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## DOXIL when combined with Withaferin A (WFA) targets ALDH1 positive cancer stem cells in ovarian cancer

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### Abstract

Ovarian cancer is a highly aggressive and deadly disease. Currently, the treatment for ovarian cancer entails cytoreductive surgery followed by chemotherapy, mainly cisplatin or carboplatin combined with paclitaxel. Although this regimen is initially effective in a high percentage of cases, unfortunately, after few months of initial treatment, tumor relapse occurs due to platinum-resistance. DOXIL (liposomal preparation of doxorubicin) is a choice of drug for recurrent ovarian cancer. However, its response rate is very low and is accompanied by myocardial toxicity.

Resistance to chemotherapy and recurrence of cancer is primarily attributed to the presence of cancer stem cells (CSCs), a small population of cells present in cancer. Effect of DOXIL and withaferin A (WFA), both alone and in combination, was investigated on cell proliferation of ovarian cancer cell line A2780 and tumor growth in SCID mice bearing i.p. ovarian tumors. ALDH1 cells were isolated from A2780 using cell sorter, and effect of DOXIL and WFA both alone and in combination on tumorigenic function of ALDH1 was studied using spheroids formation assays in vitro. Western blots were performed to examine the expression of ALDH1 and Notch 1 genes. In our studies, we showed, for the first time, that DOXIL when combined with withaferin A (WFA) elicits synergistic effect on inhibition of cell proliferation of ovarian cancer cells and inhibits the expression of ALDH1 protein, a marker for ALDH1 positive cancer stem cells (CSCs), and Notch1, a signaling pathway gene required for self-renewal of CSCs. Inhibition of expression of both ALDH1 and Notch1 genes by WFA was found to be dose dependent, whereas DOXIL (200 nM) was found to be ineffective. SCID mice, bearing i.p. ovarian tumors, were treated with a small dose of DOXIL (2 mg/kg) in combination with a sub-optimal dose of

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#### COMPETING INTERESTS

The authors declare that they have no competing interests.

#### AUTHORS' CONTRIBUTION:

SSK designed and performed most of the experiments and drafted the manuscript. CAW performed sorting of ALDH1 positive and negative cells from A2780 cell line. ZW performed histo-pathological analysis of the tumors. KC performed some experiments, performed analysis of data and editing of the manuscript. MR helped SSK in designing of the experiments. PG generated the i.p. tumors in SCID mice and performed injection of drugs, collection of tumors and other tissues at the time of sacrificing the animals. All authors read the final version of the manuscript and approved the contents.

WFA (2 mg/kg) which resulted in a highly significant (60% to 70%) reduction in tumor growth, and complete inhibition of metastasis compared to control. In contrast, WFA treatment showed a significant reduction in tumor growth but no change in metastasis compared to control. DOXIL showed non-significant reduction in tumor growth and no change in metastasis compared to control. Isolated ALDH1 positive CSCs treated with the combination of DOXIL and WFA resulted in a significant reduction in spheroids formation (tumorigenic function of CSCs) and expression of ALDH1 protein. WFA when used alone at a concentration of 1.5  $\mu$ M was found to be highly effective in suppression of ALDH1 expression, whereas DOXIL at a concentration of 200 nM was found to be ineffective. DOXIL in combination with WFA elicits synergistic effects, targets cancer stem cells, and has potential to minimize induction of drug resistance and reoccurrence of cancer. Based on our studies, we conclude that the combination of DOXIL with WFA has the potential to be an effective therapy for ovarian cancer and may ameliorate DOXIL related side effects as well as recurrence of ovarian cancer leading to increase in patients' survival rate.

### Keywords

cancer stem cells; ovarian cancer; DOXIL; withaferin A; combination therapy; ALDH1

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## INTRODUCTION

Ovarian cancer is the major cause of death in women with gynecological malignancies [1]. Currently, ovarian cancer treatments are based on a surgical cytoreduction followed by cisplatin or platinum/taxane combination chemotherapy [2]. Initially, patients with ovarian cancer respond positively in 70 to 80% of the cases [3], however, within few months of treatment, patients develop platinum-resistance resulting in the recurrence of cancer [4, 5]. Resistance to platinum based chemotherapies have been associated with number of mechanisms; such as increase in glutathione [6], metallothionein levels [7], decrease in drug uptake [8,9], increase in DNA repair mechanisms [10–12], tolerance to the formation of platinum-DNA adducts [13], and changes in status of p53 gene which alters the sensitivity of tumors to cisplatin therapy [14, 15]. DOXIL (liposomal preparation of doxorubicin) is used as a major drug for the treatment of patients with recurrence cancer. However, DOXIL response rate is very low (< 20%) [16], therefore, approximately 70% of patients diagnosed with recurrence ovarian cancer die within 5 years of their diagnosis.

For the last few years, presence of CSCs in tumors has become very clear and these cells have been reported to be the cause of development of drug-resistance and recurrence of cancer. CSCs are a minor sub-population of tumors and possess the capacity for self-renewal and give rise to heterogeneous cancer lineages that comprise the tumor of origin [17]. The presence of CSCs have been reported in ovarian cancer cell lines, ovarian cancer and patients' ascites [18–22]. CSCs are isolated based on the presence of certain extracellular markers. The most common markers used for ovarian cancer stem cells include CD24, CD34, CD44, CD117, CD133, ALDH1, Oct4, Myd88 and EpCAM. An increase in the number of CSCs in ovarian cancer correlates with a poor prognosis, including shorter overall life and disease free survival [21–29]. In recent studies, Abubaker et al [30] using two ovarian cancer cell lines (epithelial OVCA433 and mesenchymal HEY) demonstrated

enrichment for a population of cells with high expression of CSC markers at protein as well as mRNA levels after treatment of cells with cisplatin, paclitaxel and the combination. In our recent study, we showed a significant increase in CD24, CD34, CD44, CD117 and Oct4 positive cells in tumors collected from mice bearing implanted orthotopic ovarian cancer after treatment with cisplatin [31]. These results clearly demonstrate that cisplatin, paclitaxel or carboplatin combination when used as first line chemotherapy for ovarian cancer suppress tumor growth by targeting cancer cells but spare CSCs which undergo enrichment resulting in drug-resistance, ultimately leading to recurrence of cancer. Therefore, developing a chemotherapy that targets both cancer cells and CSCs is mandatory and an appropriate approach to avoid recurrence of ovarian cancer.

