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Synergistic cytotoxic action of cisplatin and withaferin A on ovarian cancer cell lines

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ABSTRACT

Cisplatin derivatives are used as the mainline treatment of ovarian cancer, despite their severe side effects and development of resistance. We developed a novel combination therapy by combining cisplatin with withaferin A. Treatment of ovarian cancer cell lines with combination therapy acted synergistically to induce cell death, thus required a lower dose of cisplatin to achieve the same therapeutic effect. WFA and cisplatin combination induced cell death through the generation of reactive oxygen species (ROS) for WFA, while DNA damage for cisplatin, suggesting that cisplatin binds directly to DNA to form adducts while WFA indirectly damages DNA through ROS generation. Our results for the first time suggest that combining low dose of cisplatin with suboptimal dose of WFA can serve as a potential combination therapy for the treatment of ovarian cancer with the potential to minimize/eliminate the side effects associated with high doses of cisplatin.

1. Introduction

The mainline treatment of ovarian cancer is cytoreductive surgery followed by platinum-based chemotherapy, namely carboplatin in combination with paclitaxel [1,2]. Initially, ovarian cancer responds positively in 70 to 80% of the cases [3]. However, approximately 70% of patients develop recurrent cancer and eventually succumb to their disease, which is attributed to the carcinomas having become platinum-resistant [3]. If the relapse occurs within 6 months of treatment, the carcinomas are considered platinum-resistant [3]. Despite the frequency of relapse, platinum-based chemotherapy remains the main stream for treatment of ovarian cancer [3], in which after five years only 30% of women survive [2]. The poor survival rate for women with platinum-resistant ovarian carcinomas points to an urgent need for an alternative treatment strategy.

Cis-diamminedichloroplatinum(II) (best known as cisplatin) is a platinum-based compound that has clinical activity against a wide spectrum of solid cancers including ovarian, testicular, bladder, colorectal, lung, and head and neck [4]. While cisplatin itself is inert, it spontaneously undergoes an aqueous reaction, resulting in the replacement of one or both *cis*-chloro groups with water leading to the generation of highly reactive mono- and bi-aquated cisplatin forms [5], which avidly bind DNA and cause formations of protein-DNA complexes and DNA-DNA inter-and intra-strand adducts [5]. In addition, aquated cisplatin interacts with cytoplasmic targets, such as reduced glutathione, to cause oxidative stress [4] in addition to generating superoxide anions and hydroxyl radicals [6]. The use of cisplatin is mainly limited by chemo-resistance [3,4], which can be intrinsic or acquired [7]. Side effects associated with cisplatin include nausea, vomiting, myelosuppression, hepatotoxicity, neurotoxicity, and ototoxicity [4,7]. However, the main limiting factor is cumulative nephrotoxicity as a result of ROS production inducing apoptosis [8,9].

Recently, to reduce the side effects and resistance caused by cisplatin-based chemotherapy a number of combinations with other compounds have been explored. Some of these include *N*-acetylcysteine [10], naltrexone [11], glutathione ester [12], vitamin E and losartan [13], melatonin [14], quercetin [15], metformin [16,17], and rehmannia [18]. However, none of the combinations have rendered the desired outcome of leading to clinical application.

Withaferin A (WFA) is a bioactive, cell permeable compound isolated from the plant *Withania somniferia* that has been a part of Indian traditional medicine for centuries and is now available as an over-the-counter dietary supplement in the US. It is being used to treat various disorders due to its anti-inflammatory, antibacterial, and cardio-protective properties. Recently, WFA has been suggested as a potential anti-cancer compound shown to prevent tumor growth, angiogenesis, and metastasis [19,20]. Several biological functions have been influenced by WFA including induction of apoptosis through inactivation of Akt and NF-κB [21] as well as decrease of pro-survival protein Bcl-2 [22,23], induction of Par-4 [24], inhibition of Hsp90 and Notch-1 [25], G2/M cell cycle arrest

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[19], FOXO3a and Bim regulation [26], generation of ROS [27,28], and down regulation of expression of HPV E6 and E7 oncoproteins [29]. However, the effect of WFA on ovarian cancer has not been studied, nor has the combined effects of WFA with cisplatin been explored.

We propose that WFA when combined with cisplatin will elicit a synergistic effect on the suppression of ovarian tumor growth, hence, will reduce the dosage requirement of cisplatin resulting in minimizing/eliminating the side effects, and induction of drug resistance associated with high doses of cisplatin. To test our hypothesis, we studied the combined effect of cisplatin and WFA on cisplatin-sensitive ovarian epithelial cancer cell line A2780, cisplatin-resistant variant A2780/CP70, and p53 mutant ovarian epithelial cell line CAOV3. Ovarian cancer cells treated with WFA (0.5 μM) in combination with low dose of cisplatin (20 μM) exhibited a synergetic effect on cell death through the generation of ROS leading to DNA damage and culminating in apoptosis.

