

All-trans retinoic acid and genistein induce cell apoptosis in OVCAR-3 cells by increasing the P14 tumor suppressor gene

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Abstract

In this study, we evaluated the effects of all-trans retinoic acid (ATRA) alone or in combination with genistein (GEN) in p14 tumor suppressor gene and subsequent apoptosis of human ovarian carcinoma cells (OVCAR-3). The cells were treated with ATRA or GEN at concentrations of 50 and 25 μ M respectively, either alone or in combination for 24 and 48 h. The cell viability was evaluated using 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay. The percentage of cell apoptosis was determined using flow cytometry and p14 gene expression was measured using real time PCR. The MTT results showed that in both ATRA and GEN treated groups, the cell viability in group treated for 48 h was significantly lower than group treated for 24 h. The flow cytometry results showed that the percentage of apoptotic cells in groups that treated with ATRA and GEN in combination for 24 h and 48 h was significantly more than all other tested groups. The real time results showed that the mRNA level of p14 in cells treated with both drugs for 48 h was significantly higher than all other groups. In conclusion, we confirm that GEN in combination with ATRA is an effective strategy to up regulate the p14 tumor suppressor gene and induce cell apoptosis in OVCAR-3 cell line.

Keywords: ATRA; Genistein; p14; OVCAR-3

INTRODUCTION

Ovarian cancer is the second most common gynecological malignancy and the main cause of death from cancer in women. Every year about 22000 cases of ovarian cancer are diagnosed in the USA (1). In recent years surgery and chemotherapy are the main therapeutic methods for many types of cancers. Statistics show that only 45 percent of women who have been diagnosed with ovarian cancer survived more than 5 years. In some cases of ovarian cancer the disease returns 15 months after the initial treatment (2). Therefore, finding of effective new approaches for ovarian cancer treatment is necessary.

It is clearly recognized that 90–95% of cancers are caused by epigenetic factors, while the remaining is related to genetic alterations (2,3). Previous studies have shown that the epigenetic changes of tumor suppressor genes are one of the main causes of ovarian cancers (4). One of the most important tumor suppressor genes is p14^{ARF} (5) (also called ARF tumor suppressor, ARF, p14ARF), an alternate

reading frame protein product of the CDKN2A locus and accumulates mainly in the nucleolus. P14^{ARF} can cause cell growth arrest and apoptosis via two p53-dependent and independent pathways (6,7). The p14^{ARF} promoter contains numerous CpG islands and therefore is prone to epigenetic alterations and gene down regulation. In recent years investigations have shown that the epigenetic alteration of p14^{ARF} promoter is one of the important causes of down regulation of this gene in many cancers such as colorectal (8), colon, hepatocellular carcinoma (9), esophagus (10) and urinary bladder cancers (11). Previous studies showed the down regulation of p14^{ARF} is due to epigenetic alteration in ovarian cancer cells (6).

The OVCAR-3 cell line that used in this study is a highly metastatic, drug resistant human ovarian carcinoma cell line, and previous studies have shown the down regulation of p14 gene in this cell line (12,13).

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All trans retinoic acid (ATRA) is one of the derivatives of vitamin A which can prevent carcinogenesis in 3 mechanism: cell apoptosis induction, abnormal cell growth arrest, and differentiation of abnormal cells to normal (14,15). Of these three mechanisms, the ability of ATRA in induction of apoptosis is the interest in cancer treatment. Although a number of mechanisms by which ATRA leads to apoptosis have been studied, however, the details do not fully understand (16). It seems that one of these mechanisms is modification of epigenetic alterations (9). The role of ATRA in the modification of epigenetic changes in malignancies has been shown in many studies (17). However, the use of ATRA in cancer treatment alone has some limitations (18). It seems that, ATRA in combination with other drugs which modify epigenetic alterations is the appropriate method for the treatment of various cancers including ovarian cancer.

Genistein (GEN) may be a suitable choice for use with ATRA that modifies epigenetic alteration in p14^{ARF}. This isoflavone can be found in large quantities in soybean (19), and its effects have been studied in various reactivate tumor suppressor genes in many cancers such as uterus, brain and breast (3). Choi, *et al.* showed the apoptotic effects of GEN in ovarian SK-OV3 cell line (20). This study was performed to investigate the effects of ATRA and GEN on p14 tumor suppressor gene expression and subsequent apoptosis of OVCAR-3 ovarian cancer cell line.

