

RESEARCH ARTICLE

Arsenic Trioxide Promotes Paclitaxel Cytotoxicity in Resistant Breast Cancer Cells

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Abstract

A partial response or resistance to chemotherapeutic agents is considered as a main obstacle in treatment of patients with cancer, including breast cancer. Refining taxane-based treatment procedures using adjuvant or combination treatment is a novel strategy to increase the efficiency of chemotherapy. PPM1D is a molecule activated by reactive oxygen species, whose expression is reported to modulate the recruitment of DNA repair molecules. In this study we examined the impact of arsenic trioxide on efficacy of paclitaxel-induced apoptosis in paclitaxel-resistant MCF-7 cells. We also investigated the expression of PPM1D and TP53 genes in response to this combination treatment. Resistant cells were developed from the parent MCF-7 cell line by applying increasing concentrations of paclitaxel. MTT assays were applied to determine the rate of cell survival. DAPI staining using fluorescent microscopy was employed to study apoptotic bodies. Real-time RT-PCR analysis was also applied to determine PPM1D mRNA levels. Our results revealed that combination of arsenic trioxide and paclitaxel elevates the efficacy of the latter in induction of apoptosis in MCF-7/PAC resistant cells. Applying arsenic trioxide also caused significant decreases in PPM1D mRNA levels ($p < 0.05$). Our findings suggest that arsenic trioxide increases paclitaxel-induced apoptosis by down regulation of PPM1D expression. PPM1D dependent signaling can be considered as a novel target to improve the efficacy of chemotherapeutic agents in resistant breast cancer cells.

Keywords: Combination therapy - arsenic trioxide - resistant breast cancer - taxanes - Wip1

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Introduction

Breast cancer, the most common cancer among women, is the second cause of death after lung cancer, with approximately 40,000 occurrence in American females (Siegel et al., 2013). Despite marked improvement in diagnosis and long term disease-free survival because of improvement in current treatment; the high rate of death extremely caused by the resistance to chemotherapy (Zekri et al., 2013; Sabzichi et al., 2014).

Paclitaxel is an anti-mitotic agent which is usually used against a wide range of solid tumors include locally advanced and metastatic breast cancer (Bauer et al., 2010). Paclitaxel as a microtubule-stabilizing agent induces apoptosis in cancer cells by activation of the mitotic check points and subsequent mitotic blockage, which is resulted in inhibition of cancer cell proliferation (Burns et al., 2003; Herbst and Khuri, 2003; Ikui et al., 2005). Studies showed that paclitaxel can induce DNA single strand breaks too (Branham et al., 2004).

As the acquired resistance increased; many patients

that receive paclitaxel as the first or secondary-line treatment, have not responded completely to the agent. Different molecular mechanisms can be complicated the taxol resistance, including changes in metabolism of the chemotherapeutic agents (Kavallaris, 1997), modifications in the dynamics of microtubules (Goncalves et al., 2001), over expression of multidrug resistance gene products (Gottesman, 2002) and alteration in expression of genes, which are participated in cellular responses to DNA damage like PPM1D (Bauer et al., 2010).

PPM1D was identified as the protein phosphatase, which has a p53-dependent expression in response to DNA-damage (Fiscella et al., 1997; Rossi et al., 2008). It regulates several different networks in the cell cycle by selectively inactivating some central tumor suppressor pathways (Takekawa et al., 2000; Lu et al., 2008). This phosphatase has main activity in destruction of DNA damage-induced apoptosis (Ali et al., 2012). It has been shown that PPM1D as a growth-promoting phosphatase plays a crucial role in tumor progression, poor prognosis and resistance to various chemotherapeutic agents,

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including doxorubicin and cisplatin (Ali et al., 2012; Rivas et al., 2012; Wang et al., 2012).

