RESEARCH ARTICLE

Combined gossypol and zoledronic acid treatment results in synergistic induction of cell death and regulates angiogenic molecules in ovarian cancer cells

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ABSTRACT. In the present study, we aimed to evaluate the possible synergistic, cytotoxic effects of combination treatment of gossypol and zoledronic acid, in human ovarian cancer cell lines, OVCAR-3 and MDAH-2774, and to elucidate the role of this novel combination treatment on angiogenesis-related molecules in ovarian cancer. The XTT cell viability assay was used for showing cytotoxicity. Both DNA fragmentation by ELISA assay and caspase 3/7 activity measurement were used for demonstrating apoptosis. To elucidate the angiogenic molecules affected by combination treatment, mRNA levels of angiogenic molecules were measured using the Human Angiogenesis RT^2 Profiler^{TM} PCR Array (SuperArray, Frederick, MD) in ovarian cancer cell lines, OVCAR-3 and MDAH-2774.The combined treatment resulted in significant, synergistic cytotoxicity, and induced apoptosis. This effect was observed to happen in a dose- and time-dependent manner. Moreover, the combination treatment of 10 μM gossypol and 5 μM zoledronic acid resulted in significant down-regulation (≥ thee-fold) in mRNA levels of some pivotal angiogenic molecules in OVCAR-3 and MDAH-2774 cells as compared to the untreated control. However, this effect was different in the two ovarian cancer cell lines observed. Gossypol, in combination with zoledronic acid, may provide a rational treatment option for ovarian cancer, not only by direct inhibition of cell proliferation, but also inhibition of angiogenesis-related molecules.

Keywords: gossypol, zoledronic acid, OVCAR-3, MDAH-2774, angiogenic molecules

Ovarian cancer is the most fatal gynecologic malignancy among female cancers [1]. Standard chemotherapy for previously untreated patients with advanced ovarian cancer consists of the combination of a taxane and a platinum compound. However, the disease becomes chemorefractory within approximately two years, and second-line treatment options do not provide significant survival advantage [2]. Thus, new treatment options are needed in this area. Like many other solid tumors, angiogenesis is a crucial step for the development of ovarian cancer, and new treatment strategies are now focused on blocking this step at different stages [3, 4].

Gossypol is a yellowish compound extracted from the cotton plant (*Gossypium* species) and the tropical tree *Thespesia populnea*. In the late 1960's, following several studies that had been undertaken on gossypol's antifertility action in Chinese men, it attracted the attention of many investigators trying to elucidate the mechanisms

underlying this property [5]. However, more importantly, gossypol had been shown to inhibit cellular growth and proliferation in a number of human tumor cells. Tuszynski and Cossu were the first to demonstrate that gossypol had anti-cancer effects against several tumor cell lines, the most sensitive of which being melanoma and colon carcinoma cells [6]. The anti-proliferative effect of gossypol and its optical isomers on various human cell lines of reproductive and nonreproductive tissue origin was also studied. Although the mechanism for the cytotoxic effect of gossypol on cancer cells has been widely studied, there are limited data for the angiogenic properties of gossypol [7-9]. We have previously shown that gossypol potently inhibits some of the angiogenic cytokines released from hormone- and drug-refractory prostate cancer cell lines at clinically achievable doses [10]. Zoledronic acid is the most potent member of the new generation, nitrogen-containing bisphosphonate family.

In addition to preventing bone loss, there is a substantial amount of preclinical and early clinical evidence showing that zoledronic acid has potent anti-tumoral properties. These promising findings have led to several ongoing studies that should ascertain the benefit of combining zoledronic acid with chemotherapy regimens [11-15]. The latest trials investigating the efficacy of zoledronic acid directly on cancer cells have demonstrated that zoledronic acid also has diverse anti-tumor effects via multiple mechanisms, including the inhibition of angiogenesis [16-18]. Moreover, there is a strong body of *in vitro* evidence that the bisphosphonates, particularly zoledronic acid, may be used as the enhancers of the anti-tumor effect of a number of cytotoxic agents used for cancer treatment [15, 17].

Although gossypol has been shown to have anti-tumor activity in ovarian cancer cells, with a negligible toxicity profile, to the best of our knowledge, there are no *in vitro* or *in vivo* studies investigating the effect of treatment with a combination of zoledronic acid and gossypol in ovarian carcinoma cell lines, OVCAR-3 and MDAH-2774, particularly investigating antiangiogenic potency [19]. Since both agents have potent anti-tumoral effects, with an acceptable side-effect profile as compared to conventional cytotoxic treatments, we investigated whether zoledronic acid, in combination with gossypol, might be a treatment option particularly for those elderly patients who are not eligible for standard treatments.

METHODS AND MATERIALS

Cell lines and reagents

Human OVCAR-3 and MDAH-2774 ovarian cancer cells were obtained from ICLC (Genova, Italy). The cells were grown as monolayers in adherent cell lines, and were routinely cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine, 1% penicillin-streptomycin in 75 cm^2 polystyrene flasks (Corning Life Sciences, UK), and maintained at 37°C in a humidified atmosphere with 5% CO_2. Growth and morphology were monitored, and cells were passaged when they had reached 90% confluence. Cell culture supplies were obtained from Biological Industries (Kibbutz Beit Haemek, Israel). Zoledronic acid was a generous gift from Novartis Pharmaceuticals Inc. (Basel, Switzerland). Gossypol (98% > purity) was obtained from Sigma Chemical Co., (USA). The stock solution of zoledronic acid (10 mM) was prepared in distilled water and gossypol was prepared in DMSO (10 mM). The final dilutions were made immediately before use, and new stock solutions were made for each experiment. The DMSO concentration in the assay did not exceed 0.1% and was not cytotoxic to the tumor cells. All other chemicals, unless mentioned, were purchased from Sigma.