2. Materials and methods

2.1. Materials

RPMI, DMEM, FBS, penicillin/streptomycin, insulin, cisplatin, withaferin A, N-acetyl-L-cysteine, and DMSO were purchased from Sigma. Human epithelial ovarian tumor cisplatin-sensitive (A2780) cell line was obtained from Dr. Denise Connolly (Fox Chase Cancer Center, Philadelphia, PA). The cisplatin-resistant (A2780/CP70) cell line was obtained from Dr. Christopher States (University of Louisville, Louisville, KY). CAOV3 cell line was purchased from American Type Culture Collection (ATCC).

2.2. Cell culture and treatment with cisplatin and WFA

A2780 and A2780/CP70 cells were cultured in RPMI with 10% FBS, 1% penicillin/streptomycin, and 0.05% (v/v) Insulin. CAOV3 cells were cultured in DMEM with 10% FBS and 1% penicillin/streptomycin. Cells growing in log phase were trypsinized and seeded into 96 wells plates (approximately 5000 cells/well). After 24 h of plating, cells were treated with various concentrations of cisplatin and WFA both alone or combination of cisplatin/WFA. Treatments of cells were performed in 5% FBS medium by adding cisplatin (final concentration of 2, 5 10, 20, 50 or 100 μM) solubilized in DMSO and/or WFA (final concentration of 0.1, 0.5, 1, 1.5, 2, 3, or 5 μM) solubilized in DMSO to a concentration of 0.2% (v/v). DMSO (0.2% v/v) was used as a vehicle control.

2.3. Cell proliferation assays

A2780, A2780/CP70, and CAOV3 cells were seeded into 96-wells plates. After 24 h of plating, cells were treated in triplicates with cisplatin and WFA alone or combination of cisplatin/WFA for 24 h, 48 h, or 72 h as described above. Twenty microliters of MTT reagent from cell proliferation assay kit (Promega) was added to each well and cell proliferation was measured as described previously [30].

2.4. Isobologram analysis

A2780 cells were treated in triplicates for 48 h using 6 different concentrations of cisplatin and WFA at a constant ratio. Viable cells were quantitated with MTT assays as described above and fraction affected was calculated from percent inhibition. Fraction affected was then used in CalcuSyn software to generate an isobologram.

2.5. Measurement of cell apoptosis using flow cytometry for Annexin V

A2780 cells were treated with cisplatin and WFA both alone and in combination of cisplatin/WFA for 24 h and dissociated with versene (Invitrogen). Cells were resuspended in Annexin V binding buffer to a concentration of 1×10^6 cells/ml. Annexin V-FITC (2 μl, BD Biosciences) was incubated for 15 min in the dark in 100 μl of cells suspension. Propidium iodide (PI) was then spiked into 400 μl of Annexin V binding buffer and then was added immediately to cell suspension and used on a FACSCaliber (BD Biosciences) and analyzed with FlowJo software.

2.6. Measurement of the generation of ROS

A2780 cells (20,000/dish) were seeded on glass bottom 35 mm^2 dishes overnight followed by treatment with cisplatin and WFA as described above for 24 h. Medium was replaced with fresh medium containing 2 μM H_2DCFDA (Invitrogen) and incubated for 30 min at 37 °C. Cells were washed with PBS, and examined under confocal microscopy [31]. Relative fluorescence (RF) of ROS positive cells was quantified at green channel using NIS-AR Elements analysis software (Nikon). RF values were measured from 8 representative fields from 2 independent experiments.

2.7. Measurement of DNA damage (TUNEL assay)

A2780 cells were plated on chamber slides and treated with cisplatin and WFA as described above. Cells were then assayed for DNA damage using Dead End Fluorometric TUNEL assay kit (Promega) according to the manufacturer's instructions. RF was quantified at green channel as described above.

2.8. Statistical analysis

Standard error of mean (SEM) and level of significance (*P* value) were calculated using unpaired non-parametric Mann–Whitney *t*-test using Graph Pad Prism software.

3. Results

3.1. WFA synergistically enhances the antitumor effects of cisplatin

Patients treated with cisplatin-based chemotherapy present with serious side effects and eventually develop resistance to cisplatin. To overcome these problems, we combined WFA with cisplatin to minimize/eliminate the side effects associated with high doses of cisplatin. Two cisplatin-sensitive ovarian cancer cell lines A2780 and CAOV3 and one cisplatin-resistant ovarian cancer cell line A2780/CP70 were treated with various concentrations of cisplatin and WFA, both alone and in combination for 24, 48, and 72 h. Cell death induced was determined by MTT assays. Both cisplatin and WFA induced cell death in a time- and dose-dependent manner. After 48 h of treatment, IC_{50} values for cisplatin to inhibit cell proliferation of A2780, A2780/CP70, and CAOV3 cells were found to be 40, 32 and 40 μM respectively (Fig. 1), which decreased significantly to 10, 6 and 12 μM upon combination with WFA 1.5 μM (Fig. 1A, C, E). IC_{50} values for WFA alone was found to be 6, 4.5 and 5 μM respectively, which decreased significantly to 0.8, 0.6, and 1 μM respectively upon combination with cisplatin 20 μM (Fig. 1B, D, F). These results indicate that interaction between cisplatin and WFA is synergistic in inducing cell death. Isobologram analysis using 6 different concentrations of WFA and cisplatin at a constant ratio (as little as 1:5) of WFA to cisplatin demonstrated that cisplatin and WFA acted synergistically (Supplementary Fig. S1).