In the past few years, many efforts have been devoted to develop drugs that can increase response rate and reduce chemo-resistance and recurrence of cancer including different combinations of DOXIL with other currently used chemo-drugs. Even though some of these combination showed enhanced effects and increase in sensitivity to DOXIL in vitro and in patients with recurrence ovarian cancer [32–34], but no study showing targeting of CSCs by these combinations has been reported. In this context, we explored the combination of DOXIL with withaferin A (WFA) to study its effect on ovarian cancer cell proliferation in vitro and tumor growth in nude mice and its effect on isolated ALDH1 positive cancer stem cells, which has been reported as a major population of CSCs in ovarian cancer [35]. WFA, a bioactive compound isolated from the plant *Withania somnifera*, is available as an over-the-counter dietary supplement in the U.S. It has been purported to possess anticancer, anti-inflammatory, anti-angiogenic and cardio-protective effects [36–40]. However, its clinical application in combination with DOXIL to treat cancer has not been explored. In our previous studies, we showed that WFA when used alone or in combination with cisplatin to treat mice bearing orthotopic human ovarian tumor reduced tumor growth by 60 to 70% and prevented metastasis to other organs [31], in addition to eliminating CSCs as well as CSCs enhanced by cisplatin. In our present study, we showed that when a low dose of DOXIL is used in combination with suboptimal dose of WFA, it synergistically inhibits proliferation of ovarian cancer cells, and reduces tumor growth and metastasis in SCID mice. In addition, treatment of isolated ALDH1 positive CSCs from A2780 cell line with DOXIL/WFA combination significantly inhibits the spheroid formation (characteristics of cancer stem cells) and ALDH1 expression. Thus, our studies, for the first time, demonstrate that a combination of low dose of DOXIL with suboptimal dose of WFA is highly effective in suppressing tumor growth as well as eliminating putative CSCs. Therefore, this combination has the potential to be an effective therapy for ovarian cancer and may ameliorate DOXIL related side effects as well as recurrence of ovarian cancer, leading to increase in patients' survival rate.

## MATERIAL AND METHODS

### Ethical Statement

Animals work reported in the manuscript was performed after approval of the protocol by the University of Louisville Animal Care Use Committee (IACUC).

## Cell Culture

Human epithelial ovarian cancer cell line (A2780) was obtained as a gift from Denise Connolly (Fox Chase Cancer Center, Philadelphia, PA). The cell line was originally generated from human ovarian cancer patient prior to treatment [41]. The cisplatin-resistant (A2780/CP70) cell line was obtained as a gift from Dr. Christopher States (University of Louisville, Louisville, KY). This cell line was derived from A2780 cell line after treatment with cisplatin [42]. The third cell line (CaOV3), was purchased from American Type Culture Collection (ATCC). Both A2780 and A2780/CP70 cell lines were cultured in RPMI medium containing 10% fetal bovine serum (FBS), 1% Penicillin/Streptomycin, and 0.05% (v/v) Insulin (Sigma). CaOV3 cell line was cultured in DMEM medium containing 10% FBS and 1% Penicillin/Streptomycin. Withaferin A and DMSO were purchased from Sigma. DOXIL was obtained from Ortho Biotech.

## Treatment of cells with DOXIL and WFA

For treatment of ovarian cancer cells with DOXIL and WFA both alone and in combination, cells growing in log phase were harvested using trypsin and plated at 3,000 cells/well into 96 well plates, and allowed to grow for 24 hours before treatment in triplicates with various concentrations of DOXIL, WFA or combination of DOXIL and WFA. Where necessary, DMSO was used as a vehicle control for untreated cells. Cells were incubated for 24 to 72 hr before quantitating by MTT assays as described previously [43]. Color development was assayed by an ELISA reader at 492 nm. DOXIL was diluted in serum free medium whereas WFA was solubilized in DMSO.

## Isobologram analysis

A2780 cells were treated in triplicate with 7 different concentrations of DOXIL and WFA both alone and in combination at a constant ratio as described above. The cell proliferation was quantitated using MTT assays and fractions affected were calculated from percent inhibition. Fractions affected were then used in CalcuSyn software to generate dose-dependent curve and isobologram as described previously [43].

## SDS-PAGE and Western blot analysis

Cells were plated in 6-well plates and treated with WFA and DOXIL both alone and in combination. After 48 h of treatment, cells were rinsed with PBS and suspended in chilled lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% NP-40, 1 mM  $\text{Na}_3\text{VO}_4$ , and 1 mM NaF] supplemented with complete Protease Inhibitor Cocktail (Sigma). To prepare cell lysate, cells were suspended in lysis buffer, followed by sonication for 15 to 20 sec. Protein concentrations for each sample were determined using Bradford method (Bio-Rad Laboratories) according to supplier's instructions. Forty  $\mu\text{g}$  of protein from each sample was mixed with SDS-PAGE buffer and heat-denatured at 95°C for 5 min and separated on SDS-PAGE. The proteins were transferred to Hybond nitrocellulose membrane (GE HealthCare) and blocked with 5% non-fat milk in TBST buffer [10 mM Tris, 150 mM NaCl and 0.1% (v/v) Tween-20, pH 7.4] for 1 hr at room temperature followed by incubation with primary antibody diluted in non-fat milk at 4°C for overnight. The antibody for ALDH1 (SC-166362), was purchased from Santa Cruz Biotechnology and  $\beta$ -actin (cat # A3854) was

obtained from Sigma-Aldrich. Membranes were washed with TBST (three times, 5 min each) and then incubated with appropriate secondary antibody conjugated with horseradish peroxidase (Sigma, diluted 1:2000). Membranes were washed with TBST (three times, 5 min each) and the immuno-reactive bands were visualized by enhanced chemiluminescence (ECL) (GE Healthcare). Membranes were stripped off for 10 min with methanol containing H<sub>2</sub>O<sub>2</sub> and probed with  $\beta$ -actin antibody conjugated with horseradish peroxidase in order to serve as an internal control as described previously [31].

### **Generation of intraperitoneal ovarian tumor and treatment with DOXIL and WFA**

To determine the efficacy of DOXIL in combination with WFA on tumor growth and metastasis in vivo, we generated intraperitoneal (i.p.) tumors in SCID mice followed by treatment with DOXIL and WFA both alone and in combination. i.p. tumors were generated by injecting 1X10<sup>6</sup> A2780 cells, suspended in serum and antibiotics free RPMI medium/ mouse directly into peritoneal cavity of 5 to 6 weeks old SCID mice. After 10 days of post-cell injections, mice were treated with: 1) vehicle control (10% DMSO and 90% glyceryl trioctanoate), 2) DOXIL (2 mg/kg doxorubicin concentration), 3) WFA (2 mg/kg), and 4) DOXIL (2 mg/kg) plus WFA (2 mg/kg). Randomly three animals were included in each group. DOXIL in saline was injected i.p. once a week, whereas WFA was injected in 10% DMSO and 90% glyceryl trioctanoate on every third day. Following three weeks of treatment, animals were sacrificed, visible tumors and other tissues such as ovaries, kidney, liver, adrenal and lungs were collected from each mouse. Tumors were weighed at the time of collection. The tumors and other tissues were divided into two parts; one part was immediately frozen in liquid nitrogen, and the second part was fixed in 10% buffered formalin. The animals' experiments were approved by the University of Louisville, Institutional Animal Care and USE Committee (IACUC) (protocol # 12063).