Arsenic trioxide, one form of arsenicals, has approved as anticancer components against acute promyelocytic leukemia (Liu et al., 2012a). Its inhibitory effect on the tumor cell growth and induction of apoptosis can be observed in malignant solid tumors as well as acute promyelocytic leukemia (Baj et al., 2002; Cheng et al., 2008; Li et al., 2009). Arsenic trioxide induces a paclitaxel-like effect in promotion tubulin polymerization and cell cycle arrest in most solid tumors (Ling et al., 2002).

Combination of various chemotherapeutic agents can be an appropriate regimen for overcoming chemoresistance. Multiple agents, whether have the same mechanism or have antagonistic effects on the cell cycle can participate with each other in cancer treatment (Ghanbari et al., 2014; Samadi et al., 2014). Several studies suggest that combination of multiple agents in comparison with single drug, make cancer cells more sensitive (Skidan et al., 2009). Moreover, increasing in the dosage of chemotherapeutic agents could raise their toxicity and side effects, therefore, employment of combination therapies can be a useful solution to obtain the maximum effect with the minimum dosage (Duan et al., 2010; Samadi et al., 2011).

Because of widely administration of paclitaxel in breast cancer patients, finding new strategies, to overcome chemoresistance will greatly maximize the therapeutic benefit for individual breast cancer patients.

In this study, we investigated the combination effects of arsenic trioxide and paclitaxel in paclitaxel-resistant breast cancer cells. We further studied the role of PPM1D and TP53 in induction of chemoresistance against paclitaxel by comparing PPM1D and TP53 expressions in single and combined treatment of resistant cells. Results from our study showed that combination treatment with arsenic trioxide and paclitaxel promotes apoptosis and decreases resistance of the cells to paclitaxel. Down regulation of PPM1D expression via using arsenic trioxide in combination of paclitaxel, increased the sensitivity of the resistant cells to paclitaxel. Our results suggest that combination treatment increases the efficacy of taxans. We predict that identifying breast cancer patients with high PPM1D expression and then inhibiting this molecule could provide an important adjuvant for improving the efficacy of paclitaxel in these patients.

Materials and Methods

Materials

Paclitaxel and arsenic trioxide were purchased from Ebetaxel®, EBEWE Pharma (Unterach- Austria) and Sina Darou

(Tehran-Iran), respectively. RPMI-1640 medium and Fetal Bovine Serum (FBS) were obtained from Gibco® (Invitrogen, USA).

3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; 5 mg) and penicillin/streptomycin were obtained from Sigma-Aldrich (St Louis, MO, USA). Primers were purchased from Anaspec (Canada). RNXTM-Plus Kit and REVERTA-L RT reagent's kit were obtained

from CinnaGen (Tehran, Iran) and Central Institute of Epidemiology (Moscow, Russia), respectively. Power SYBR®Green PCR Master Mix was obtained from Applied Biosystems (Warrington, UK).

Cell culture

Human breast cancer MCF-7 cells were obtained from Pasteur Institute Cell Culture Collection (Tehran, Iran). Cells were grown in RPMI 1640 containing 10 % FBS and 100 units/ml penicillin/streptomycin and incubated at 37°C in 5% CO₂. Cultured cells at 70-80% confluency were washed with pre-warmed phosphate buffered saline (PBS) and detached from the flask using 500 µl trypsin-EDTA with incubation at 37°C for 3-5 minutes, followed by addition of culture media containing 10% FBS to neutralize the excess trypsin activity. The cell suspension was then centrifuged, and the cell pellet was re-suspended in fresh culture media.

