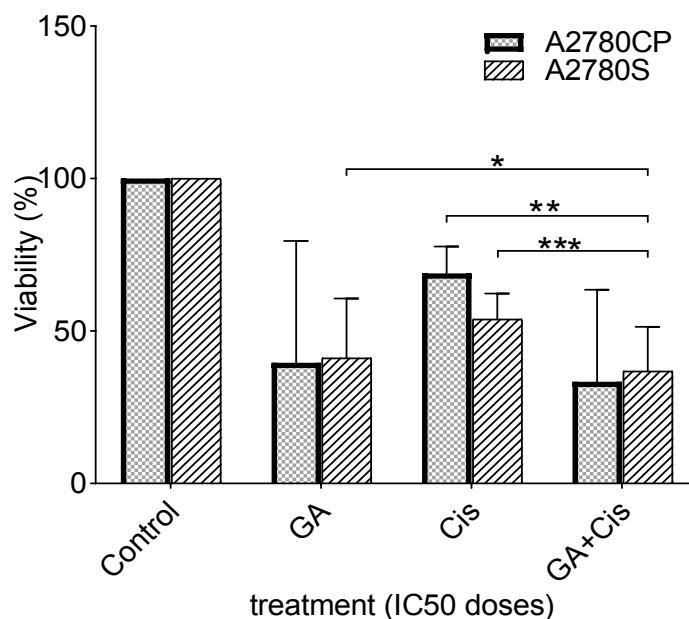

Figure 4. The Effect of Cisplatin, GA, and a Combination of both (Cis+GA) on A2780S and A2780CP. Cells were exposed to each drug's IC_{50} values for 24 hours. Data represent the means ± SD from three independent experiments. All data were analyzed by one-way ANOVA, followed by Dunnett's 2-sided Post Hoc test. *P < 0.05, **P < 0.01, ***P < 0.001.
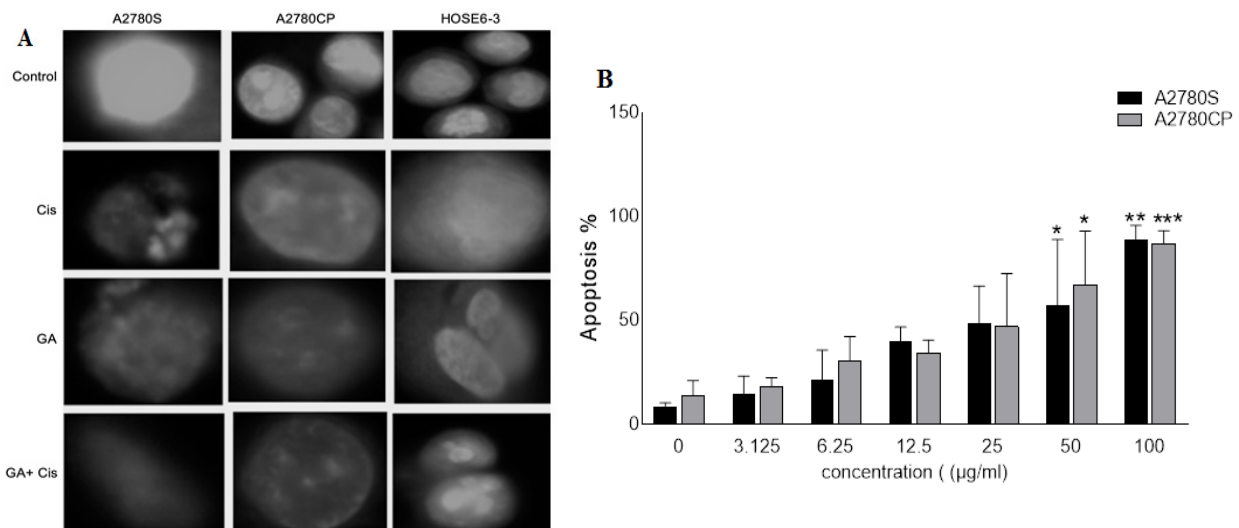
Figure 5. (A) Morphology of the nucleus in ovarian cancer cells and in normal ovarian cells treated with Cisplatin, Gallic acid, and Gallic acid plus Cisplatin combined. Fluorescence of Hoechst stain was visualized with a fluorescence microscope at magnification (40x). (B) The percentage of apoptotic cells in A2780-S and A2780-CP lines following exposure to different concentrations of GA (0, 3.125, 6.25, 12.5, 25, 50, and 100 µg/mL) for 24 hours. *P < 0.05, **P < 0.01, ***P < 0.001

repeated three times for consistency.