### **Isolation of ALDH1 positive cancer stem cells from A2780 cell line and treatment with DOXIL and WFA**

Aldehyde dehydrogenase 1 (ALDH1) is a cancer stem cell marker, and ALDH1 positive cancer stem cells have been reported to play clinical significance role in ovarian cancer [44]. To determine the effect of DOXIL and WFA both alone and in combination on ALDH1 positive cancer stem cells, we isolated ALDH1 positive cancer stem cells from ovarian cancer cell line, A2780. Cells growing in log phase were rinsed with PBS and then dislodged by incubating with non-enzymatic cell dissociation solution (Sigma) in CO<sub>2</sub> incubator at 37°C for 60 min. The cells were pelleted by centrifuging at 2,000 rpm for 2 min and suspended in assay buffer (from Adelfluor kit purchased from Stem Cell Technologies) at 2X10<sup>6</sup> cells/ml. The cells were incubated in Adelfluor substrate (1  $\mu$ M/1X10<sup>6</sup> cells) and incubated for 45 min at 37°C. One sample was treated with 50 mM of diethylaminobenzaldehyde (DEAB, an aldehyde inhibitor), as a negative control. After incubation, cells were centrifuged at 2,000 rpm for 2 min and resuspended in assay buffer. The highly bright fluorescent ALDH1-expressing (ALDH1<sup>+</sup>) and ALDH1<sup>-</sup> cells were detected in the green fluorescent channel (520–540 nm) using Beckman Coulter MoFlo XDP and collected in RPMI medium containing 10% FBS.

To determine tumorigenic potential of ALDH1 positive cancer stem cells and examine the effect of DOXIL and WFA, alone and in combination, on tumorigenic potential of ALDH1 positive cancer stem cells, standard spheroid formation assays were performed according to Zhang et al. [45] with some modifications. Initially, isolated ALDH1 positive cancer stem cells or ALDH negative cancer stem cells ( $1 \times 10^3$ ) were suspended in RPMI medium supplemented with 5  $\mu\text{g/ml}$  insulin (Sigma), 20 ng/ml human recombinant epidermal growth factor (EGF; Sigma), 10 ng/mL basic fibroblast growth factor (bFGF; Sigma), and 1% FBS. The cells were plated into 6-well ultra-low attachment plates (Corning Costar) and incubated at  $37^\circ\text{C}/5\% \text{CO}_2$  in the incubator. Fresh insulin, bFGF and EGF were added to the medium on every third day. Spheroids that formed within 3 to 4 days after plating were counted and photographed. Spheroids diameters  $> 50 \mu\text{m}$  were counted as a single positive colony. Several fields for each well were counted under a phase contrast microscope. For all spheroid formation experiments, a minimum of two wells were used for each condition, and experiments were repeated three times.

### Statistical analysis

Statistical comparison of data was carried out by the student's t test (for single comparison). Probability of  $p = 0.05$  was determined from the two-sided test and was considered significant. The statistical analysis was carried by using SPSS 10.0 software.

## RESULTS

### DOXIL when combined with WFA elicits synergistic effects on inhibition of ovarian cancer cell proliferation

Liposomal preparation of doxorubicin "DOXIL" is preferred over doxorubicin as a second line option for the treatment of recurrent ovarian cancer due to its substantial better toxicity profile. However, it has very low response rate ( $< 20\%$ ) and is still associated with myocardial toxicity [16, 46]. To minimize the side effects as well as to increase its response rate, we explored the possibility of using DOXIL/WFA combination. Treatment of three ovarian tumor cell lines (cisplatin-sensitive, A2780 and CaOV3; and a cisplatin-resistance cell line, A2780/CP70) with various concentrations of DOXIL and WFA both alone and in combination showed a time- and dose-dependent inhibition of cell proliferation. Only results from A2780 cell line are shown (Figure 1). When DOXIL and WFA were used alone, the  $\text{IC}_{50}$  values for DOXIL and WFA for A2780 cells (after 48 h of treatment) were found to be approximately 1  $\mu\text{M}$  and 2.5  $\mu\text{M}$  respectively. On co-treatment of cells with DOXIL and WFA,  $\text{IC}_{50}$  values for both the agents decreased significantly (Figure 1 A and B). Similar synergistic results were obtained with other two cell lines (CaOV3 and A2780/CP70). Isobologram analysis using both the agents at a constant ratio confirmed the synergistic effect on combination of DOXIL and WFA (Figure 1 C). The combination of WFA with DOXIL requires several-fold lower dose of DOXIL to achieve the same level of cell death compared to DOXIL alone. Therefore, it is expected that the side effects associated with high dose of DOXIL will be eliminated or reduced.

### **DOXIL when combined with WFA suppresses tumor growth and metastasis in nude mice**

To access the efficacy of WFA/DOXIL combination on tumor growth and metastasis in vivo, we tested the effect of DOXIL and WFA, both alone and in combination, on tumor growth and metastasis in SCID mice bearing i.p. human ovarian tumors, as described in materials and methods. Beginning from 10 days after injection of A2780 cells ( $10^6$  cells/mouse), mice were treated with vehicle, DOXIL (2 mg/kg), WFA (2 mg/kg) or combination of DOXIL/WFA (2 mg/kg DOXIL + 2 mg/kg WFA). After 3 weeks of treatment, animals were sacrificed. We observed that the control vehicle-treated animals developed highly vascularized and large tumors (Figure 2A) which metastasized to the ovaries (formed bilateral ovarian cancer) (Figure 2B), intestine, omentum and liver (results not shown). Treatment of animals with WFA (2 mg/kg) alone showed a significant reduction in tumor weight but no change in metastasis (Figure 2B and C). Treatment of animals with DOXIL (2 mg/kg) alone showed some reduction in tumor weight but was found to be non-significant compared to control (vehicle-treated) animals (Figure 2C). However, no change in metastasis in these animals was observed. In contrast, animals treated with DOXIL and WFA combination (2 mg/kg each) showed a highly significant reduction (60 to 65%) in tumor weight compared to control animals and interestingly no visible metastasis to any organ was observed (Figure 2A, 2B and 2C). H&E staining of ovarian tissues followed by histo-pathological analysis of the intestine and ovaries collected from the control, as well as treated animals confirmed metastasis to intestine and ovaries in control animals as well as DOXIL or WFA alone treated animals, whereas no cancer cell or metastasis was observed in ovaries collected from animals treated with DOXIL/WFA combination (Figure 3). These results suggest that combination of low dose of DOXIL (2 mg/kg) with suboptimal dose of WFA (2 mg/kg) is highly effective in suppressing tumor growth and metastasis of i.p. ovarian tumor in SCID mice.