Development of paclitaxel-resistant MCF-7 cells

Resistant cells was established as we described previously (Sharifi et al., 2014). MCF-7 cells with about 20-30% confluency were treated with increasing concentrations of paclitaxel. Initial treatment concentration was one-tenth of IC₅₀ (0.5 nM) which determined by MTT assay. Paclitaxel-resistant MCF-7 cells were established by treating cells with continues and stepwise paclitaxel concentrations (0.5 to 64 nM). Culture medium for growth of MCF-7/PAC resistant cells were enriched with 20% FBS and 10% conditioned medium. Conditioned medium was prepared as the supernatant of cultured non-resistant MCF-7 cells with about 80% confluency. Cells can be a subculture when become confluent then dose of paclitaxel was increased to 1.5 time of previous dose, and this process continued to reach 64 nM paclitaxel concentration. Generally, cells will tolerate the lower doses of paclitaxel, when the cells look not to have tolerated a drug treatment, it is unwise to repeat another round of drug treatment so cells maintained in the previous dose for more days. MTT assay was applied to confirm the resistance cells to each dose of paclitaxel.

Assessments of cell viability using MTT assay

Single Therapy: to determine the drug efficacy in induction of apoptosis after a single exposure, MCF-7/PAC resistant cells were seeded in 96-well plates with seeding density of 12,000 cells/well. Then, increasing concentrations of paclitaxel (up to 1000 nM) and arsenic trioxide (up to 9 µM) were applied. Resistant cells were incubated with media containing these agents for 24 and 48 h.

Combined treatment

To determine the effects of paclitaxel/arsenic trioxide combinations in cell death, MCF-7/PAC resistant cells were seeded at the density of 12,000 cells/well in 96-well plates. Subsequently, the cells were incubated with variable concentrations of paclitaxel and arsenic trioxide.

MTT Assay

The media in each well was replaced with 200 µl

fresh media containing 50 µl of MTT. Then the cells were incubated for 4 h at 37°C. After incubation period, media/MTT mixture was removed and 200 µl of DMSO plus 25 µl of Sorenson's glycine buffer (0.1 M glycine and 0.1 M NaCl, pH 10.5) was added to each well. The absorbance of each well was measured at 570 nm after shaking for 10 minutes, employing a microplate reader (Biotek, ELx 800, USA).

MTT solution with DMSO (without cells and medium) was used as blank control.

Data analysis

Plots of cytotoxicity index ($\%CI = (1 - ((OD_{treated}) / (OD_{control})) \times 100$) versus different concentrations of each chemotherapeutic agents were drawn. IC₅₀ was determined from each plot by calculating the slope and intercept.

The combination index (CI) was calculated using the following equation (Eq. 1): $CI = (D_1) / ((D_{50})_1) + (D_2) / ((D_{50})_2) + \alpha ((D_1) (D_2) / ((D_{50})_1 (D_{50})_2))$

Where $(D_{50})_1$ and $(D_{50})_2$ are the concentrations of drug 1 or 2 alone giving a 50% reduction in cell viability compared to a control. $(D)_1$ and $(D)_2$ are the concentration of drug 1 and 2 in combination producing a 50% reduction in cell viability compared to a control. α is 0 when 1 and 2 are mutually exclusive or 1 when they are mutually non-exclusive (Chou and Talalay, 1984). $CI < 1$ was considered synergistic, $CI = 1$ was considered additive and $CI > 1$ was considered an antagonistic interaction.

Assessment of apoptotic cells using DAPI staining

DAPI is known to form fluorescent complexes with natural double-stranded DNA. Binding of DAPI to DNA enhances its fluorescence strongly. DAPI staining was performed as we established previously (Samadi et al., 2009). Cells were seeded in 6-well plates, and after single and combination treatment for 24 h, cells were fixed with 4 % paraformaldehyde. After 15 minutes, cells were washed with phosphate-buffered saline (PBS) and then permeabilized with 0.1 % Triton-X-100 for 10 minutes. Cells then were stained with DAPI (1:500 dilution in PBS) for 10 minutes. Cells were evaluated as normal or apoptotic depending on morphological characteristics. Normal nuclei (smooth nuclear) and apoptotic nuclei (condensed or fragmented chromatin) were easily distinguished. Triplicate samples were prepared for each treatment and at least 300 cells were counted in random fields for each sample and apoptotic nuclei were identified.