XTT viability assay

After verifying cell viability using the trypan blue dye exclusion test and the Cellometer automatic cell counter (Nexcelom Inc.,USA.), cells were seeded at approximately 1x10^4/ well in a final volume of 200 μL in 96-well, flat-bottomed microtiter plates with or without various concentrations of drugs. Plates were incubated at 37°C in a 5% CO_2 atmosphere incubator for the indicated time periods. At the end of incubation, 100 μl of XTT (2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide) (Roche Applied Science, Mannheim, Germany) were added to each well, and plates were incubated at 37°C for another four hours. Absorbance was measured at 450 nm against a reference wavelength at 650 nm using a microplate reader (Beckman Coulter, DTX 880 Multimode Reader). The mean of triplicate experiments for each dose was used to calculate the IC_{50} and the combination index (CI) values.

Evaluation of drug interaction effect

The median dose-effect analysis by Chou and Talalay was used to assess the interaction between agents [20]. Determination of the synergistic versus additive versus antagonistic cytotoxic effects of the combined treatment of cells with gossypol and zoledronic acid was assessed by Biosoft CalcuSyn program (Ferguson, MO, USA). The CI was used to express synergism (CI < 1), additive effect (CI = 1), or antagonism (CI > 1).

Evaluation of apoptosis

Apoptosis was evaluated by enzyme-linked immunosorbent assay (ELISA) using the Cell Death Detection ELISA Plus Kit (Roche Applied Science, Mannheim, Germany), in accordance with the instruction manual. The relative amounts of mono- and oligonucleosomes generated from the apoptotic cells were quantified using monoclonal antibodies directed against DNA and histones and an ELISA. Briefly, cytoplasmic fractions of the untreated control, and gossypol and/or zoledronic acid and the combination of both treated cell lines were transferred onto a streptavidin-coated plate and incubated for two hours at room temperature with a mixture of peroxidase-conjugated, anti-DNA and biotin-labeled anti-histone. The plate was washed thoroughly, incubated with 2,29-azino-di-[3-ethylbenzthiazoline sulfonate] diammonium salt (ABTS), and absorbance measured at 405 nm with a reference wavelength at 490 nm (Beckman Coulter, DTX 880 Multimode Reader).

Measuring caspase 3/7 enzyme activity

Detection of apoptosis was verified by measuring caspase 3/7 enzyme activity. Briefly, OVCAR-3 and MDAH-2774 cells, at a concentration of 10^4 cells/well, were plated onto a 96-well plate in 100 μL of culture medium in the presence or absence of gossypol and zoledronic acid or a combination of both, for the desired period of time. Then, 100 μL of Caspase-Glo 3/7 (Promega, Madison, WI, USA) reagent was added to each well and the plates were incubated at room temperature for one more hour. Finally, the luminescence of each sample was measured with a luminometer (Beckman Coulter, DTX 880 Multimode Reader). All experiments were repeated in triplicate.

Cell treatment and RNA isolation

OVCAR-3 cells were treated with 5 μM of zoledronic acid, or 10 μM of gossypol alone, or the combination of both, whereas MDAH 2774 cells were treated with 2.5 μM of zoledronic acid or 5 μM of gossypol in the same way for 72 h. Total RNA from each sample was then isolated by TridityG (Applichem), followed by chloroform, according to the manufacturer's instructions. Samples were vigorously shaken for 15-20 seconds and were incubated for 15 minutes at room temperature to allow separation of the aqueous layer with isopropanol, followed by a final wash in 75% ethanol. RNA pellets were air-dried and resuspended in RNase-free water. The RNA yield was determined spectrophotometrically by measuring the optical density at 260 nm, and quality was determined by running samples on a 2% agarose gel and inspecting for distinct 18S, 28S and tRNA bands, indicating lack of degradation. Samples were frozen at - 80°C until use in cDNA synthesis.

$RT^2 Profiler^{TM}$ PCR array human angiogenesis first strand cDNA synthesis

Five μg of total RNA was reverse transcribed in a final reaction mix of 20 μL using RT^2 First Strand Kit (SuperArray Bioscience) according to the manufacturer's instructions. cDNA was diluted by adding RNase-free water. The PCR was carried out using the Light Cycler 480 (Roche Applied Science, Mannheim, Germany). For one 96-well-plate of the PCR array, 2 550 μL of PCR master mix containing 2 x SuperArray RT^2 qPCR Master Mix and 102 μL of diluted cDNA was prepared, and aliquots of 25 μL were added to each well. Universal cycling conditions (10 min at 95°C, 15 s at 95°C, 1 min 60°C for 40 cycles) were used.

Data normalization and analysis

Five endogenous control genes; beta-2-microglobulin (B2M), hypoxanthine phosphoribosyltransferase (HPRT1), ribosomal protein L13a (RPL13A), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and β-actin (ACTB) present on the PCR array were used for normalization. Each replicate cycle threshold (C_T) was normalized to the average C_T of five endogenous controls on a per plate basis. The comparative C_T method was used to calculate the relative quantification of gene expression. The following formula was used to calculate the relative amount of the transcripts in the drug-treated samples and the control group, both of which were normalized to the endogenous controls: $\Delta\Delta C_T = \Delta C_T$ (drugs treated) $- \Delta C_T$ (control) for RNA samples.