### **DOXIL when combined with WFA inhibits tumorigenic potential of ALDH1 positive CSCs**

CSCs are capable of forming characteristic compact circular colonies with cobblestone appearance and can survive numerous passages. These spheroid clusters have the potential to be highly tumorigenic and possess the capability to propagate and reconstitute original tumor architecture when injected into permissive hosts [20, 47, 48]. To test that DOXIL and WFA when used in combination inhibits tumorigenic function of CSCs, we isolated ALDH1 positive CSCs from A2780 cell line using Aldefluor kit as described in materials and methods. Approximately 0.6% to 1% of the cells were found to be ALDH1 positive (Figure 4). Approximately 2000 ALDH1 positive, as well as negative cells were plated on ultra-low attachment plates (corning), respectively. As shown in Figure 5, ALDH1 positive cells, when plated on ultra-low adhering plates, formed large spheroids (colonies) within one week of plating, whereas ALDH1 negative cells did not develop colonies or formed a few and very small size colonies, suggesting tumorigenic characteristic of ALDH1 positive CSCs.

To determine the effect of DOXIL/WFA combination on tumorigenic potential of ALDH1 positive CSCs, spheroids were collected, dispersed mechanically, and plated again on 6 well ultra-low attachment plates. After 24 h of plating, small spheroids formed and were treated with DOXIL and WFA, both alone and in combination. After three days of treatment, spheroids were observed under phase contrast microscope, counted and photographed. As

shown in Figure 6 A, a dose dependent deleterious (apoptotic) effect on spheroids formation was observed when treated with WFA alone compared to control. DOXIL also inhibited the spheroid formation. The effects of both WFA (1.5  $\mu$ M) and DOXIL, when used alone, were found to be significant in reducing the number as well as size of colonies compared to control (Figure 6A and B). However, combination of DOXIL (200 nM) with WFA (1.5  $\mu$ M) was found to be highly toxic and enhanced the inhibition of colonies formation to a greater extent. Few small disintegrated colonies were observed after treatment with DOXIL and WFA combination especially at higher concentration 1.5  $\mu$ M of WFA (Figure 6A and B), suggesting that combination of DOXIL with WFA is highly effective in targeting the CSCs and hence may reduce or eliminate the drug-resistance and recurrence of ovarian cancer.

#### **DOXIL when combined with WFA suppresses the expression of ALDH1 protein**

Both DOXIL and WFA were found to inhibit spheroid formation by isolated ALDH1 CSCs. Therefore, to examine the effect of DOXIL and WFA both alone and in combination on the expression of ALDH1 protein, we examined the effect of DOXIL and WFA, both alone and in combination, in A2780 cells and ALDH1 positive isolated CSCs. We treated both A2780 cells and isolated ALDH1 positive cells with DOXIL and WFA, both alone and in combination, as described above. Treatment of A2780 cells with DOXIL (200 nM) and WFA (1.5  $\mu$ M) combination showed a significant suppression of ALDH1 gene expression compared to control untreated cells. Both DOXIL and WFA showed a non-significant suppression of ALDH1 expression (Figure 7A). In contrast, isolated ALDH1 positive cells when treated with WFA at a concentration of 1.5  $\mu$ M was highly effective in suppressing the expression of ALDH1 protein, however, DOXIL was found to be ineffective at a concentration of 200 nm. Combining of DOXIL (200 nM) with WFA (1.5  $\mu$ M) showed enhanced suppression of ALDH1 protein expression (Figure 7B), suggesting that DOXIL, when used alone, is ineffective in targeting CSCs, however, when combined with WFA it enhances the effect of WFA in targeting CSCs.

#### **DOXIL when combined with WFA inhibits Notch1 signaling gene**

Self-renewal, drug resistance and differentiation are key characteristics of CSCs. Sonic Hedgehog (Shh), Notch1, Twist, Snail, Slug and Wnt1 signaling transduction pathways play major roles in the self-renewal of CSCs [49–55]. Notch 1 signaling pathway is associated with regulation of cell fate at several distinct developmental stages and has been implicated in cancer initiation and progression [51, 55, 56]. Initially, to show the effect of DOXIL and WFA, both alone and in combination, we treated ovarian cancer cell line, A2780, with different concentrations of DOXIL and WFA, both alone and in combination, for 48 hr. As shown in Figure 8, treatment of A2780 cells with various concentrations of WFA showed a dose dependent suppression of expression of Notch1. DOXIL treatment resulted in an insignificant effect. However, combining DOXIL with WFA showed an enhanced suppressive effect on Notch1 protein expression, suggesting the combination therapy targeted the signaling mechanism involved in self-renewal or CSCs, therefore, resulting in reduction or elimination of drug-resistance and hence recurrence of cancer caused by CSCs.



## DISCUSSION

Ovarian cancer is the leading cause of death from gynecological malignancies [1, 57]. The main reasons for such high mortality rate are due to the lack of symptoms accompanying this tumor, an effective screening strategy, and limited results obtained with medical treatments. The most commonly used first line chemotherapy after cytoreductive surgery is a platinum/taxane combination [2]. Although this strategy initially has high response rate, however, after few treatments, the vast majority of patients develop cisplatin resistance and require further therapy [2–4]. Many strategies have been implanted as second line chemotherapy for the patients that develop cisplatin resistance, and several new drugs have been investigated. Among these, liposomal doxorubicin (DOXIL) is a choice of drug. However, DOXIL alone is not considered an effective drug because it is associated with myocardial toxicity and has a very low response rate.

In recent years, combination of two or more clinically approved drugs for the treatment of cancer has become a common strategy with the hope to improve the clinical outcome. With this respect, to improve the efficacy of DOXIL, DOXIL in combination with various drugs including platinum (carboplatin) [58–61], oxaliplatin [62], gemcitabine [63, 64], paclitaxel [65], topotecan [66], vinorelbine [67], ifosfamide [68], olaparib [69], cyclophosphamide and 5-fluorouracil [33], checkpoint blockers antibodies such as PD-L1, PD-1, and CTLA-4 mAbs [32], and trabectedin [70] have been explored in patients with cisplatin-sensitive or cisplatin-resistance recurrent ovarian cancer. A low to moderate increase in response rate, overall survival (OS), and progression free survival (PFS) has been reported for various combinations [71]. Among these combinations, carboplatin/DOXIL has been reported to be a valid alternate in both first line and recurrent ovarian cancer, compared to actual standard options [71].