Assessment of PPM1D and p53 mRNA expression via Real-time RT-PCR

Cells were harvested 48 h after incubation with different concentrations of drugs and lysed using lysis buffer, RNXPLUS™ (RN7713C) CinnaGen Co., according to manufacturer protocol. RNA pellet was dissolved in DEPC-treated water, quantified by optical density measurement (A260/A280 ratio) with NanoDrop 1000 Spectrophotometer (Wilmington, DE, USA), checked the quality by agarose gel electrophoresis, and stored at -70°C. cDNA synthesis was done using REVERTAA-L

(RT reagents kit). Real time PCR was carried out using the SYBR Green-based PCR Master Mix and analyzed on a Corbett 6000 Rotor-Gene Thermocycler (Corbett Research). Total volume of amplification reactions was 25 µl, and each well were included 12.5 µl of SYBR Green PCR Master Mix, 1 µl of cDNA, 70-100 nM of both forward and reverse special primers. The internal control was the constitutively expressed housekeeping human beta-actin. Primers for human PPM1D were as follows: sense, 5'-GCC AGA ACT TCC CAA GGA AAG -3'; antisense, 5'-GGT TCA GGT GAC ACC ACA AAT TC -3', for human TP53 were as follows: sense, 5'-TCAACA AGA TGT TTT GCC AAC TG -3'; antisense, 5'-ATG TGC TGT GAC TGC TTG TAG ATG -3' and for human beta-actin were as follows: sense, 5'-TGC CCA TCT ACG AGG GGT ATG-3'; antisense, 5'-CTC CTT AAT GTC ACG CAC GAT TTC-3. Samples were measured in triplicate. Interpretation of the results was performed using the Pfaffle method, and the CT values were normalized with respect to beta-actin expression.

Statistical analysis

Results were presented as mean±SD from at least three independent experiments. Statistical analysis was performed using SPSS software through Student's t-test. A p-value of less than 0.05 was considered to be statistically significant.

Results

Anti-proliferative activity of paclitaxel and arsenic trioxide on paclitaxel-resistant MCF-7 cells

The effects of paclitaxel and arsenic trioxide in induction of apoptosis on resistant breast cancer cells were examined through MTT assay. We first incubated the cells with increasing concentrations of each chemotherapeutic agent for 24 and 48 h to determine the percentage of viability for each agent. Paclitaxel was not able to induce 50% cytotoxic effects in resistant cells after 24 h incubation. Maximum percentage of cytotoxicity was 44±0.19 %. The IC₅₀ value for paclitaxel after 48 h incubation was 526±0.13 nM. Arsenic trioxide induced 50% cell death in resistant cells with 5.21±0.17 and 3.4±0.06 µM after 24 and 48 h incubation, respectively ($p < 0.05$) (Figure 1a,b).

Combination treatment restores the sensitivity of resistant cells to paclitaxel

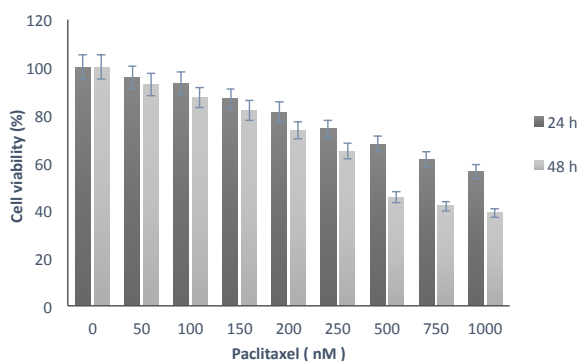
To investigate whether incubation of the cells with paclitaxel in combination with arsenic trioxide overcome chemoresistance, the inhibitory effects of different combinations of paclitaxel/arsenic trioxide were compared with single therapy. The effect of combination treatment on cellular proliferation was evaluated by MTT assay. The combined effects of paclitaxel/arsenic trioxide were calculated using isobolographic analysis method. Cells were incubated with 250, 500 and 1000 nM of paclitaxel along with variable concentrations of arsenic trioxide (1-5 µM) for 24 and 48 h. Arsenic trioxide with paclitaxel showed an additive or synergistic interaction in MCF-7/PAC resistant cells after 24h incubation (Table 1). While