Caspase3 mRNA and protein expression in treated cell lines

qPCR data revealed that cell lines treated with cisplatin displayed the highest caspase-3 expression levels than cell lines treated with gallic acid, a combination of cisplatin/ GA, and the control (Figures 6-8A). However, western blot analysis revealed different expression patterns of caspase3 in the cell lines, specifically A2780CP and HOSE6-3 cell lines (Figures 6-8B and C).

Caspase3 mRNA and protein levels were found higher in A2780S cells treated with cisplatin alone or combined with GA compared to the control (Figure 6A, B and C). Although increased content of caspase3 protein was evident in GA and cisplatin-treated cells, the difference

did not reach significance compared to the control.

In the A2780CP cell line, cisplatin treatment was shown to promote apoptosis since caspase3 was highly expressed at mRNA and protein levels. In contrast, caspase3 expression in A2780CP cells treated with GA combined with cisplatin displayed higher mRNA abundance and lower protein content than controls (Figure 7A, B and C).

Immortalized normal epithelial ovarian cancer HOSE6-3 cells treated with cisplatin showed similar results to other cell lines, where expression of caspase3 at both mRNA and protein levels was significantly displayed. However, in GA and (GA + cisplatin) treated HOSE6-3 cells, lower expression than control was recorded by qRT-PCR, whereas Western blot analysis displayed higher levels than controls (Figure 8A, B and C).
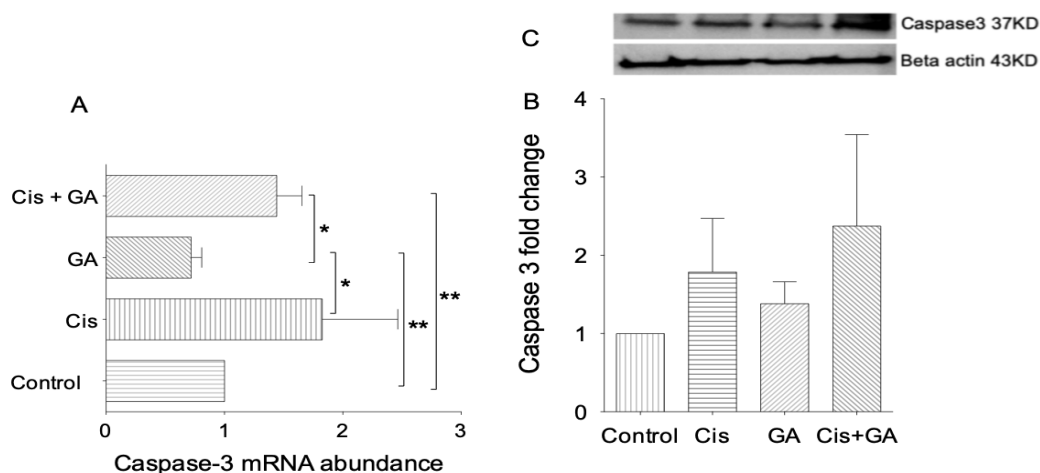

