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ORIGINAL ARTICLE

Enhancement of taxane-induced cytotoxicity and apoptosis by gossypol in human breast cancer cell line MCF-7

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Summary

Purpose: *Gossypol is a natural polyphenolic compound extracted from cotton plant (Gossypium species) which has shown potent inhibitory effect on cell growth of many types of cancers. In this study, we aimed to evaluate the interaction of gossypol with some conventional drugs known to be effective in the treatment of breast cancer, like taxanes, doxorubicin, gemcitabine, cisplatin and vinorelbine, in MCF-7 breast cancer cells.*

Materials and methods: *The XTT viability assay was used to evaluate the cytotoxicity of various cytotoxic agents alone and in combination with gossypol in MCF-7 breast cancer cells. The combination effect analysis of Chou and Talalay was used to identify the most synergistic drug combinations. The possible synergistic effects of the combination of drugs on apoptosis were also evaluated by using two different apoptosis assays.*

Results: *We identified strong synergistic cytotoxic and apoptotic activity of gossypol with taxanes among all other studied cytotoxic drugs.*

Conclusion: *This study provides proof that gossypol combined with taxanes may have potential as a novel future treatment for breast cancer.*

Key words: apoptosis, gossypol, MCF-7, synergy, taxanes

Introduction

Breast cancer is the most prevalent malignancy among women in the western world, representing the second cause of cancer-related deaths [1]. Despite major developments in early detection and treatment of breast cancer in recent years, more than one third of women with breast cancer are diagnosed in advanced stage [2]. New chemotherapeutic agents, as well as targeted treatments are widely used for treating this disease. However, like many other types of solid tumors, breast cancer initially responds well to treatment, but eventually cancer cells become resistant to these treatments [3]. One of the most common gateways to overcome the drug resistance problem in cancer is to add a second drug to the cytotoxic agents.

The estrogen receptor (ER) positive MCF-7 cell line has been studied widely as an ideal model for breast cancer cells [4]. In addition to providing basic knowledge for ER (+) breast cancer, specific key pro-and antiapoptotic regulators in signal transduction pathways that influence chemoresistance have recently been identified in MCF-7 breast cancer cells [5]. Thus, MCF-7 cell line is an ideal tool for the study of breast cancer resistance mechanisms to conventional chemotherapy.

Gossypol is a natural polyphenolic, lipid-soluble compound extracted from cotton plant (*Gossypium species*). Recently, it was identified as a toxin when used as an animal food and has long been recognized as an antispermatogenic agent in rural areas of China with negligible toxicity profile. *In vitro* studies of gossypol have been performed in tumor cells lines including breast, prostate, melanoma, glioma and adrenocortical carcinoma [6-8]. It disrupts a variety of cellular processes, including energy metabolism and other mitochondrial functions in tumor cells. It also interferes with DNA

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synthesis and repair, specifically via effects on DNA polymerase alpha and topoisomerase II [9]. Gossypol was also shown to be a potent inhibitor of Bcl-2/Bcl-X_L [10]. All these properties of gossypol, coupled with a relatively low cytotoxicity profile, suggest that this compound might be a good candidate for combination treatment with conventional cytotoxic agents in overcoming drug resistance in breast cancer treatment.

In this preclinical study, we evaluated the interaction of gossypol with taxanes (microtubule stabilizing agents), doxorubicin (topoisomerase II poison), gemcitabine (antimetabolite), cisplatin (DNA alkylating agent) and vinorelbine (semi-synthetic vinca alkaloid). We used the combination effect analysis of Chou and Talalay [11] to identify the most synergistic drug combinations and additionally we also evaluated the possible synergistic effects of the combined drugs on apoptosis by DNA fragmentation and acridine-orange assay.

Materials and methods

Cell lines and reagents

Human MCF-7 cancer cell line was 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).