paclitaxel was not able to induce 50% cytotoxicity in resistant cells after 24 h incubation, using combination treatment increased cytotoxicity of paclitaxel up to $77.3\% \pm 0.01$ after 24 h ($p < 0.05$) (Figure 2a). IC₅₀ value for paclitaxel decreased to 250 ± 0.21 nM when the cells were incubated with paclitaxel in combination with 4 μ M of arsenic trioxide after 24h incubation ($p < 0.05$). Incubation of resistant cells with the same combination regimens for 48 h, showed mostly synergistic interaction (Table 1). After 48 h incubation, 250 nM of paclitaxel along with 3 μ M arsenic trioxide was able to induced 50% cell death in resistant cells (Figure 2b).

Table 1. CI Values of Combined Treatment with Paclitaxel and Arsenic Trioxide

Drug combination (48h)	CI	Drug combination (48h)	CI
PAC* 250+ATO** 1	1.002	PAC 250+ATO 1	1.001
PAC 250+ATO 2	1.001	PAC 250+ATO 2	1.001
PAC 250+ATO 3	1.002	PAC 250+ATO 3	0.96
PAC 250+ATO 4	0.98	PAC 250+ATO 4	0.89
PAC 250+ATO 5	0.93	PAC 250+ATO 5	0.88
PAC 500+ATO 1	1.001	PAC 500+ATO 1	0.98
PAC 500+ATO 2	1	PAC 500+ATO 2	0.95
PAC 500+ATO 3	0.97	PAC 500+ATO 3	0.87
PAC 500+ATO 4	0.97	PAC 500+ATO 4	0.82
PAC 500+ATO 5	0.93	PAC 500+ATO 5	0.76

The combined cytotoxic effect of paclitaxel (250 and 500 nM) with arsenic trioxide (1-5 μ M) was determined by: CI-isobologram using COMPUSYN software; CI > 1, = 1 and < 1 indicate antagonistic, additive and synergistic effects, respectively; *PAC: Paclitaxel; **ATO: Arsenic trioxide



Combination of paclitaxel and arsenic trioxide promotes the formation of apoptotic nuclei in resistant cells

To study the effects of single or combination treatment in induction of apoptosis response, cells were stained with DAPI and examined under a fluorescence microscope. Minimum 300 cells were examined for each treatment, and the percentage of apoptotic nuclei, fragmented nuclei, and condensed chromatin, was calculated. Our results from DAPI staining showed that 24 h incubation of the cells with 250, 500 and 1000 nM paclitaxel induced 21, 28 and 39% apoptosis, respectively. Furthermore, after incubation of resistant cells with 3 and 5 μ M of the arsenic trioxide, a population of 29 and 40% apoptotic cells was observed, respectively ($p < 0.05$) (Figure 3a-c). Incubation of the cells with combined paclitaxel (250, 500 and 1000 nM) and arsenic trioxide (3 μ M) for 24 h increased the percentage of apoptotic cells up to 39, 48 and 56%, respectively ($P < 0.05$) (Figure 3d). Our results from DAPI staining indicated that combination of two agents could enhance the percentage of apoptotic nuclei significantly.