MATERIALS AND METHODS

Cell toxicity studies

The OVCAR-3 cell line was purchased from National Cell Bank of Iran Pasteur Institute and was transferred to the cell culture laboratory. For proliferation, the cells were seeded in T₇₅ flasks and the Roswell Park Memorial Institute (RPMI 1640) (Sigma, USA), containing 10% fetal bovine serum (Sigma, USA), 1% antibiotics including 10,000 units/mL penicillin G sodium (Sigma, USA), 10,000 µg/mL streptomycin sulfate and 25 µg/mL amphotericin B (Sigma, USA) and 1% sodium pyruvate (Sigma, USA) were added into the flasks and then incubated at

37 °C in a humidified atmosphere with 5% CO₂ / 95% air. The media were changed every 48 h. The sufficient number of cells to perform assessment procedure was achieved. Both ATRA (Sigma, USA) and GEN (Sigma, USA) were dissolved in dimethyl sulfoxide (DMSO) (Sigma, USA). The DMSO concentration in the medium was less than 0.1% and was not cytotoxic to the tumor cells. The cells were divided in 7 groups, two groups were treated with ATRA for 24 h or 48 h (ATRA24 and ATRA48), two groups with GEN (GEN24 and GEN48), two groups with combination of both drugs (AG24 and AG 48), and the untreated group as the control.

Determination of IC₅₀ values

MTT was purchased from Sigma (St. Louis, MO, USA). The cells were harvested using trypsin digestion from flasks and centrifuged in 1400 RPM/5 min. After washing with phosphate buffered saline (PBS), 5×10^5 cells were counted and placed into each well of the two 24-well culture plates. The next day after 24 h, different concentrations of ATRA (5, 10, 15, 25, 50, and 75 µM) and GEN (1, 2.5, 5, 10, 25, and 50 µM) were added to the medium. After 24 and 48 h of treatment, the MTT survival assay was then carried out for determination of the cell viability with different drug concentration. Cell viability was measured spectrophotometrically at 570 nm. All experiments were repeated three times, with at least three measurements (triplicates).

Measurement of cell viability by MTT assay

To measure cell viability MTT assay was used. Briefly, the cells in the 24 well-plates were treated with ATRA (with concentration of 25 µM) and GEN (with concentration of 50 µM), separately and in combination for 24 and 48 h. Then MTT solvent was added for 4 h at 37 °C. The formazan compound derived from MTT by mitochondrial dehydrogenases of living cells were dissolved in DMSO and quantified by measuring absorbance at 550 nm.

Measurement of apoptosis by flow cytometry

For determination of apoptotic cell percentage, firstly 5×10^5 cells in various groups were seeded in each well of 24 well-

plates and then treated for 24 and 48 h by ATRA (with concentration of 25 μ M) and GEN (with concentration of 50 μ M), separately and in combination together. After washing with PBS, the cell suspension was centrifuged (1400 rpm and 8 min). Then the cells were suspended in binding buffer (1 \times 5 μ L) and AnnexinV-FITC was added to the cell suspension. Analysis was carried out according to the manufacturer's protocol (BMS500F1/100CE AnnexinV-FITC, eBioscience, USA). In the next step the apoptotic cells were counted by FAC Scan flow cytometry (Becton Dickinson, Heidelberg, Germany).

Real-time quantitative RT-PCR

Real-time PCR was performed to quantitatively evaluate the P14 gene expression after the treatment of cell groups with ATRA, GEN, and their combinations. Total RNA was isolated by RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol and in then treated with RNase free DNase (Qiagen, Hilden, Germany) to eliminate the genomic DNA.

The RNA concentration was determined using a Biophotometer (Eppendorf). Total RNA (100 ng) was reverse transcribed to CDNA by using the RevertAid™ First Strand CDNA Synthesis Kit (Fermentas, USA) according to the manufacturer's instructions. Real-time RT-PCR was done with the Maxima SYBR Green RoxqPCR master mix kit (Fermentas, USA) according to the manufacturer's instructions. The Maxima SYBR Green Rox qPCR master mix kit (Fermentas, USA) was used for real-time RT-PCR. P14 primers were designed by oligo V7.56 software and shown in Table 1. Real-time PCR reactions were performed using Step one plus (Applied Biosystem, version 2.3). The PCR amplification

conditions was 10 min at 95 °C followed by 40 cycles of denaturation step at 95 °C for 15 Sec and annealing and extension for 1 min at 60 °C. Data were analyzed using the Comparative CT ($\Delta\Delta$ ct) method.