Docetaxel, cisplatin and gossypol (>98% purity) were obtained from Sigma Chemical Co (USA). The stock solution of docetaxel (10 mM), cisplatin (10 mM) and gossypol (10 mM) were prepared in DMSO. The DMSO concentration in the assay did not exceed 0.1% and was not cytotoxic to the tumor cells. Paclitaxel (7 mM), gemcitabine (10 mM), vinorelbine (13 mM) and doxorubicin (3.5 mM) were obtained from commercial stocks. The final dilutions were made immediately before use, and new stock solutions were made for each experiment. All other chemicals, unless mentioned, were purchased from Sigma.

XTT viability assay

After verifying cell viability using trypan blue dye exclusion test by Cellometer automatic cell counter (Nexcelom Inc., USA), cells were seeded at approximately 1×10^4/ well in a final volume of 200 µl in 96-well flat-bottom microtiter plates. To evaluate the effect of each cytotoxic agent and gossypol alone on the growth of breast cancer cells, MCF-7 cells were exposed to increasing concentrations of each drug for 24, 48 and 72 h. Plates were incubated at 37° C in a 5% CO_2 incubator for the indicated time periods. At the end of incubation, 100 µl of XTT (Roche Applied Science, Mannheim, Germany) were added to each well, and plates were incubated at 37° C for another 4 h. Absorbance was measured at 450 nm against a reference wavelength at 650 nm using a microplate reader (DTX 880 Multimode Reader, Beckman Coulter). The mean of triplicate experiments for each dose was used to calculate the IC_{50} and the combination index (CI) values.

Detection of apoptosis

A. DNA fragmentation analysis

After the detection of synergistic cytotoxic activity of gossypol with taxanes, the possible synergistic effects of combination of gossypol with paclitaxel or docetaxel, as compared to any agent alone on inducing DNA fragmentation as a marker of cell death has been investigated. We quantified the levels of mono-oligonucleosome fragments using cell death detection plus ELISA kit (Roche Applied Science, Mannheim, Germany) according to the instruction manual. We treated MCF-7 cells in different concentrations of gossypol or paclitaxel or docetaxel and the combination of both for 72 h before analyzing DNA fragmentations. The relative amounts of mono- and oligonucleosomes generated from the apoptotic cells were quantified using monoclonal antibodies directed against DNA and histones by ELISA. Briefly, the cytoplasmic fraction of the cells after the appropriate time of drug(s) exposure and the combination-treated cells were transferred onto a streptavidin-coated plate and incubated for 2 h at room temperature with a mixture of peroxidase-conjugated anti-DNA and biotin-labeled antihistone. The plate was washed thoroughly and incubated with 2,29-azino-di-[3-ethylbenzthiazolinesulfonate] diammonium salt (ABTS); then, absorbance was measured at 405 nm with a reference wavelength at 490 nm (DTX 880 Multimode Reader, Beckman Coulter). Samples were measured in triplicate and a positive control was provided with the kit.

B. Acridine-orange assay

The frequency of apoptotic induction of MCF-7 cells following exposure to combination of taxanes with gossypol was determined by the use of a Live/Dead Viability-Cytotoxicity Kit (Molecular Probe Inc., OR). The medium was removed from each well and the cells rinsed 1X with D-

PBS. The working buffer, which was used to make the dilute dye, was D-PBS. Ethidium homodimer-1 (dead stain) was applied at a concentration of 10 M/ml and Calcein AM (live stain-esterase substrate) was applied at 12 M/ml in a 10 ml D-PBS working buffer. Once mixed, 150 µl of the diluted dye were added to each well of a 96-well plate. The plates were kept at room temperature for at least 1 h. Digital photographs were taken using fluorescent microscopy. Viable normal cells displayed bright green nuclei with intact structure; viable apoptotic cells were bright green with highly condensed or fragmented nuclei; non-viable normal cells had bright orange chromatin with organized structure; non-viable cells with apoptotic nuclei exhibited bright orange chromatin which was highly condensed or fragmented.