Combination of paclitaxel with arsenic trioxide decreases PPM1D expression

PPM1D, a serine/threonine protein phosphatases, is a known negative regulator of cell stress response pathways including those regulated by p53 and p38 MAP kinase (Lu et al., 2008). As shown in Figure 4a, compared to untreated cells, paclitaxel alone caused a slight decrease in PPM1D expression. Incubation of resistant cells with 250 nM paclitaxel along with 3, 4 and 5 μ M arsenic trioxide

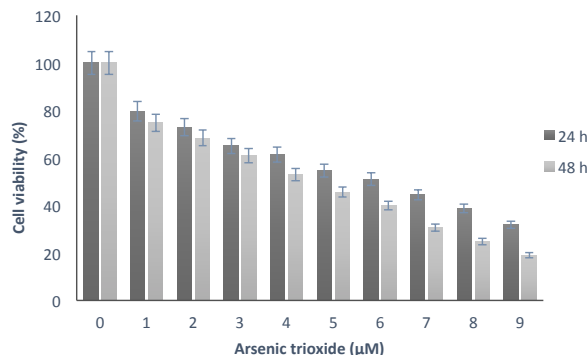


Figure 1. Cytotoxic Effects of the Paclitaxel (a) and Arsenic Trioxide (b) in Paclitaxel Resistant Cells After 24 and 48h Incubation. Results are shown as mean \pm SD for at least three independent experiments

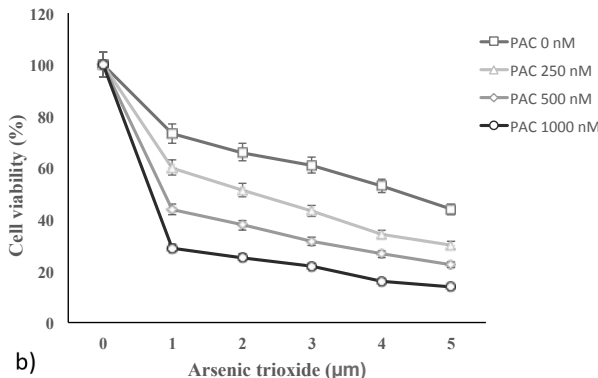
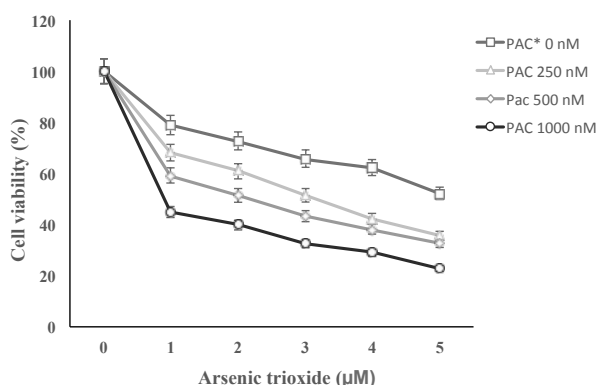


Figure 2. Combination Treatment of Resistant MCF-7 Cells with Paclitaxel and Arsenic Trioxide. Treatment of resistant cells with variable concentrations of paclitaxel (PAC) along with arsenic trioxide (1-5 μ M) for 24 h (a) and 48 h (b). Data are shown as mean \pm SD for at least three independent experiments. *PAC: Paclitaxel