Figure 6. The Level of Caspase3 in A2780S Control and Treated Cells with Cisplatin, GA, and Cisplatin Combined with GA. The relative caspase-3 mRNA abundance plot (A) resemble the data from two independent experiments while caspase-3 protein content data (B,C) were from three independent experiments . The Mann Whitney t-test was used to analyze all data. *P < 0.05, **P < 0.01
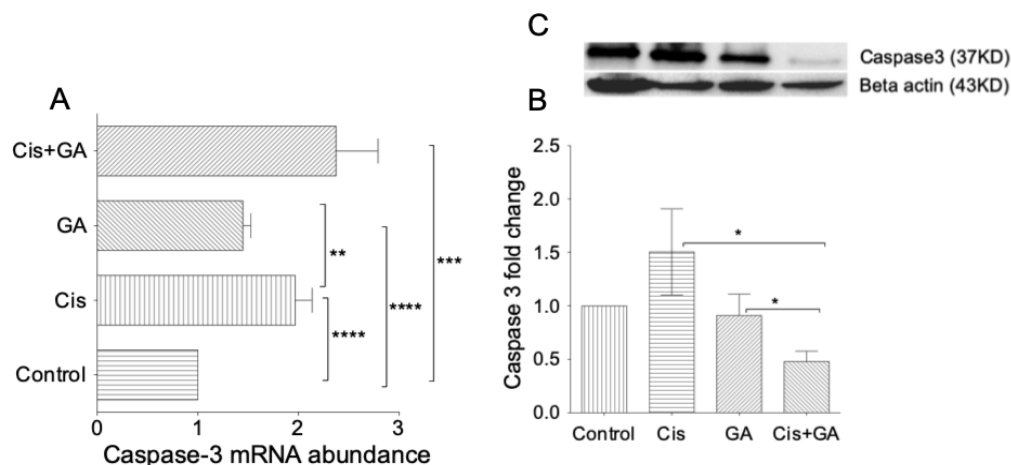
Figure 7. The Level of Caspase3 in A2780CP Control and Treated Cells with Cisplatin, GA, and Cisplatin Combined with GA. The caspase-3 mRNA abundance (A) resemble the data from two independent experiments while caspase-3 protein content (B, C) were from three independent experiments . The Mann Whitney t-test was used to analyze all data. *P < 0.05, **P < 0.01, ***P < 0.001
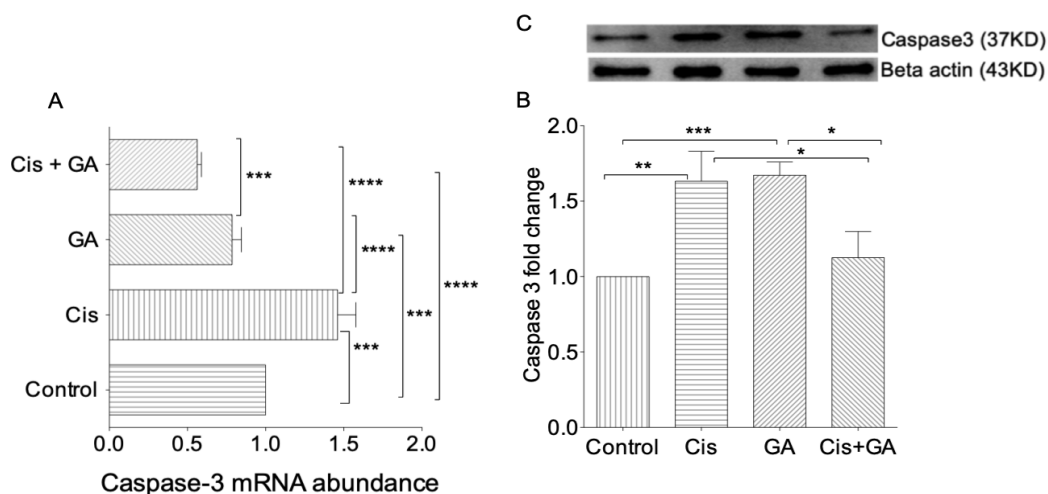

Figure 8. The Level of Caspase3 in HOSE6-3 Control and Treated Cells with Cisplatin, GA, and Cisplatin Combined with GA. The RNA expression plots (A) resemble the data from two independent experiments while protein expression data (B,C) were from three independent experiments. The Mann Whitney t-test was used to analyze all data. *P < 0.05, **P < 0.01, ***P < 0.001

Discussion

GA monohydrate produced significant cytotoxicity in cisplatin-sensitive and cisplatin-resistant cell lines. GA monohydrate was significantly more cytotoxic than the 6 congeners (1-6). GA produced cytotoxicity, at least in part, by inducing apoptosis. When compared with cisplatin, there was no significant difference in the cytotoxic activity; however, when cisplatin was added to GA, a significant increase in cytotoxicity was observed, suggesting synergistic action.

GA was selected for this study because our previous screening of anticancer compounds from Omani marine organisms demonstrated a potent cytotoxic activity against the MCF-7 breast cancer cell line (Dobretsov et al., 2016). Commonly, GA is isolated from plant origin, but it is also found in seaweeds (Neethu et al., 2017; He

et al., 2019; Wekre et al., 2019) and mangroves (Dobretsov et al., 2016). Cytotoxicity of GA has been demonstrated against the cancers of the lung (You and Park, 2010), prostate (Kaur et al., 2009), uterine cervix (You et al., 2010), stomach (Lin et al., 2007), and the ovary (He et al., 2016). While, the anticancer activity of GA is not novel, anticancer activity of GA in combination with cisplatin, especially the synergistic effect against cisplatin-resistant ovarian cancer cell line, has been shown for the first time in this study.

To understand the effect of activating or deactivating groups on GA, compounds 1-6 were tested against the A2780S, A2780CP, and HOSE6-3 cell lines. The results showed that cytotoxicity decreases as the electron-donating OH group is removed or replaced with electron-withdrawing fluorine (F) group from the benzene ring (Figure 3 and Figure S7). The results also showed that