Assessment of drug interaction

Median dose effect analysis, a measure of synergism or antagonism, was determined by the method of Chou and Talalay, using their computer program (Biosoft CalcuSyn, Ferguson, MO, USA) to assess drug interaction. We chose this method because it takes into account both the potency of each drug or combination of drugs and the shape of dose-effect curve. CalcuSyn software which is based on this method was used to calculate the CI. Synergy, additivity and antagonism were defined as CI <1, CI =1, CI>1, respectively, where CI $\leq$ 0.5 characterizes strong synergy. For this analysis, concentrations of gossypol and other cytotoxic chemotherapeutic agents were chosen as clinically achievable concentrations and below the IC_{50} values. Each experiment provided triplicate data points for each concentration and was repeated at least 3 times. The resulting dose-response curves were averaged, creating a single composite dose-response curve for each combination. Fa is defined as the fraction of cells affected, and a plot of log dose vs. log (Fa/1- Fa) gives parallel slopes if no biologic interaction between drugs is present or converges if there is an interaction between drugs, thus suggesting the appropriate model to determine the CI.

Statistical analysis

Statistical analysis and p-values determinations were conducted by the Student's *t*- test. The data were analyzed using GraphPad PRISM software (version 5, CA, USA).

Results

Activities of agents alone

We first determined the chemosensitivity of MCF-7 cell line to each individual agent mentioned above. The experiments were done at 24, 48 and 72 h. The highest cytotoxicity was obtained at 72 h by using various agents. Table 1 illustrates the drug concentrations needed *in vitro* to cause 50% growth inhibition (IC_{50}) vs. untreated control in MCF-7 cells after 72 h of incubation by XTT viability assay. Gossypol demonstrated single-agent cytotoxicity at 10 µM concentration in MCF-7 cells, and this effect was also time-dependent with longer incubation times associated with greater cytotoxicity (Figure 1).

Effects of gossypol in combination with various cytotoxic chemotherapeutic agents on the growth of MCF-7 cell line

Concentrations of gossypol and other cytotoxic chemotherapeutic agents were chosen at clinically achievable concentrations and below the IC_{50} values. As an example, for the determination of synergistic cytotoxic effects, MCF-7 cells were exposed for 72 h to 10 µM of gossypol in combination with 5 µM paclitaxel, 1 nM docetaxel, 3 µM doxorubicin, 1.5 µM

Table 1. IC_{50} doses of each single cytotoxic agent including gossypol against MCF-7 cells *in vitro*

Drug	*IC_{50} value (µM) ± standard deviation*
Paclitaxel	19.6 ± 1.1
Docetaxel	0.008 ± 2.6
Doxorubicin	5.5 ± 0.4
Vinorelbine	29 ± 0.6
Cisplatin	39.6 ± 1.6
Gemcitabine	1.5 ± 0.6
Gossypol	10 ± 3.5

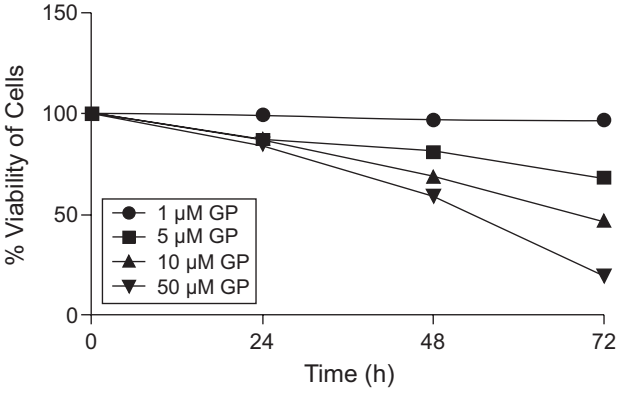

Figure 1. Antiproliferative effect of gossypol (GP) in MCF-7 cells at 24, 48 and 72 h. The figure shows that the antiproliferative effect is time- and dose-dependent.