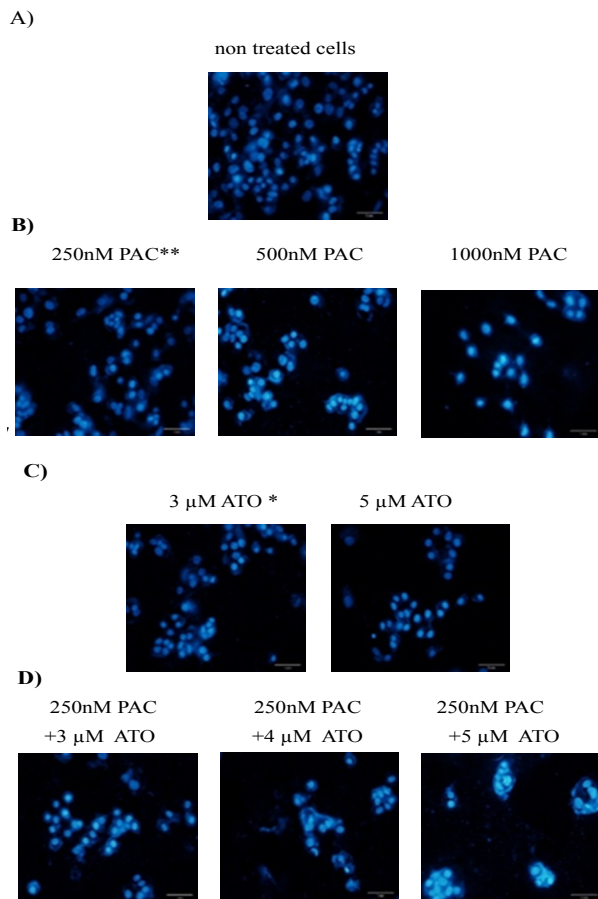


Figure 3. Determination of Apoptotic Response in Resistant Cells Upon Treatment of the Cells with Different Concentrations of Agents. DAPI images of: a) untreated cells, b) paclitaxel, c) arsenic trioxide and d) combination treated cells have been shown. The results are expressed as mean \pm SD values for at least three independent experiments.*ATO: Arsenic trioxide; **PAC: Paclitaxel

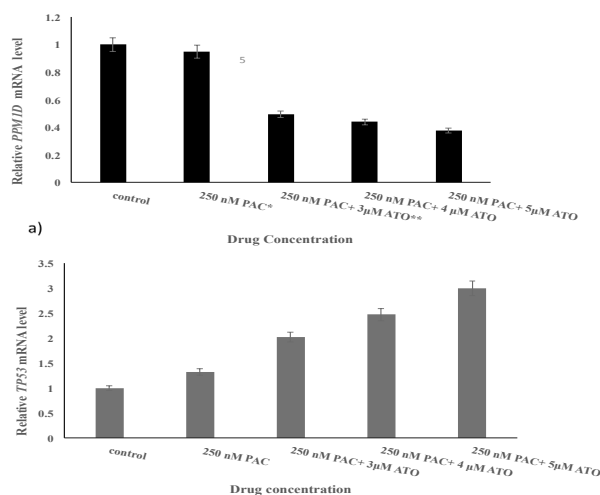


Figure 4. Determination of a) PPM1D and b) TP53 mRNA Level in Resistant Cells Upon Combination Treatment with Different Concentration of Paclitaxel (PAC) and Arsenic Trioxide (ATO). The results are expressed as mean \pm SD values for at least three independent experiments.*PAC: Paclitaxel; **ATO: Arsenic trioxide

for 48 h, caused a marked decrease in PPM1D expression. Consistent with this result, compared to untreated and

paclitaxel-only treated cells, the TP53 expression was increased considerably by combination treatment of both agents (Figure 4b).

Discussion

Resistance to chemotherapeutic agents results in poor responses and high incidence of relapse in patients with metastatic breast cancer (McGrogan et al., 2008; Samadi et al., 2009). Therefore, understanding about the mechanisms by which cancer cells evade apoptosis and developing new approach to overcome chemo-resistance remain one of the main obstacles in breast cancer treatment (Melet et al., 2008). Using the combination of two or more drugs can be an effective strategy to overcome resistance by affecting signaling pathways in cancer cells to promote apoptosis. p53 protein has a central role in response to cellular stress, including DNA damage (Wahl and Carr, 2001; Vousden and Lu, 2002). The leading function of p53 protein as a tumor suppressor is to act as a transcription factor for both key downstream tumor suppressors and oncogenes (Kong et al., 2009). Inexistence in p53 function can be the result of mutation or depletion of the TP53 gene. On the other hand, tumor cells could use negative feedback to suppress p53 activities (Momand et al., 1998; Bulavin et al., 2002).