Development of cisplatin resistance or chemo-resistance in patients with currently used drugs is a major clinical problem and has been reported due to the presence of cancer stem cells, a small population of cells present in tumors. These cells are chemo-resistant, capable of self-renewal and differentiation and responsible for recurrent cancer [21]. Currently used drugs, including DOXIL or its combination with other commonly used drugs, has not been tested for targeting CSCs. There has been an increasing support for natural compounds when developing new treatments for cancer to enhance the therapeutic effect of an anti-neoplastic agent. This allows a lower dose to be used while achieving the same anti-neoplastic effect and reducing the side effects. WFA is a bioactive, cell permeable compound isolated from the plant *Withania somnifera* is an anticancer and anti-inflammatory compound and possesses cardio-protective properties [36–40]. In our previous studies, we showed that WFA when combined with doxorubicin elicits synergistic effects on inhibition of cell proliferation of ovarian cancer cells (A2780, A2780/CP70 and CaOV3) and tumor growth in nude mice [43]. In our other studies, we showed for the first time, that WFA alone or in combination with cisplatin (CIS) target CD24, CD34, CD44, CD117 and Oct4 positive CSCs in tumors collected from nude mice treated with WFA or WFA and CIS combination, suggested that WFA target cancer stem cells in addition to cancer cells [31]. DOXIL has a lower toxicity profile compared to doxorubicin, therefore, in our present studies, we tested its efficacy in combination with WFA on ovarian cancer cell proliferation in vitro, tumor

growth in SCID mice and targeting of CSCs. As shown in Figure 1, DOXIL when combined with WFA elicits synergistic effect on inhibition of ovarian cancer cell proliferation, tumor growth and metastasis in SCID mice (Figures 2 and 3). These results are consistent with our previous findings for WFA and doxorubicin combination [43]. We examined the effect of DOXIL alone and in combination with WFA on targeting of ALDH1 positive CSCs. ALDH1 positive CSCs have been reported in ovarian tumors as well as in ascites from patients with ovarian cancer. In addition, ALDH1 has been shown to have clinical significance in progression and recurrence of ovarian cancer [44]. DOXIL, when used alone to treat ovarian cancer cell line A2780, was found to be ineffective in downregulation of ALDH1 protein expression. However, combination of DOXIL with WFA showed a significant suppression of ALDH1 protein expression (Figure 7A). Treatment of isolated ALDH1 positive CSCs with DOXIL and WFA both alone showed a significant inhibition of spheroids formation (tumorigenic function of CSCs) and such effects are enhanced significantly on combination of DOXIL with WFA (Figure 7B). Combination of a small dose of DOXIL (200 nM) with suboptimal concentration of WFA (1.5  $\mu$ M) was found to be highly effective in inhibiting tumorigenic function of ALDH1 (Figure 6 A and B) and its expression (Figure 7), suggesting that DOXIL alone is not significantly effective in inhibiting ovarian cancer cell proliferation or targeting CSCs. However, when combined with WFA, DOXIL is highly efficacious in targeting cancer cells as well as CSCs. Combining DOXIL with WFA also showed a significant suppression of Notch1 gene, a signaling molecule involved in self-renewal of CSCs. Experiments to test the targeting of other CSCs by WFA and DOXIL combination in vivo on tumor growth by isolated CSCs are in process. Based on these results, we conclude that combining a small dose of DOXIL with suboptimal dose of WFA is highly effective in targeting CSCs, which may lead to reduction in development of drug resistance and recurrence of ovarian cancer. Application of a small dose of DOXIL in combination with suboptimal dose of WFA is expected to reduce unwanted side effects caused by high doses of DOXIL used.