The relative expression level of P14 expression was calculated by determining a ratio between the amount of this gene and that of endogenous control. Melting curve was used to determine melting temperature of specific amplification products and primer dimmers.

Statistical analysis

All the quantitative data are presented as the mean \pm standard deviation. One-way analysis of variance (ANOVA) with Tukey post hoc test was performed to determine statistical significance among different groups using SPSS software package 20.0. Significance was accepted at a level of $P < 0.05$.

RESULTS

IC₅₀ assay

The effect of ATRA and GEN on cell viability in OVCAR-3 cell line was evaluated using MTT according to standard protocol. The results showed that both ATRA and GEN inhibit the cell growth significantly in all treatment groups and the essential drug concentration to obtain the IC₅₀ in OVCAR-3 cells at 24 h was 50 μ M for ATRA and 25 μ M for GEN (Fig. 1). The effect of ATRA and GEN on cell toxicity was found to be concentration dependent dependent.

MTT assay

The effect of drugs on cell proliferation was evaluated using the MTT proliferation assay in OVCAR-3 cell line. The concentrations of the drugs were used based on their IC₅₀.

Table 1. Primers used in real-time PCR

Primers ID	Primers Sequences
P14 forward	GAAGGTCCCTCAGACATCCC
P14 reverse	GAAAGCGGGGTGGGTTGT
GAPDH forward	AAGCTCATTTCCTGGTATG
GAPDH reverse	CTTCCTCTGTGCTCTTG

After treatment with ATRA and GEN, separately or in combination, cell proliferation was determined 24 h and 48 h after the treatment period.

Untreated Cells were considered as the control group. Cell viability in all treated groups was significantly lower than that of the control group. In both ATRA- and GEN-treated groups, the cell viability in groups treated for 48 h was significantly lower than cells treated for 24 h.

The cell viability in AG48 was significantly lower than ATRA24 or GEN24, but the differences in the cell viability between AG48 with ATRA48 and GEN48 was not significant (Fig. 2).

Flow cytometry assay

Flow cytometry was performed to determine the percentage of apoptotic cells visualized using Annexin V-FITC and/or PI staining. 4×10^5 cells/mL was analyzed for each group. The flow cytometry results indicated that the percentage of apoptosis in all treated groups was significantly more than that

of the control group except for GEN24 and ATRA24. The percentage of apoptotic cells in the GEN48 was significantly more than that of the ATRA48 group. The percentage of cell apoptosis in groups AG24 and AG48 was significantly more than the other groups (Figs. 3A and 3B).

Real time PCR

To examine the effect of ATRA and GEN at various times on the expression of p14 gene in OVCAR-3 cells, real-time quantitative PCR was employed.

The expression of p14 gene in all groups was significantly more than that of the control group. Significant differences in mRNA levels of p14 were observed for AG24 compared to that of the control, ATRA24 and AG48. The p14 gene expression in the AG48 group was significantly higher than that of all other groups.

There was a significant difference between the expressions of p14 gene in the GEN24 group compared to that of the ATRA24 group (Fig. 4).