the removal of OH group from the ring has no significant change in cytotoxicity. The cytotoxicity of all congeners (1-6) was comparable to each other. In congeners 1-6, benzene ring carries only one OH group either at position 3 or 4 or 5. Gallic acid (7) has three OH group at positions 3, 4, 5 and this increases the cytotoxicity against A2780S cells and the least cytotoxic effect on the normal cell line HOSE6-3. These results are concordant with the previously reported GA selective inhibitory effect on ovarian cancer cell lines OVCAR-3 and A2780/CP70 compared to the normal IOSE-364 ovarian cell lines (He et al., 2016). The inhibitory effect of GA on the OVCAR-3 cells was more pronounced than the observed inhibition on A2780/CP70 cells. At 40 μM GA, A2780/CP70 viability was reduced to 30%, whereas on OVCAR-3, it was reduced to 2.1% (He et al., 2016). Also, 3,4-Dihydroxybenzoic acid was shown to decrease the proliferation of human gastric adenocarcinoma cells in a dose and time-dependent manner (Lin et al., 2007). However, human gastric adenocarcinoma cells were exposed to much higher doses of 3,4-Dihydroxybenzoic acid (0.5, 1.0, 2.0, 4.0, 6.0, and 8.0 mM), and IC_{50} was approximately 7.3 mM (Lin et al., 2007), which is higher than the dose used in the present study (103 μM in A2780S and 189 μM in A2780CP). Similarly, 4-Hydroxybenzoic acid derivatives were also tested on human K-562 leukemia cells and decreased the cell proliferation by approximately 20-40% at 100 μM dose after 24 hours of treatment (Seidel et al., 2014).

One of the major issues in the treatment of ovarian cancer is the resistance to platinum. Almost 80% of high-grade EOC respond to cisplatin at the time of diagnosis, and even at the time of relapse, if the relapse occurs more than one year after the last platinum dose (McGuire and Markman, 2003), called the platinum-sensitive relapse (Eisenhauer et al., 1997). Nevertheless, subsequently, almost all patients develop resistance to platinum compounds, and this is the major cause of ovarian cancer-related mortality. One of the important aims of the study was to see whether the addition of GA would overcome the resistance to cisplatin. Hence, a cell line was chosen with its cisplatin-resistant counterpart. The A2780 human ovarian cancer cell line was established from the tumour of an untreated patient with endometroid cancer. The cisplatin-resistant cell line A2780CP was developed by chronic exposure of A2780 to cisplatin; A2780CP had a 6-7 fold resistance to cisplatin (IC_{50} = 23.4 μM), compared with A2780S cells (IC_{50} = 3.7 μM (Pan et al., 2002)). The mechanism underpinning this resistance is an increased activity of general efflux mechanisms, especially the intracellular copper ion transporters, ATP7A and ATP7B, which decrease the intracellular impact of cisplatin (Kalayda et al., 2008). It has been suggested that A2780CP is able to sequester and traffic the bound cisplatin to cellular lysosomes, away from genomic DNA (Kalayda et al., 2008). Protein profiling comparisons between cisplatin-sensitive parent strains and their resistant counterparts found similar proteomes between A2780 and A2780CP, suggesting the mechanism for cisplatin resistance occurs as part of post-translational modification (Zhu et al., 2005). Our results demonstrate that when cisplatin-sensitive and cisplatin-resistant cells were exposed to either cisplatin or GA, a modest degree of cytotoxicity was observed. However, when GA was added to cisplatin, cytotoxicity was significantly increased, especially in the cisplatin-resistant-cell line. There are several limitations of this study. While an increased cytotoxicity was observed in both A2780S and A2780CP cells treated with cisplatin combined with GA, the expression of caspase3 did not increase accordingly suggesting alternate mechanisms of GA-induced cytotoxicity. We did not check for the cleaved caspase-3 since the primary objective of this study was to show the effect of GA on the viability of cisplatin-resistant cells and whether adding GA to cisplatin will enhance cytotoxicity despite cisplatin resistance.

In conclusion, we report potent cytotoxic activity of GA in both cisplatin-sensitive and cisplatin resistant cell lines. Addition of GA to cisplatin increases the cytotoxicity. This suggests that addition of GA to cisplatin may be a potential treatment for cisplatin-resistant cancer patients, and is worth to be explored further.

Author Contribution Statement

IAB, YT, SIH, IA, SAB and SD made the concept and design of the study. NAB, NA, BAD, IAB, YT, SD, SAB, and SIH performed the investigation, data collection and analysis. NAB, IAB, YT, and SIH wrote the original draft. YT, IAB, SIH, SD, SAB and BKT did the critical revision of the manuscript. IAB, YT, and IA acquired funds for this project. BKT provides the cell line. All authors have read and approved the final manuscript.

Acknowledgments

Availability of data and materials
The datasets generated and/or analysed during the current study are available from the corresponding author on request.

Ethics Approval
Not applicable. This study doesn't require any ethical approval from the ethical committee.

Conflict of Interest
All the authors declare no conflict of interests.

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