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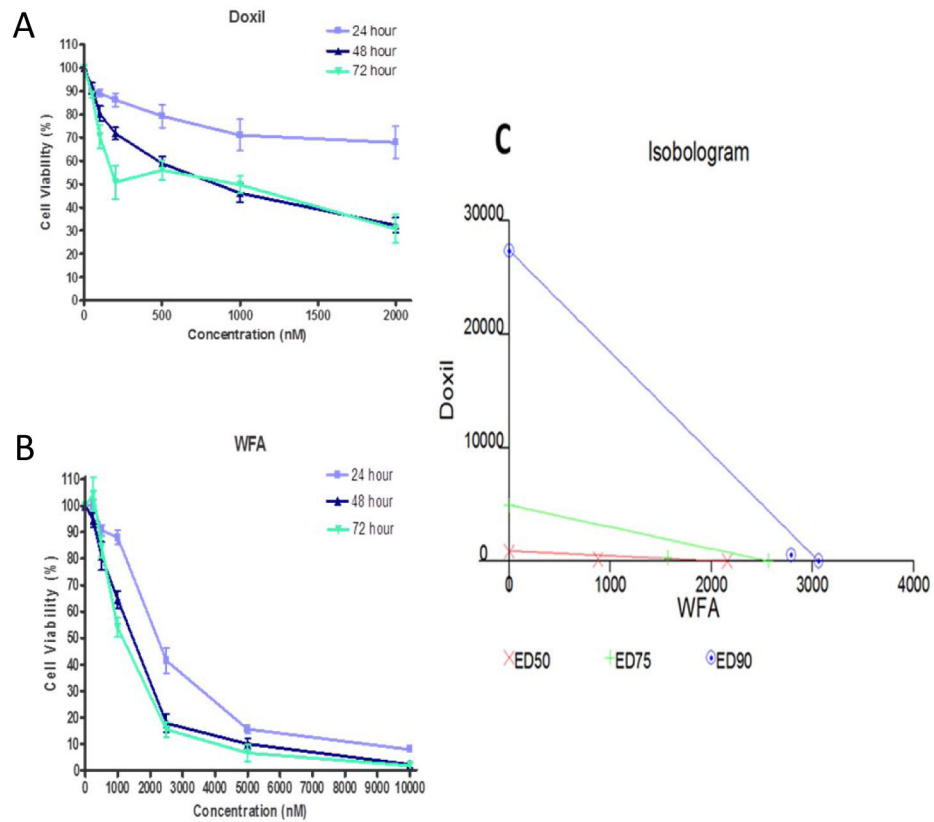
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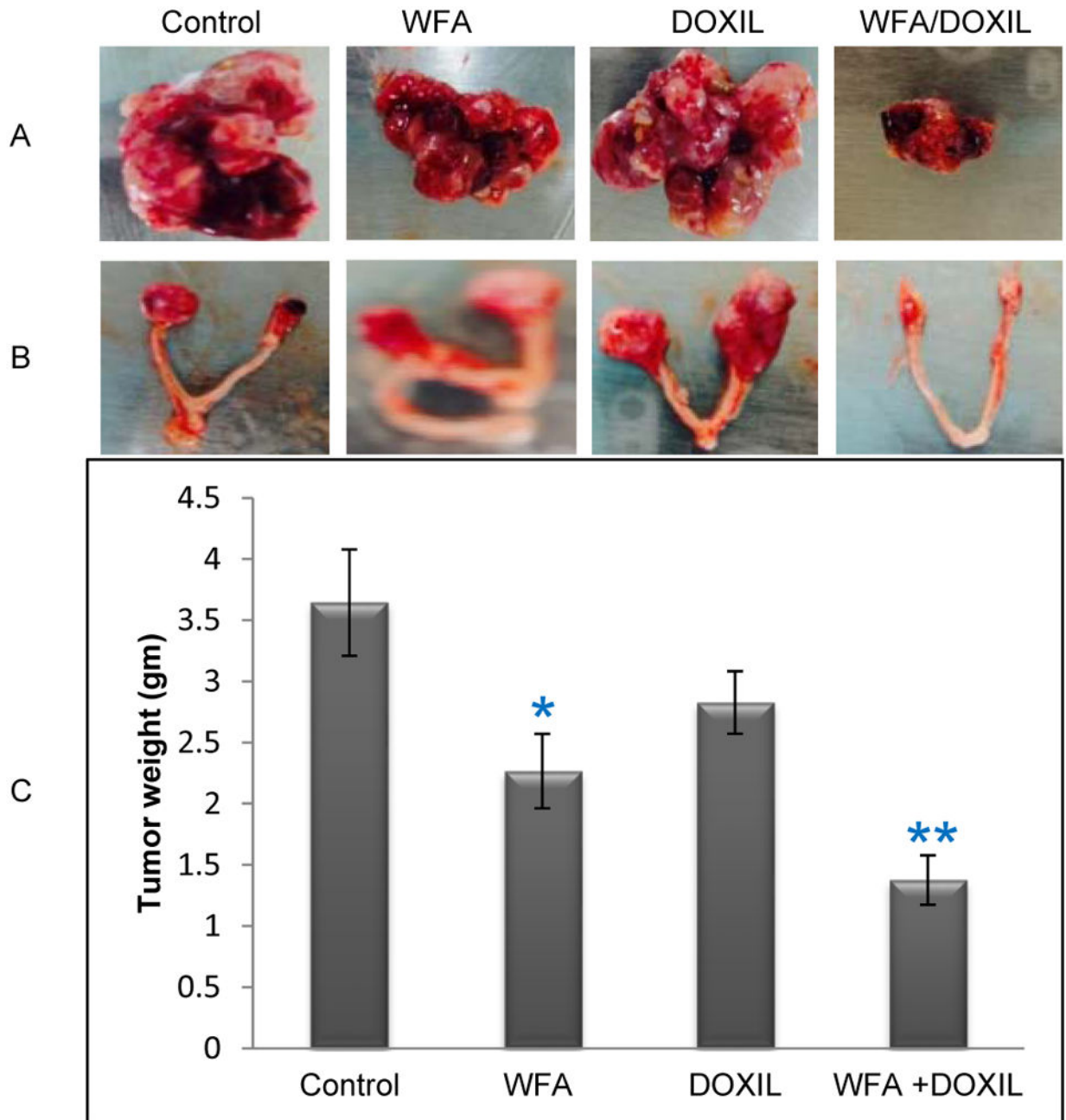
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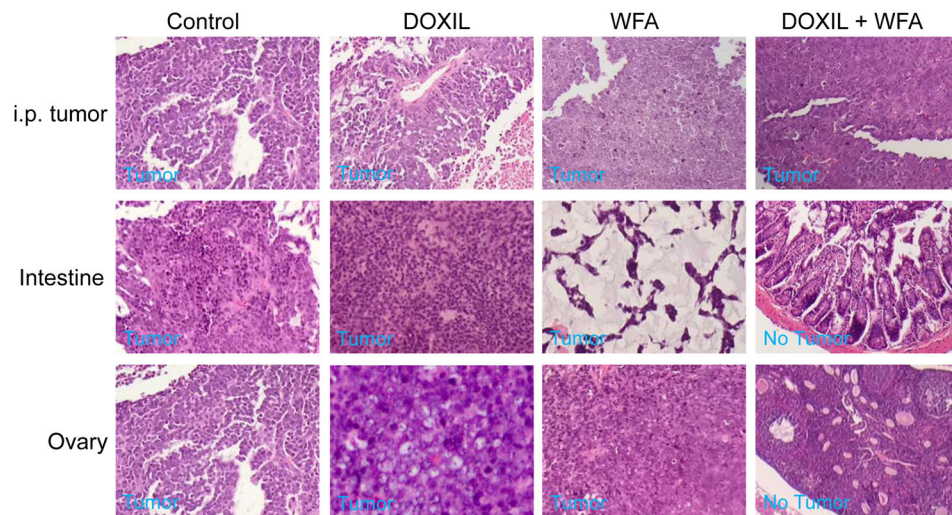
**Figure 1.** Effect of WFA and DOXIL both alone and in combination on A2780 cell proliferation. A2780 cells were treated with DOXIL (A) and WFA (B) both alone and in combination for 24 h, 48 h or 72 hr. Cell proliferation was measured using MTT assays (A and B). Isobologram analysis using DOXIL in combination with WFA at constant ratio (C).