gemcitabine, 20 μM cisplatin and 10 μM vinorelbine. Data for gossypol and paclitaxel combination are presented in Figure 2, including the composite dose-response curves, median effect, and combination index plots. The results indicate that the combination of 5 μM gossypol and 10 μM paclitaxel had strong synergistic effect with CI value of 0.409. In addition, among all the combinations studied in MCF-7 cancer cells, gossypol (5 μM) - docetaxel (1 nM) were also found to be strongly synergistic with CI value of 0.119 (Table 2). Other drug combinations (doxorubicin, gemcitabine, cisplatin, vinorelbine) with gossypol showed antagonistic effect with CI values as shown in Table 2. To demonstrate that this strong synergistic effect of gossypol together with paclitaxel and docetaxel in MCF-7 cells was not just growth inhibition but represented cell death, the combination of gossypol with taxanes was also evaluated by DNA fragmentation and acridine-orange methods.

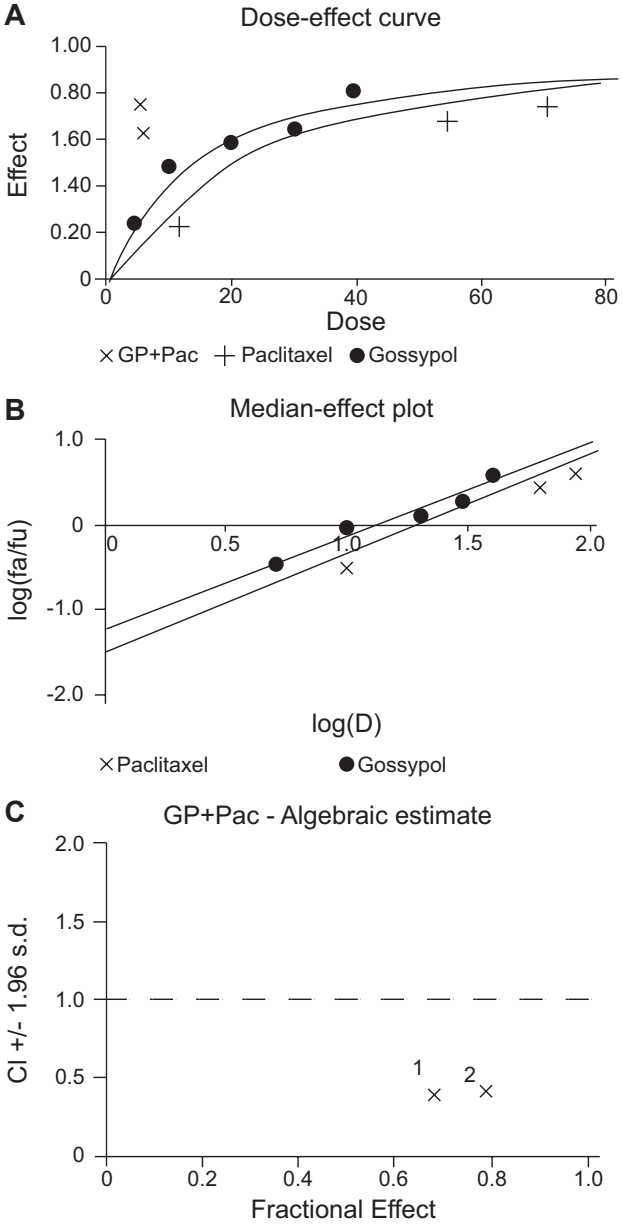

Figure 2. Interaction and synergy of gossypol with paclitaxel in MCF-7 cells. **A:** composite dose-response curves for antiproliferative activity of gossypol (●), paclitaxel (+), or the combination (×) of both were evaluated *in vitro* in MCF-7 cells as described in materials and methods. **B:** median plot for the interaction of gossypol + paclitaxel in **A. C:** combination index (CI) for the interaction as a function of level of effect. X1 and X2 represent CI values of two different drug combinations of gossypol and paclitaxel (fractional effect= 0.5 is the IC_{50}).