The aim of this study was to examine whether the combination of paclitaxel and arsenic trioxide can suppress resistance to paclitaxel treatment and explore the potential role of PPM1D phosphatase as one of the central negative regulators of DNA damage signaling pathways. In this work, we investigated the anti-proliferatives and apoptotic effects of paclitaxel (up to 1000 nM) and arsenic trioxide (up to 9 μ M) in paclitaxel-resistant MCF-7 cells. Treating resistant cells with 526 nM paclitaxel showed a percentage of cell death up to 55% after 48 h incubation. The IC₅₀ value for arsenic trioxide after 24 and 48 h incubation was 5.21 and 3.4 μ M, respectively. These results indicated that arsenic trioxide and paclitaxel have cytotoxic effect in breast cancer resistant cells at a time- and dose-dependent manner, which is consistent with results from previous studies (Liu et al., 2012b). Cells were also incubated with different combinations of paclitaxel and arsenic trioxide. Treating MCF-7/PAC resistant cells with the combination of two agents increased cytotoxicity by 77.3% after 24 h. While we showed that paclitaxel was not able to induce 50% cytotoxic effects in resistant cells after 24 h incubation, combination treatment is more successful in induction of apoptosis in resistant cells.

In traditional Chinese medicine arsenic trioxide has been shown to have anticancer activity, and approved by the FDA for the treatment of acute myeloid leukemia (Liu et al., 2012a). The effect of using arsenic trioxide in brain, ovarian, prostate, and some other types of cancers, either as a single therapy or in combination with other anticancer agents, has been implicated (Platanias, 2009; Emadi and Gore, 2010). Various studies suggested that arsenic trioxide has an activity like paclitaxel, against cell proliferation in human solid tumors (Ling et al., 2002). Some studies showed that arsenic trioxide produces mitotic arrest probably because of targeting tubulin before induction of apoptosis in human breast cancer

cells (Halicka et al., 2002; Cai et al., 2003). Our results indicated that arsenic trioxide has a significant impact on the efficacy of paclitaxel in induction of apoptosis in resistant cells. The combinatorial treatments led to elevated apoptosis response and decreased cell viability compared with treatment with paclitaxel alone.

It has been demonstrated that arsenic trioxide can accumulate apoptosis in some type of cancer cells by alteration of the oncogenic Wip1 (Yoda et al., 2008; Meng et al., 2011). PP2C serine-threonine phosphatase Wip1 (PPM1D gene) is one of the main negative regulators of the p53 by affecting p53 stability through post translational modifications or changing Mdm2/p53 interaction (Lu et al., 2005; Lu et al., 2007). Amplification of PPM1D has been detected in several different cancers particularly in breast cancer which is usually associated with a poor prognosis, resistance to radiotherapy and some type of chemotherapies (Fiscella et al., 1997; Parssinen et al., 2007; Parssinen et al., 2008; Wang et al., 2012).

In this study, we examined whether combination of arsenic trioxide and paclitaxel can augment p53 expression because of down regulation of PPM1D expression. We found that treatment of resistant cells by the combination of two agents decreases the PPM1D mRNA level significantly; while using paclitaxel alone did not show the same effect. We also revealed that combination regimens of these agents could expressively increase the TP53 mRNA level. These results are constant with the previous research, which are reported that arsenic trioxide made a difference in PPM1D expression (Meng et al., 2011; Dogra et al., 2015).

In summary, our data demonstrate that combination of the arsenic trioxide and paclitaxel is effective in suppressing the tumor proliferation by modulation Wip1/TP53 expression in paclitaxel-resistant breast cancer cells. Arsenic trioxide is more efficient than paclitaxel in induction apoptosis in resistant cells. Our data suggest that arsenic trioxide can be applied as an adjuvant to increase the efficacy of the therapeutic agents for paclitaxel-resistant breast cancer patients.

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