ΔC_T is the $\log^2$ difference in C_T between the target gene and endogenous controls by subtracting the average C_T of controls from each replicate. The fold-change for each treated sample relative to the control sample $= 2^{-\Delta\Delta CT}$.

Sensitivity detection and identification expressed genes

PCR array quantification was based on the C_T number. C_T was defined as 35 for the ΔC_T calculation when the signal was below detectable limits. A list of differentially expressed genes was identified using a 2-tailed, Student's *t*-test. Changes in gene expression between drug-treated cells and untreated controls were illustrated as a fold-increase/decrease. The criteria were: a p value less than 0.05 and a mean difference equal to or greater than a three-fold change in expression levels after treatment. The statistical calculation was based on the web-based program of $RT^2 Profiler^{TM}$ PCR Array Data Analysis. Alterations in mRNA levels that fitted the criteria above were considered to be up- or down-regulated. The experiments were repeated three times.

Statistical analysis

All experiments were conducted in triplicate and the results expressed as the mean $\pm$ SD, with differences assessed statistically; p values were determined by Student's t-test.

RESULTS

OVCAR-3 cells are more resistant to the effects of gossypol and zoledronic acid as compared to MDAH 2774 cells

To evaluate the effects of gossypol and zoledronic acid on the growth of human ovarian cancer cells, OVCAR-3 and MDAH 2774 cells were exposed to increasing concentrations of gossypol (from 5- to 40 μM) and zoledronic acid (from 2.5- to 40 μM), for 24, 48 and 72 h. Both gossypol and zoledronic acid inhibited cell proliferation in cells in a time- and dose- dependent manner (data not shown). The highest cytotoxicity for each agent was observed at 72 h. As shown in *figure 1A*, there were decreases in cell proliferation of 20%, 48%, and 68% with 5, 15, and 40 μM of gossypol applied to OVCAR-3 cells when compared to untreated controls at 72 h. However, there were decreases in cell proliferation of 35%, 61% and 75% with 5, 10, and 20 μM of gossypol applied to MDAH 2774 cells, as compared to untreated controls at 72 h *(figure 1B)*. The IC_{50} values for gossypol in OVCAR-3 and MDAH 2774 cells were calculated from cell proliferation plots and were found to be 18 μM and 7.5 μM, respectively.

We conducted the same set of experiments with zoledronic acid and our results showed that in OVCAR-3 cells exposed to 10, 20, and 40 μM of zoledronic acid, there were decreases in cell proliferation of 28, 65 and 82%, as compared to untreated controls. The IC50 value for zoledronic acid was 14 μM in OVCAR-3 cells *(figure 2A)*. As shown in *figure 2B*, when MDAH 2774 cells were exposed to 2.5, 10 and 20 μM of zoledronic acid, there was a decrease in cell proliferation of 25%, 48% and 70% respectively. The IC_{50} value for zoledronic acid was 12 μM in MDAH 2774 cells. Trypan blue dye exclusion tests revealed similar results using either agent in OVCAR-3 and MDAH 2774 cells.

These results showed that OVCAR-3 cells are more resistant to the cytotoxic effects of both gossypol and zoledronic acid in comparison to MDAH-2774.

Exposure to a combination therapy of gossypol and zoledronic acid resulted in strong synergistic cytotoxicity as compared to either agent alone in human OVCAR-3 and MDAH 2774 ovarian cancer cells