Effects of the sequential treatment

The previous findings demonstrated that tumor cells with gossypol along with paclitaxel or docetaxel resulted in significant synergy at 72 h. We examined the effect of sequential treatment of MCF-7 cells with gossypol or paclitaxel/ docetaxel and subsequent treatment with the second agent. Pretreatment of tumor cells with gossypol for 36 h and wash and then treatment for an additional 36 h with paclitaxel resulted in synergistic cytotoxicity in MCF-7 cells. Also, pretreatment of tumor cells with paclitaxel for 36 h and wash and then treatment for an additional 36 h with gossypol resulted in synergistic cytotoxicity in MCF-7 cells (data not shown). The same set of experiments was repeated for gossypol and docetaxel and results revealed that significant synergistic effect of the combination treatment was observed in both combinations, no matter which agent was applied first (data not shown).

Combination of gossypol with either paclitaxel or docetaxel-induced DNA fragmentation as compared to any agent alone in MCF-7 cells

The results showed that when MCF-7 cells were

Table 2. Median dose effect analysis determined by the method of Chou and Talalay which is used to calculate the combination index (CI) values. Synergy, additivity and antagonism are defined as CI <1, CI =1, CI>1, respectively. CI ≤ 0.5 characterizes strong synergy

Drug combinations with gossypol	*Combination index value ± standard deviation*
Paclitaxel	0.4 ± 0.1 (strong synergy)
Docetaxel	0.1 ± 0.2 (strong synergy)
Vinorelbine	1.3 ± 0.1 (antagonism)
Cisplatin	1.7 ± 0.1 (antagonism)
Gemcitabine	1.5 ± 0.3 (antagonism)
Doxorubicin	1.4 ± 0.2 (antagonism)

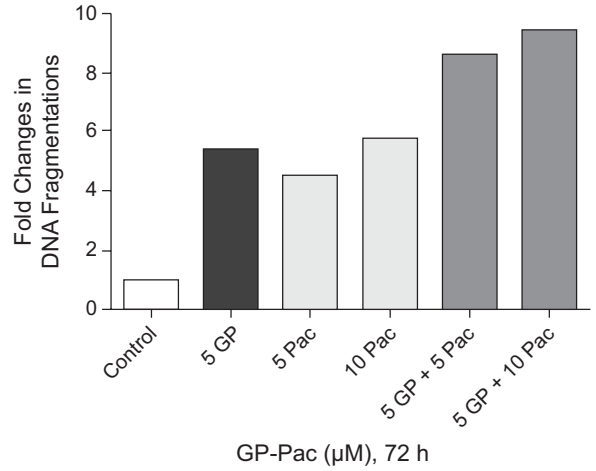

Figure 3. Apoptotic effects of gossypol (GP) and paclitaxel (Pac) alone or in combination in MCF-7 cells through DNA fragmentation analyses. The results are the means of 3 independent experiments. p=0.01 for 5 μM GP + 5 μM Pac, and p=0.03 for 5 μM GP + 10 μM Pac.

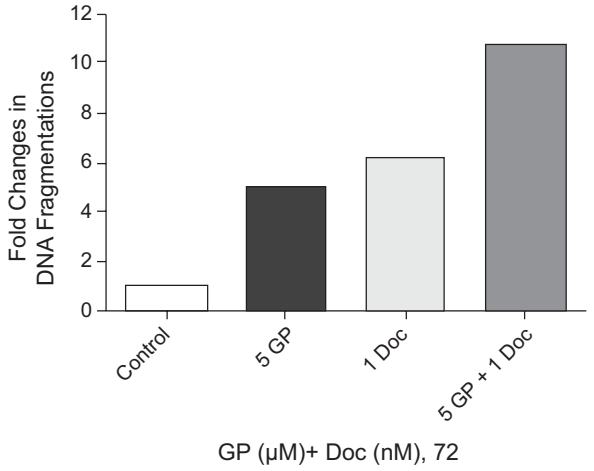

Figure 4. Apoptotic effects of gossypol (GP) and docetaxel (Doc) alone or in combination in MCF-7 cells through DNA fragmentation analyses. The results are the means of 3 independent experiments. p=0.01 for 5 μM GP + 1 nM Doc.