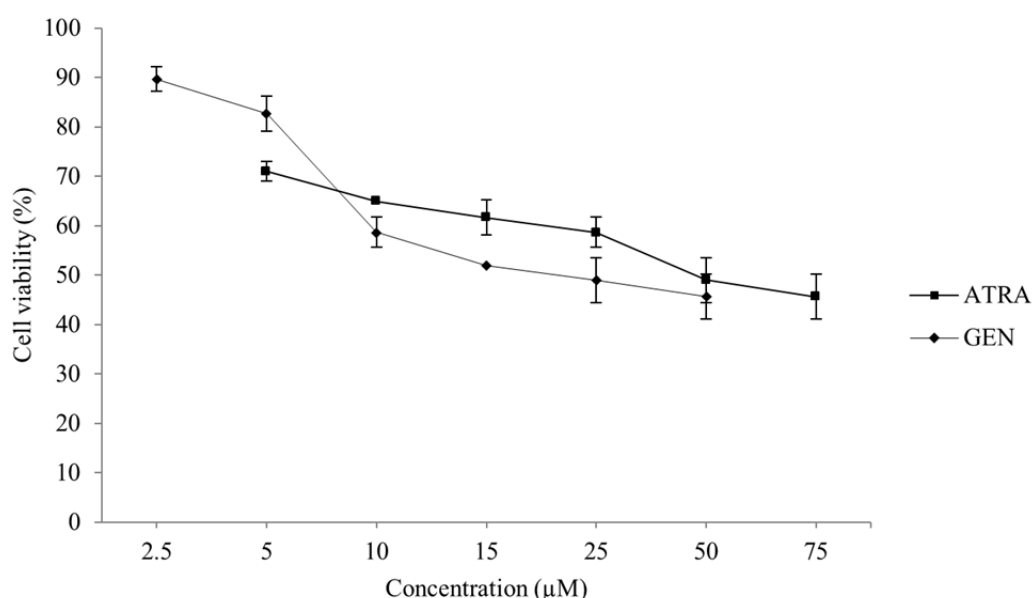


Fig. 1. IC₅₀ assay of ATRA and GEN in OVCAR-3 cancer cell lines. Cells incubated with/without the drug in various concentrations and the relative amount of viable cells determined by measuring the absorbance of MTT solution. Graph of viability versus drug concentration used to calculate IC₅₀ values.

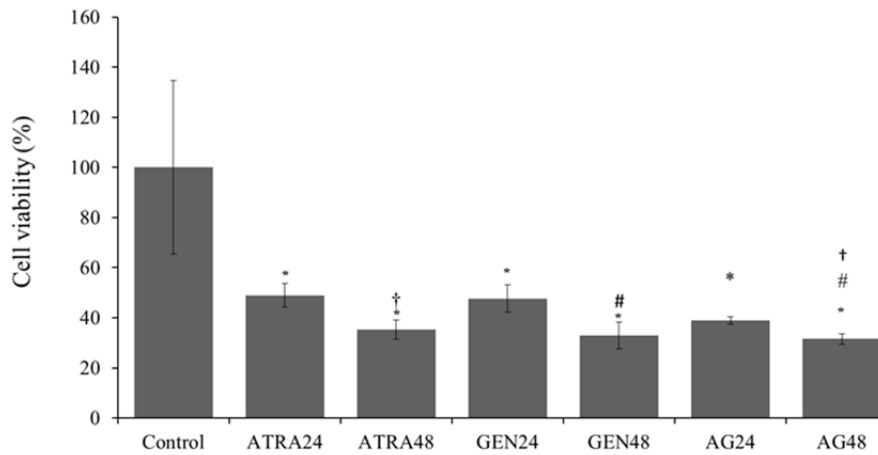


Fig. 2. MTT assay at IC₅₀ concentrations of ATRA and GEN alone and in combination at 24 and 48 h after the treatment. *, Significant difference vs control group ($P < 0.05$). †, Significant difference of ATRA24 vs ATRA48 and AG48. #, Significant difference of GEN24 vs GEN48 and AG48.