To study the possible synergistic/additive effects a combination of gossypol and zoledronic acid, OVCAR-3 and MDAH- 2774 cells were exposed to different concentra-
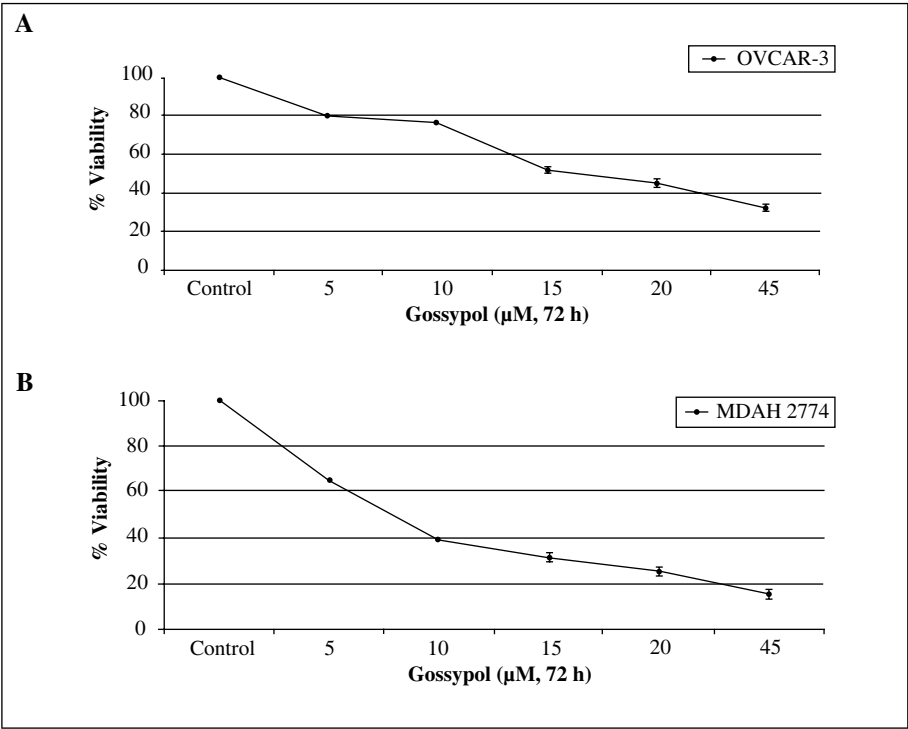
Figure 1
Effect of gossypol on proliferation of OVCAR-3 (**A**) and MDAH-2774 cells after 72 h in culture (**B**). The data represent the mean of three different experiments ($p < 0.05$). The error bars represent the standard deviations, and when not seen, they are smaller than the thickness of the lines on the graphs.
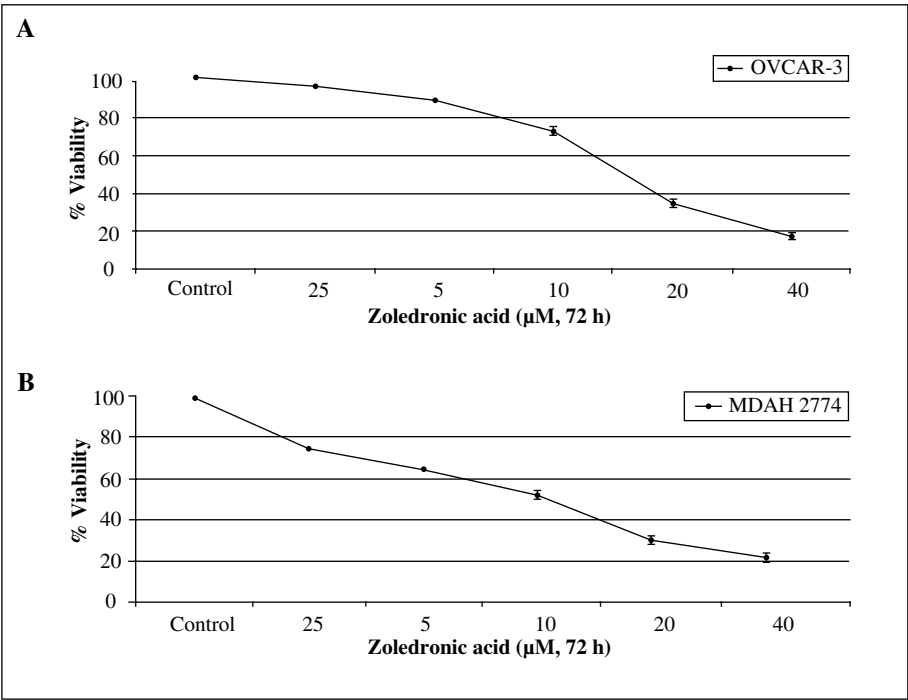

Figure 2
Effect of zoledronic acid on proliferation of OVCAR-3 (**A**) and MDAH-2774 cells after 72 h in culture (**B**). The data represent the mean of three different experiments ($p < 0.05$). The error bars represent the standard deviations, and when not seen, they are smaller than the thickness of the lines on the graphs.

tions of gossypol or zoledronic acid alone, or different combinations of both for 72 hours. The synergism or additivity was calculated *via* the combination index (CI) using Biosoft Calcusyn Program® where CI = 1 indicates an additive effect, CI > 1 indicates antagonism, CI < 1 indicates synergism and CI < 0.5 shows strong synergy. The combination of different concentrations of gossypol and zoledronic acid were evaluated at different time points (data not shown).

Results showed significant synergistic toxicity in OVCAR-3 cells, at 72 h, as compared to any agent alone as shown in *table 1*. Our results indicate that 10 μM of gossypol and 5 μM of zoledronic acid show a 24% and 12% decrease in cell proliferation respectively in OVCAR-3 cells, but the combination of both resulted in a 79% decrease in cell proliferation *(figure 3A, B)*.

As shown in *figure 3*, the results also revealed that while 5 μM of gossypol and 2.5 μM of zoledronic acid resulted in 35% and 25% decreases in proliferation of MDAH 2774 cells respectively, the combination of both drugs

Table 1
Combination index (CI) values of gossypol/zoledronic acid in OVCAR-3 and MDAH-2774 cells

OVCAR-3		
Concentration of drugs	**CI value**	**Interpretation**
GP (10 μM) + ZA (5 μM)	0.252	Strong synergism
GP (15 μM) + ZA (5 μM)	0.286	Strong synergism
MDAH-2774		
Concentration of drugs	**CI value**	**Interpretation**
GP (5 μM) + ZA (2.5 μM)	0.152	Strong synergism
GP (5 μM) + ZA (5 μM)	0.186	Strong synergism

Combination index values of gossypol or zoledronic acid alone and their combination, for growth inhibition of OVCAR-3 and MDAH-2774 cells. Combination index (CI) values were calculated from the XTT cell proliferation assays, according to CalcuSyn® software. The CI was used to express synergism (CI < 1), additive effect (CI = 1), or antagonism (CI > 1), where CI < 0.5 represents strong synergism.