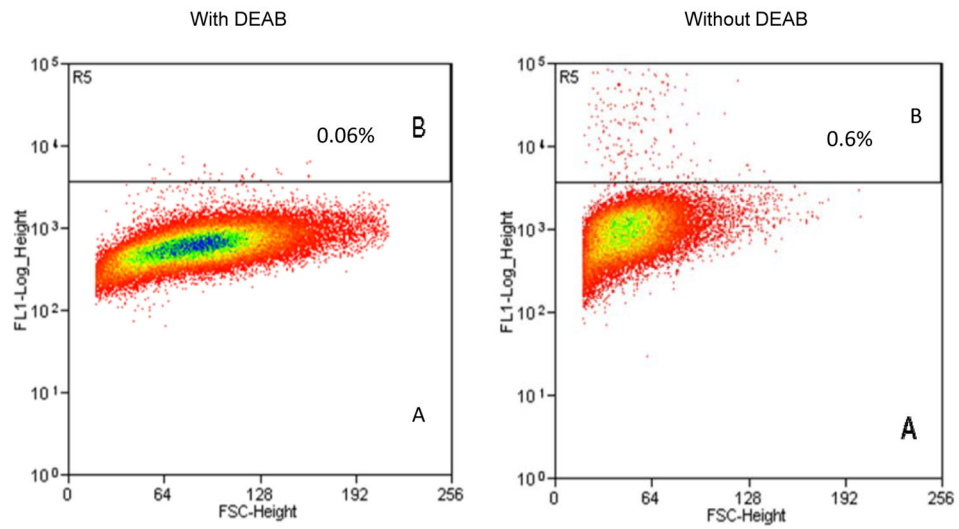
**Figure 2.**

Effect of WFA and DOXIL treatment on tumor growth and metastasis in SCID mice. i.p. tumors were generated in SCID mice by injecting A2780 cells (i.p.) followed by treatment with vehicle (control), DOXIL (2 mg/kg), WFA (2 mg/kg) or DOXIL (2 mg/kg) plus WFA (2 mg/kg). After three weeks of treatment, mice were sacrificed and tumors were weighted and photographed. A = i.p. tumors shown are representative from each group. B = Metastatic ovarian tumors. C = Tumors weights plotted from each group. Results are mean  $\pm$  SEM (vertical bar). \* represents significant at  $p < 0.05$  and \*\* represents significant at  $p < 0.001$  compared to control.

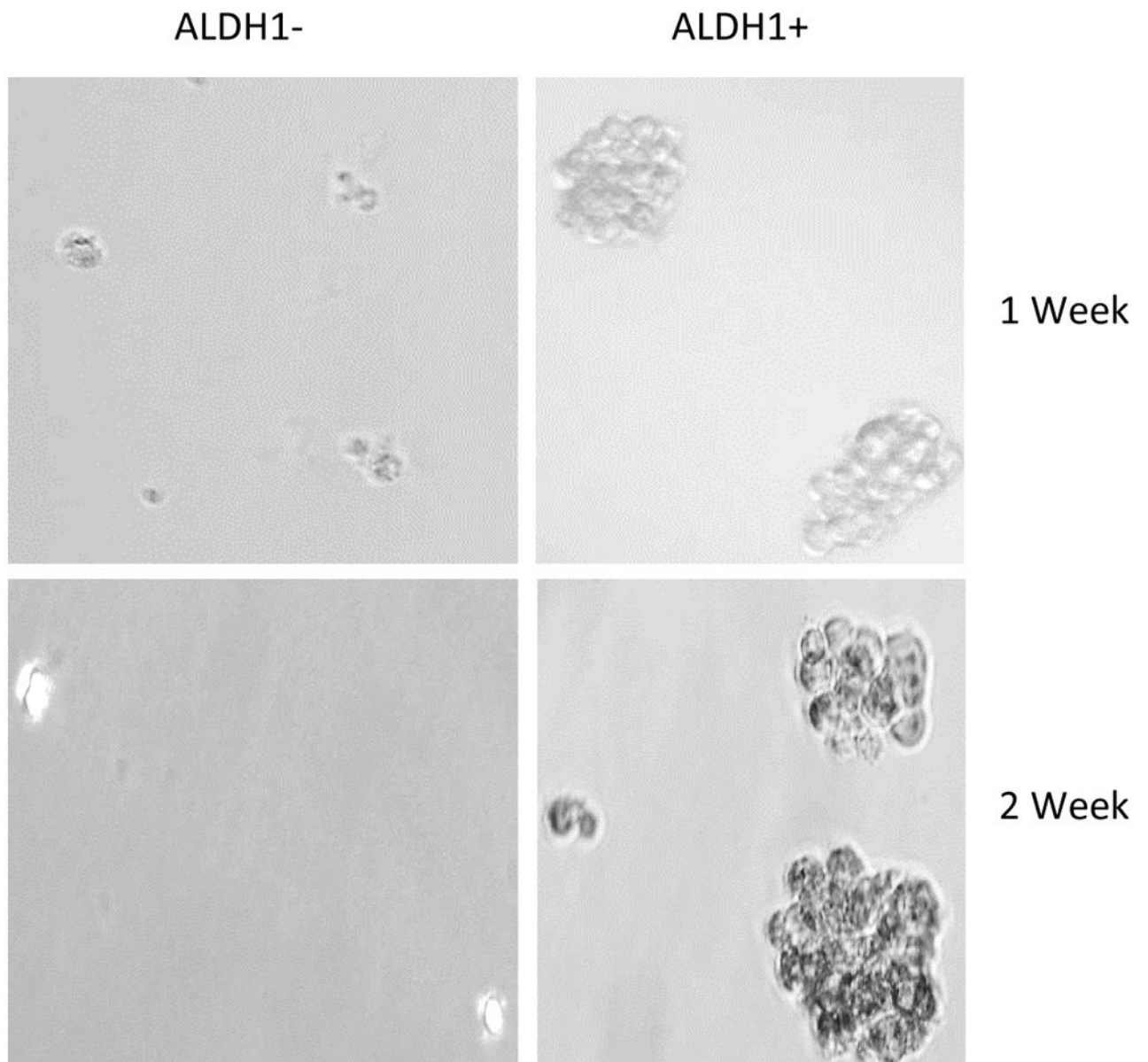




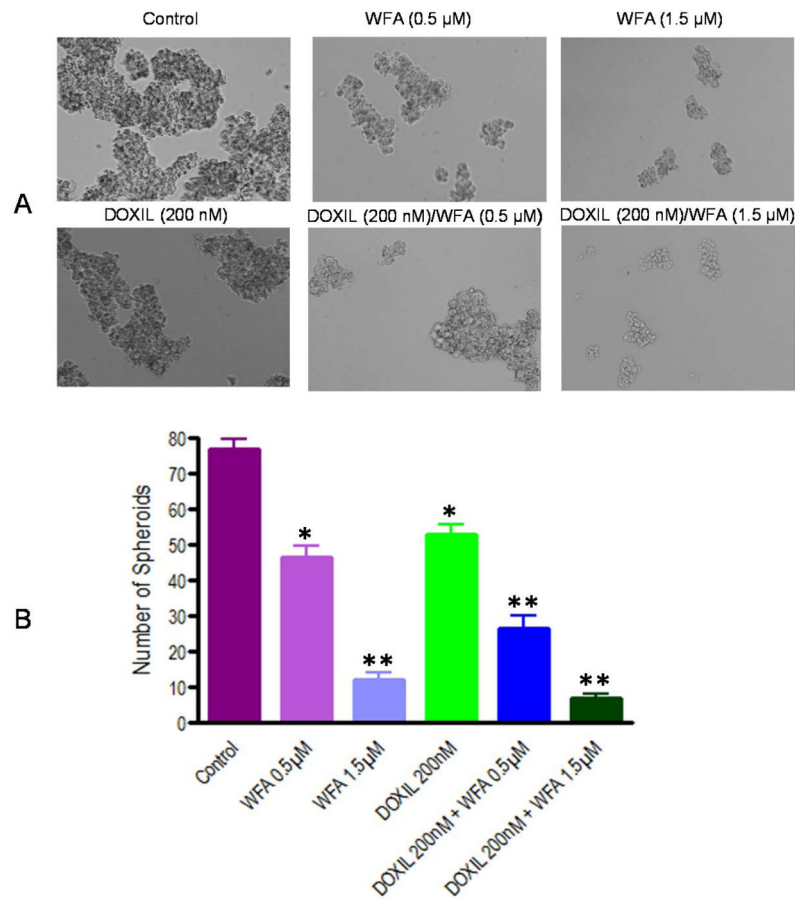
**Figure 3.** Histo-pathological analysis of tumor tissues. Visible tumors, intestine and ovarian tissues from control animals (vehicle treated) and animals treated with DOXIL, WFA or DOXIL plus WFA were collected at the time of sacrificing the animals and processed immediately for immunohistochemical analysis. Tissues sections were stained with H&E staining and analyzed by a trained pathologist for the analysis of metastasis to various organs. In control animals, animals treated with DOXIL or WFA, tumor cells metastasized to intestine and ovaries. No metastasis was observed in intestine or ovaries collected from animals treated with DOXIL and WFA combination.