exposed to 5 μM gossypol and 10 μM paclitaxel, there were 5.4- and 5.8-fold increase in DNA fragmentation, respectively. However, the combination of both induced 9.4-fold increase in DNA fragmentation as compared to untreated controls (Figure 3). When MCF-7 cells were exposed to 5 μM gossypol and 1 nM docetaxel, there was 4.9- and 6.2-fold increase in DNA fragmentation, whereas the combination of both induced DNA fragmentation 10.7 times more as compared to untreated controls (Figure 4).

Enhancement of apoptosis in MCF-7 cells exposed to combination of gossypol with taxanes also verified by acridine-orange method

Apoptosis was also verified using a mixture of acridine orange and ethidium bromide (100 mg/ml). MCF-7 cells exhibited condensed chromatin, fragmented nuclei and appearance of apoptotic bodies resulting after exposure to gossypol-docetaxel combination (Figure 5). Both apoptotic and necrotic changes

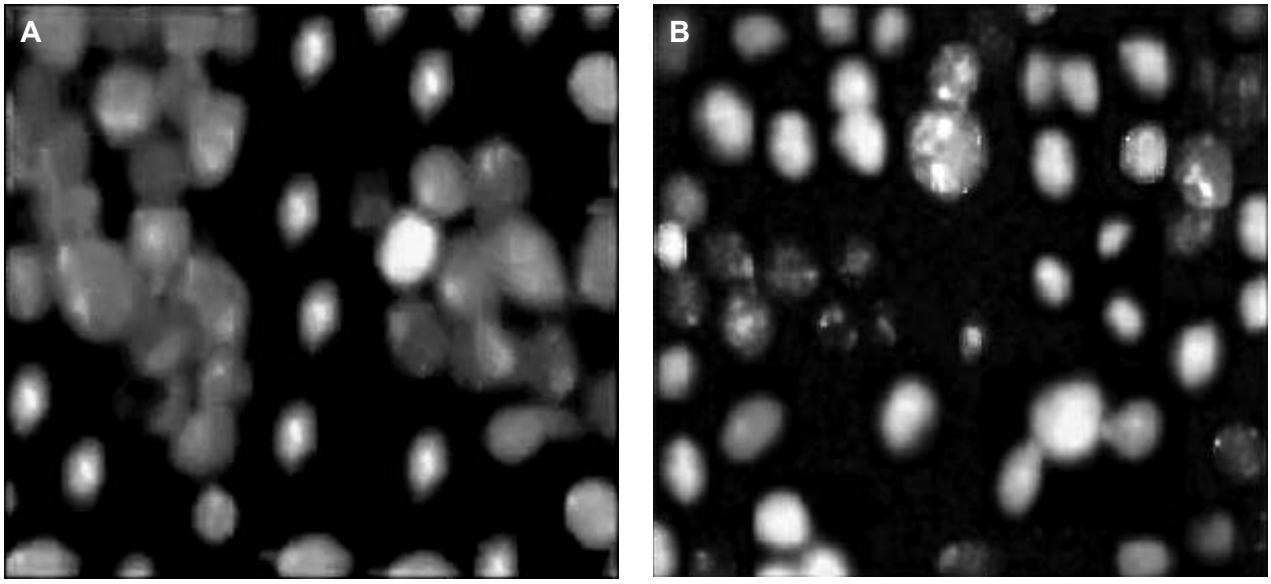

Figure 5. Under fluorescent microscope, the cells of the untreated group displayed fluorescence evenly **(A).** Uneven and denser fluorescence could be observed in large proportion of the nuclei of cells treated with gossypol in combination with docetaxel, indicating that the cells underwent apoptosis **(B).**

were achieved by synergistic doses of gossypol and docetaxel (Table 2), as measured by fluorescent DNA-microscope.