Table 2
Fold-change in angiogenesis-related genes in OVCAR-3 after drug exposure

Fold-change in OVCAR-3 cells			
Gene symbol	Gossypol	Zoledronic acid	Combination
CXCL-1	- 2.31	- 1.36	- 7.36
TP	- 1.82	+ 5.13	- 18,4
EPHB4	- 1.79	+ 1.62	- 32,9
FGF2	- 5.29	+ 2.16	- 17,0
FGFR3	- 3.90	+ 1.57	- 18,1
ID1	- 4.35	+ 1.34	- 26,5
LAMA5	- 1.71	+ 2.25	- 10,1
MDK	- 3.44	- 1.00	- 13,1
PDGFA	+ 1.75	+ 2.26	- 5,4
VEGF	- 1.11	+ 1.16	- 6,1
Fold-change in MDAH-2774 cells			
Gene symbol	Gossypol	Zoledronic acid	Combination
CCL2	- 3.82	- 1.30	- 6,7
CXCL1	- 2.32	- 1.28	- 3,3
TP	- 1.82	+ 1.88	- 3,1

Changes in levels of mRNA of angiogenesis-related genes in OVCAR-3 and MDAH-2774 cells exposed to a combination or mono-treatment with gossypol-zoledronic acid. Change ≥ 3-fold was accepted as significant according to a web-based program of RT²Profiler™ PCR Array Data Analysis.
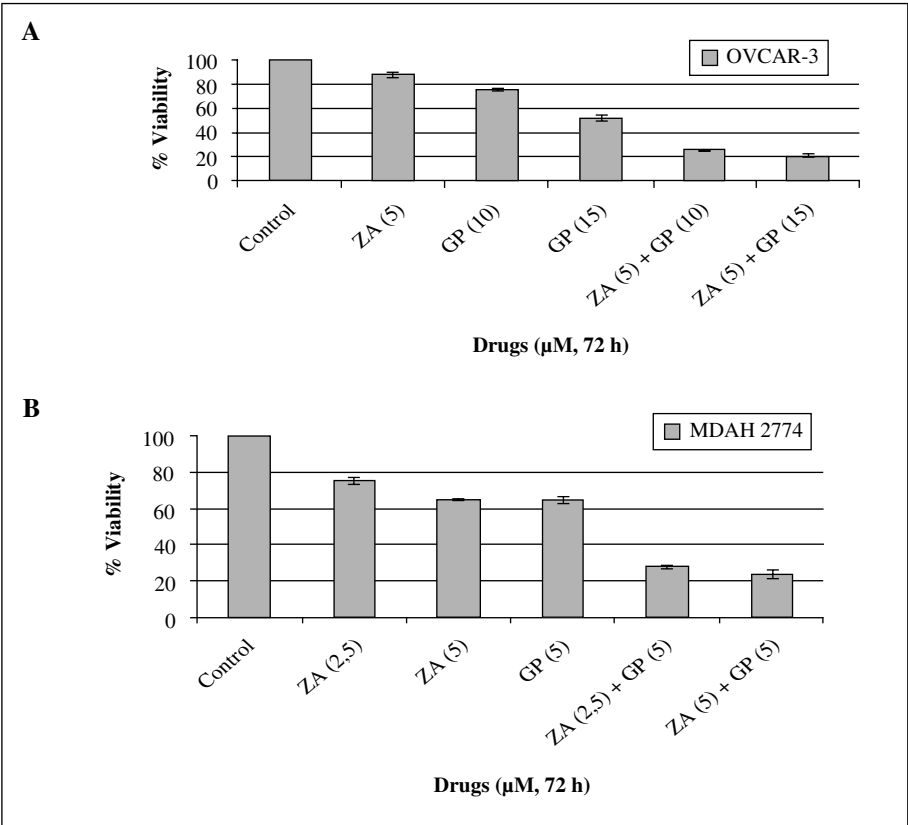
at the same doses caused 76% decrease in cell proliferation as compared to untreated controls, indicating strong synergy. Concentrations of gossypol and zoledronic acid were chosen at clinically achievable concentrations and below the IC_{50} values as clearly shown in *figure 3A, B*.

Effect of sequential treatment

The previous findings demonstrated significant synergy at 72 h in tumor cells treated with a combination of gossypol and zoledronic acid. We examined the effect of sequential treatment with either gossypol or zoledronic acid and subsequent treatment with the second agent in both cell lines. Pretreatment of tumor cells with gossypol for 36 h, washing, and then treatment for an additional 36 h with zoledronic acid resulted in synergistic cytotoxicity in both OVCAR-3 and MDAH 2774 cells. Also, pretreatment of tumor cells with zoledronic acid for 36 h, washing and then treatment for an additional 36 h with gossypol resulted in synergistic cytotoxicity in both OVCAR-3 and MDAH 2774 cells (data not shown). Thus, significant synergistic cytotoxicity of the drugs was observed regardless of which agent was applied first.