**Figure 4.** Analysis of ALDH1 positive cancer stem cells from ovarian cancer cell line A2780. ALDEFLUOR Assay Kit from Stem Cell Technology was used for collection of ALDH1 positive cancer stem cells using Beckman Counter MoFlo XDP. A = ALDH1 negative cells (cells treated with DEAB) and B = ALDH1 positive cells.

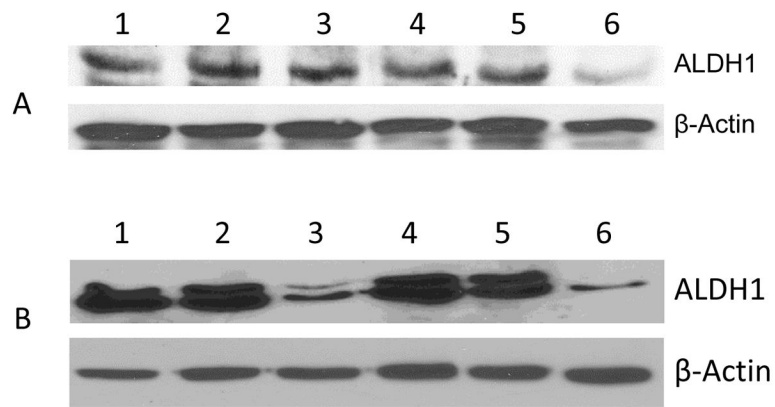


**Figure 5.** Spheroids formation by ALDH1 positive cells. Isolated ALDH1 negative and positive cancer stem cells were plated into ultra-low attachment plates. The spheroids were examined after one week of plating and photographed. ALDH1 positive cells formed large number and large size spheroids (colonies), whereas ALDH1 negative cells did not form spheroids or formed very small spheroids.



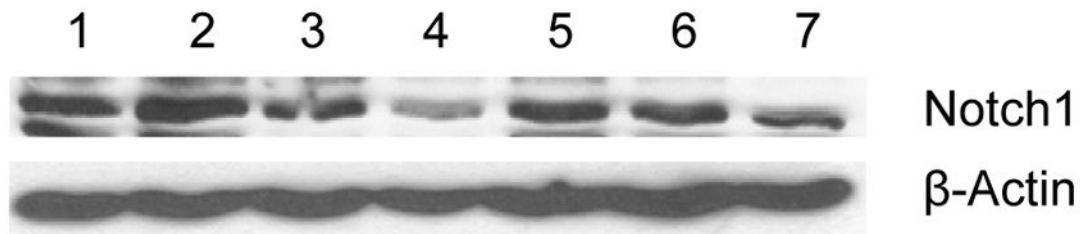
**Figure 6.**

Effect of DOXIL and WFA on ALDH1 positive cancer stem cells. ALDH1 cells were plated on ultra-low attachment plates. After one week of plating, when large size colonies were formed, colonies were collected, dispersed and plated again on 6-well ultra-low attachment plates. After 24 h of plating when small size spheroids are formed, treated with DOXIL and WFA both alone and in combination. After 3 days of treatment, spheroids formed were counted and photographed. A = spheroids formed after 3 days of treatment. B = Quantitative analysis of spheroids after 3 days of treatment. The colonies were counted in six different fields and were added. The results shown represent mean and standard deviation of three independent experiments. \* represents significant at  $p < 0.05$  and \*\* represents significant at  $p < 0.001$  compared to control.



**Figure 7.**

Western blot analysis of A2780 cells (A) and isolated ALDH1 positive cancer stem cells (B) after treatment with DOXIL and WFA, both alone and in combination. Cells/spheroids were collected after treatment and lysed using lysis buffer. Forty  $\mu\text{g}$  of protein from each sample was used for SDS-PAGE. ALDH1-specific monoclonal antibody was used for the detection of ALDH1 protein. The data shown is representative of at least two independent experiments.  $\beta$ -Actin-specific monoclonal antibody conjugated to horseradish peroxidase (Sigma) was used as internal control. Lane 1 = control untreated cells, lane 2 = cells treated with WFA (0.5  $\mu\text{M}$ ), lane 3 = cells treated with WFA (1.5  $\mu\text{M}$ ), lane 4 = cells treated with DOXIL (200 nM), lane 5 = cells treated with DOXIL (200 nM) plus WFA (0.5  $\mu\text{M}$ ), and lane 6 = cells treated with DOXIL (200 nM) plus WFA (1.5  $\mu\text{M}$ ).



**Figure 8.**

Western blot analysis of A2780 cells after treatment with DOXIL and WFA both alone and in combination. Cells were collected after treatment and lysed using lysis buffer. Forty  $\mu\text{g}$  of protein from each sample was used for SDS-PAGE. Notch1-specific antibody was used for the detection of Notch1 protein. The data shown is representative of at least two independent experiments.  $\beta$ -Actin-specific monoclonal antibody conjugated to horseradish peroxidase was used as internal control. Lane 1 = control untreated cells, lane 2 = cells treated with WFA (0.5  $\mu\text{M}$ ), lane 3 = cells treated with WFA (1.5  $\mu\text{M}$ ), lane 4 = cells treated with WFA (3  $\mu\text{M}$ ), lane 5 = cells treated with DOXIL (200 nM), lane 6 = cells treated with DOXIL (200 nM) plus WFA (0.5  $\mu\text{M}$ ), and lane 7 = cells treated with DOXIL (200 nM) plus WFA (1.5  $\mu\text{M}$ ).