Discussion

Despite major developments in the treatment of breast cancer in recent years, the management of this disease, especially in advanced stages, remains unsatisfactory with only partial responses in the majority of the patients. In the literature many studies are now focused to find novel options of treatment for many types of cancer cells [12,13]. Thus, agents with cytotoxic activity have been combined both in the hope of enhancing antitumor activity and overcoming drug resistance.

Gossypol is a potent inhibitor of growth for many types of cancer cells, including breast cancer [8,14]. Because of its very low toxicity profile in *in vivo* studies [9] it is an ideal option to combine it with various cytotoxic agents which are known to be effective in the treatment of breast cancer. In this study, we combined gossypol with taxanes, doxorubicin, gemcitabine, cisplatin and vinorelbine. Among these combinations, we found that the synergistic combination that showed significant antitumor activity in MCF-7 cells was the gossypol-taxane combination. With either paclitaxel or docetaxel, gossypol caused strong cytotoxicity in breast cancer cells, no matter which agent was applied first. The combination of taxanes with gossypol also resulted in synergistic apoptotic activity verified by DNA fragmentation and acridine-orange methods.

However, antagonistic effect was observed with other cytotoxic agents used in combination with gossypol in MCF-7 cells. In a recent study by Li et al. synergism of gossypol with various chemotherapeutic agents was studied in non-Hodgkin's lymphoma (NHL) cells; increased apoptosis and growth inhibition induced by etoposide, doxorubicin, vincristine and paclitaxel were achieved [15], and antagonistic effect was observed with gossypol-cisplatin combination. Although the different cell lines possess represent different molecular properties with different experimental conditions, the gossypol-paclitaxel combination showed also promising results for NHL, as well as breast cancer in our study.

Both paclitaxel and docetaxel are members of the taxane family and exhibit their cytotoxic effects via induction of apoptosis by stabilizing the microtubules of cancer cells [16]. Taxanes also inactivate the antiapoptotic protein Bcl-2 by phosphorylation, thereby promoting apoptosis. They are both known to be very effective agents against breast cancer, both in the adjuvant and metastatic settings. Additionally, multiple different proapoptotic effects of taxanes, including induction of p53 and effects on multidrug resistance have been described in different studies [17-19].

Although there is limited data about the molecular mechanisms induced and/or inhibited in gossypol-exposed tumor cells, it was shown that gossypol induces apoptosis through inhibition of antiapoptotic Bcl-2 family members and loss of mitochondrial membrane potential and activation of caspase-3 enzyme [10,14]. Whereas the underlying mechanism of synergy of gossypol with taxanes remains unclear, there is a rationale for combining taxanes with gossypol in the treatment of breast cancer. Since both of these drugs will act to inhibit Bcl-2 activity in MCF-7 cancer cells, the antitumor activity of the drugs might be enhanced through this mechanism by down-regulation of this pivotal antiapoptotic protein. Moreover, since bcl-2 is one of the well-known antiapoptotic protein families and is responsible for drug resistance for many types of cancer cells, these combinations might also be a hope for overcoming drug resistance which is one of the most important problems of daily oncologic practice [20-24]. These combinations might also be a hope for overcoming drug resistance.

In conclusion, we identified strong synergistic activity of gossypol with taxanes among all other studied cytotoxic drugs, in MCF-7 cells. This synergy as measured by Chou and Talalay drug synergy method was further confirmed by two different apoptosis assays. Of great interest, the combination of gossypol with taxanes was at clinically achievable doses, thus these data might argue for clinical evaluation of these drug combinations in the treatment of breast cancer. Finally, this strong synergistic activity of the drugs observed with gossypol and taxane combination raises significant questions that warrant further investigations.

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