Combination of gossypol and zoledronic acid induced DNA fragmentation significantly as compared to any agent alone in OVCAR-3 and MDAH 2774 ovarian cancer cells

To examine the possible synergistic effects of the combination of gossypol and zoledronic acid, as compared to either agent alone, on induction of DNA fragmentation as a marker of cell death, we quantified the levels of mono-oligonucleosome fragments using a Cell Death Detection Plus Elisa Kit (Roche Applied Science, Mannheim, Germany). We treated OVCAR-3 and MDAH 2774 cells with different concentrations of gossypol or zoledronic acid or the combination of both, for 72 hours before analyzing DNA fragmentation. The results showed that when OVCAR-3 cells were exposed to 10 μM of gossypol or 5 μM of zoledronic acid, there was a 2.8- and 1.7-fold increase observed in DNA fragmentation respectively, whereas the combination of both induced and 13.6-fold increase in DNA fragmentation, compared to untreated controls *(figure 4A)*. A similar set of experiment was also conducted with MDAH 2774 cells. Our results revealed that when MDAH 2774 cells were exposed to 5 μM of gossypol or 2.5 μM of zoledronic acid, there was a 3- and 1.9-fold increase observed in DNA fragmentation respectively, however the combination of both induced DNA fragmentation 11.6 times higher, as compared to untreated controls *(figure 4B)*. Thus, these results clearly indicate that the combination treatment induces apoptosis in a synergistic manner in both types of ovarian cancer cells.

A significant increase in caspase-3/7 enzyme activity was observed in response to the combination of gossypol and zoledronic acid as compared to either agent alone in human ovarian cancer cells

In order to better evaluate the possible synergistic, apoptosis-inducing effects of the combination of gossy-

Figure 3
Synergistic effects of gossypol and zoledronic acid on proliferation of OVCAR-3 (**A**) and MDAH-2774 cells after 72 h in culture (**B**).
The results are expressed as the mean of three different experiments (p < 0.05). The doses used for the synergistic effect were below each agents' IC_{50} doses.
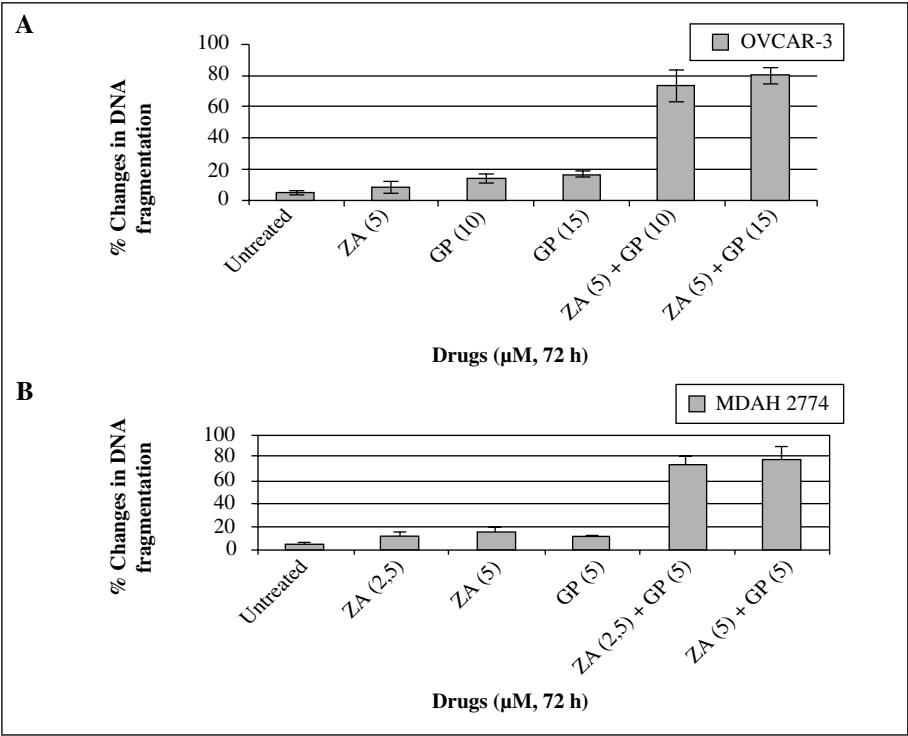

Figure 4
Apoptotic effects of gossypol or zoledronic acid alone or in combination in OVCAR-3 (**A**) and in MDAH-2774 (**B**) cells through DNA fragmentation analyses. The results are the mean of three independent experiments (p < 0.05). The error bars represent the standard deviations, and when not seen, they are smaller than the thickness of the lines on the graphs.

pol and zoledronic acid, as compared to either agent alone in human OVCAR-3 and MDAH 2774 cells, we performed a caspase 3/7 enzyme activity assay using Caspase-Glo 3/7 Assay (Promega, Madison, WI, USA). To that aim, OVCAR-3 and MDAH 2774 cells were exposed to various concentrations of gossypol or zoledronic acid alone, and in combination for 72h *(figure 5A, B)*. In parallel with the DNA fragmentation analyses, our results revealed that there was a dose-dependent increase in caspase 3/7 activation both in gossypol- and zoledronic acid-exposed OVCAR-3 and MDAH 2774 cells. As an example, in OVCAR-3 cells exposed to 10 µM of gossypol or 5 µM of zoledronic acid, there were 2- and 1.7-fold increases in caspase 3/7 enzyme activity respectively, while a combination of both resulted in a 10.6-fold increase in caspase 3/7 enzyme activity *(figure 5A)* as compared to untreated controls. Thus, we have clearly demonstrated the synergistic, apoptosis-inducing effect of combined gossypol and zoledronic acid treatment in both cell lines.

The exposure of the combination of gossypol and zoledronic acid in OVCAR-3 and MDAH-2774 cells results in significant down-regulation of mRNA levels of angiogenesis-related gene expression

The data for the changes in mRNA levels for angiogenesis-related genes after treatment with either gossypol or zoledronic acid alone or combination in OVCAR-3 and MDAH-2774 cells are shown as -fold changes in *table 2*. Changes in mRNA levels were accepted as significant if there was at least a 3-fold change in expression when compared with untreated control. The combination treatment of OVCAR-3 cells with gossypol (10 µM) and zoledronic acid (5 µM) resulted in significant downregulation of mRNA levels of some important angiogenic molecules. However, the combined treatment affected mRNA levels of angiogenesis-related genes in MDAH-2774 cells in a different manner. Treatment with gossypol (5 µM) and zoledronic acid (2.5 µM) had only affected a limited number of angiogenesis-related molecules in MDAH-2774 cells when compared to the OVCAR-3 cells. Among the angiogenic molecules affected, it was the levels of mRNA of 'CXCL-1 (Gro-α), EPHB4, ID-1, FGF2, FGFR3, TP, LAMA5, VEGF, PDGFA, MDK' and 'CXCL-1 (Gro-α), CCL-2 (MCP-1), TP' that were significantly downregulated in OVCAR-3 and MDAH-2774 cells, by the combined treatment, compared to either agent alone ($p < 0.05$). These molecules are considered to be the pivotal mediators of angiogenesis in cancer cells [21-29].

DISCUSSION

The data presented here provides evidence that the treatment of the ovarian carcinoma cell lines, OVCAR-3 and MDAH-2774, with gossypol and zoledronic acid combined, results in significant, synergistic cytotoxic activity and apoptosis. This effect was observed in a dose- and time-dependent manner. However, due to the difference in their genetic properties, OVCAR-3 and MDAH-2774 cells responded differently to the effects of the combined treatment, suggesting that OVCAR-3 cells were much more resistant [30, 31]. Further developments in combining different anti-cancer agents that can induce or enhance apoptosis seem to be a promising strategy in the treatment of cancer. Zoledronic acid has been one of
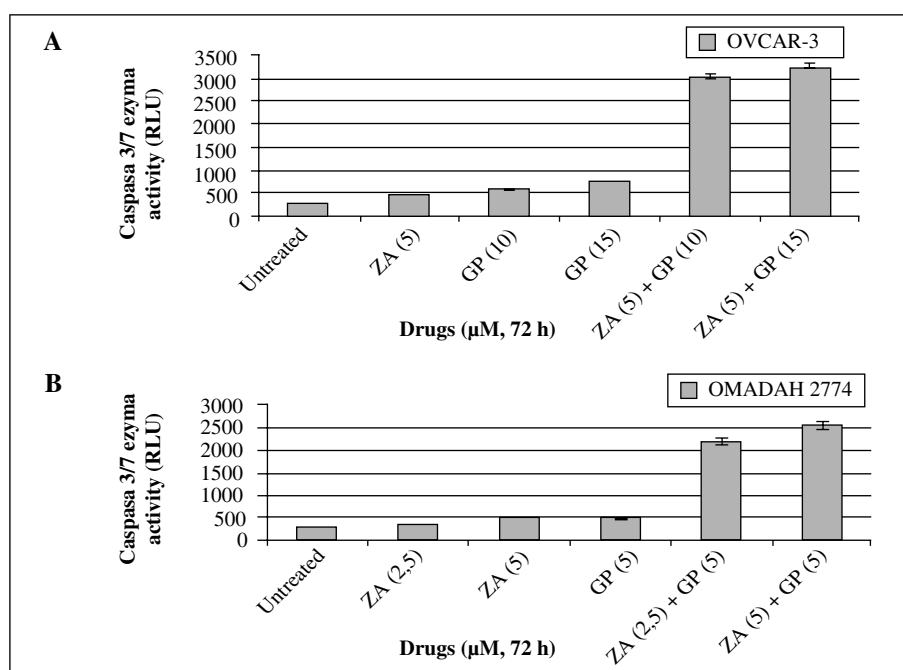

Figure 5
Relative luminescence unit (RLU) changes in caspase 3/7 enzyme activity for OVCAR-3 (**A**) and MDAH-2774 cells exposed to the gossypol and zoledronic acid combination, or either agent alone (**B**). The synergistic effect on induction of apoptosis may be seen in the graphs. $p < 0.05$ was considered significant. The error bars represent the standard deviations, and when not seen, they are smaller than the thickness of the lines on the graphs.

the most widely studied agents for the enhancement of the anti-tumoral potency of cytotoxic agents in recent years, and has shown potent activity in some cancers, even in the adjuvant setting [14, 16]. Thus, combining zoledronic acid with gossypol, a combination that has shown significant, synergistic, anti-tumoral activity against ovarian cancer cells might provide a new treatment strategy for ovarian cancer.

While searching for the underlying mechanism of the synergism of gossypol and zoledronic acid, we looked at their effect on the angiogenic molecules released from ovarian cancer cells. We found, using the PCR array method, that the combined treatment significantly downregulated mRNA levels of many pivotal, angiogenic molecules that play a crucial role in angiogenesis.

It is of interest to note that the two different cell cultures responded differently in terms of the regulation of angiogenesis-related molecules, very probably because of the differences in their genetic profiles [30, 31]. While mRNA levels of several important angiogenic molecules in OVCAR-3 cells were significantly down-regulated, this effect was markedly less in MDAH-2774 cells, showing little or no down-regulation of many of the molecules that had been affected by the combined treatment in OVCAR-3 cells.

One of the angiogenesis related genes that was down regulated in OVCAR-3 cells is inhibitor of differentiation or DNA binding (ID-1). It has been suggested that this is one of the upstream regulators of the EGFR signaling pathway, based on the fact that the down regulation of ID-1 led to the down regulation of EGF at both transcriptional and protein levels. Upregulation of ID-1 is frequently found in many types of human cancer and increased ID-1 expression levels are associated with advanced tumor stage and poor prognosis in ovarian cancer patients, indicating that ID-1 may be a target for cancer treatment [32]. In our study, the combined treatment resulted in a 26.5-fold down-regulation of mRNA levels of ID-1, as compared to untreated controls.

EPHB2/B4 has been reported to be a biomarker with negative prognostic value in ovarian carcinoma [33, 34]. Thus, downregulation of the EPBH4 gene by 32.9-fold in OVCAR-3 cells, with the combined treatment, is remarkable. Another gene that is significantly downregulated in OVCAR-3 cells is Laminin alpha-5 (LAMA-5); there was a 10.1-fold downregulation of mRNA levels following combined treatment. Binding to cells via a high affinity receptor, LAMA-5 is thought to mediate the attachment, migration and organization of cells into tissues during embryonic development by interacting with other extracellular matrix components. In many type of cancer, LAMA-5 has been observed to be highly expressed, and located at the invasive edge of the tumor, showing that this also is a pivotal molecule for targeting angiogenesis in ovarian cancer [35].

Heparin-binding activity growth factors show potent angiogenic properties and highly increased expression of these growth factors is associated with a broad spectrum of mitogenic and angiogenic activities in a number of malignant tumors, including ovarian cancer [21]. Basic fibroblastic growth factor (bFGF-2) and FGF receptor-3 (FGFR-3) are both members of this gene family, along with midkine (MDK) [36-38]. In the present study, by the combined treatment of gossypol/zoledronic acid, certain members of heparin-binding activity growth factor family, FGF2, FGFR3 and MDK are down-regulated significantly by 17-, 18.1- and 13.1-fold, respectively, as compared to untreated controls.

Platelet-derived growth factors (PDGF) are a family of disulfide-bonded dimers of structurally similar polypeptide chains. Overactivity of PDGF or PDGF receptors contributes to the development of cancer characterized by excessive cell growth, and enhanced angiogenesis [21]. In addition, thymidine phosphorylase (TP), an enzyme involved in pyrimidine metabolism, is identical to platelet-derived endothelial cell growth factor (PD-ECGF). TP is overexpressed in various tumors and plays an important role in angiogenesis, tumor growth, invasion and metastasis [22, 39]. Moreover, when TP expression is superimposed upon VEGF expression, the tumor might acquire the aggressive tumor phenotype. So, there is a strong link between the angiogenic potency of tumors and the PDGF family. In our study, following the combined treatment, TP is downregulated by 18.4-fold and 3.1-fold, in OVCAR-3 and MDAH-2774, respectively, as compared to untreated controls, whereas PDGF-A was only downregulated in OVCAR-3 cells. Data in the present study indicate that downregulation of the PDGF family of growth factors, which are strategic targets in ovarian cancer cells, might be an important route for shutting down angiogenic pathways.

Another angiogenic factor that was significantly down-regulated by the combined treatment, in both cell lines, is CXCL-1 (identical with growth-related oncogene [GRO]-α). The present data show that in both OVCAR-3 and MDAH-2774 cell lines, the mRNA levels of the gossypol/zoledronic acid-treated cells declined by 7.36-fold and 3.3-fold respectively. CXC type cytokines are present in every kind of cell, and are associated with angiogenesis. GRO-α is a member CXC-type chemokine family and is one of the main targets of anti-angiogenic treatment for ovarian cancer [23, 40, 41].

Of all the molecules that were downregulated by the gossypol/zoledronic acid combination, vascular endothelial growth factor (VEGF) is of unique importance in angiogenesis. Overexpression of VEGF mRNA in the human ovarian cancer cell line OVCAR-3 has been demonstrated previously. In addition, high VEGF expression and microvessel density have been correlated with poor, disease-free survival and overall survival in patients with early or advanced stage ovarian cancer [27, 42].

VEGF is also reported to be a chemotactic agent for monocytes. It is possible to speculate that high VEGF overexpression by tumor cells leads directly to an increased recruitment of these cells from the peripheral circulation [42, 43]. So, it is also remarkable that macrophage chemoattractant protein (MCP)-1, which can be induced in endothelial cells with VEGF treatment, is down-regulated in MDAH- 2774 cells by the combined treatment.

In conclusion, adding zoledronic acid to gossypol has been shown to be synergistically cytotoxic and apoptotic in ovarian cancer cells, causing changes in the the expression of many genes that are critically involved in the control of

angiogenesis. These findings provide basic molecular information for further investigation of the mechanisms by which gossypol and zoledronic acid exert their pleiotropic effects on ovarian cancer cells. Nevertheless, it is clear that our findings show, for the first time, that combination treatment with gossypol and zoledronic acid demonstrated efficacy in ovarian cancer cells. However, our study has some limitations, as we cannot yet provide in vivo results for the combined treatment, which will certainly be very helpful in support of our preliminary data. Further, in-depth investigations should be performed in xenograft models to confirm the efficacy of this promising new treatment for ovarian cancer.

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