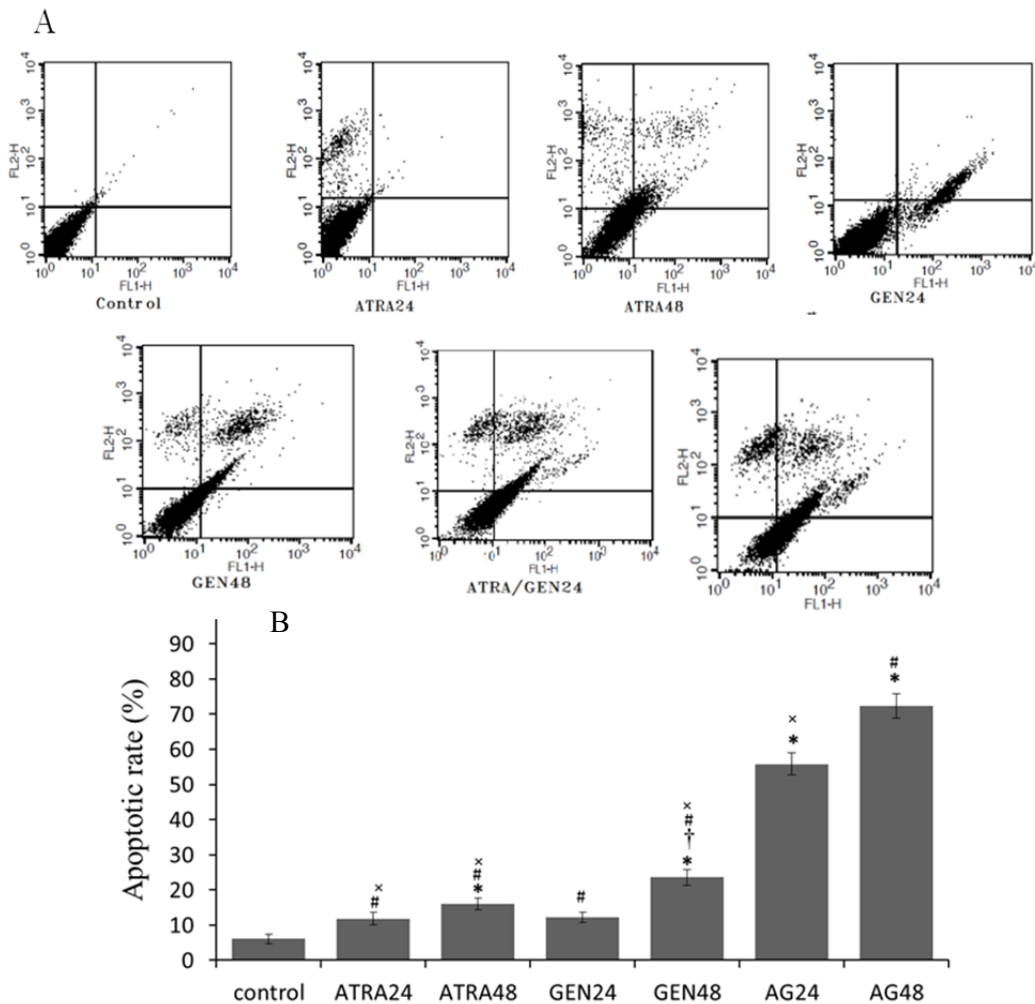


Fig. 3. (A) the apoptosis inducing effects of ATRA and GEN investigated by flow cytometric analysis on OVCAR-3 cells stained with Annexin V-FITC and/or Propidium Iodide. (B) effects of ATRA and GEN on apoptosis of OVCAR-3 cells at 24 and 48 h. *, Significant difference versus control group ($P < 0.05$). †, Significant difference of GEN48 vs ATRA48. #, Significant difference of AG24 vs all other groups. ×, Significant difference of AG48 vs all other groups.

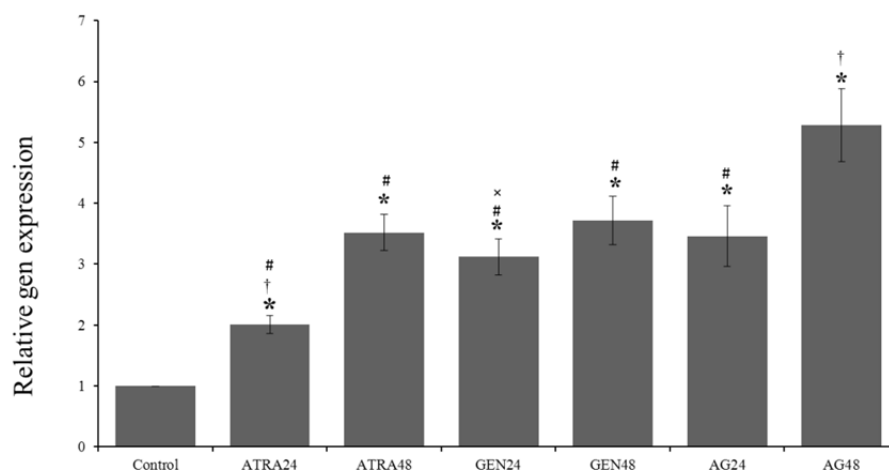


Fig. 4. Effects of ATRA and GEN on the levels of p14 expression in OVCAR-3 cells at 24 and 48 h after the treatment. *, Significant difference vs control group ($P < 0.05$). †, Significant difference of AG24 vs AG48 and ATRA24. #, Significant difference of AG48 vs other groups. ×, Significant difference of GEN24 vs ATRA24.

DISCUSSION

In many of the studies that have focused on finding new ways to treat cancer, researchers are trying to find drugs that can in different ways induce cancer cells apoptosis. Recent studies have shown that ATRA can inhibit carcinogenesis by inducing apoptosis in cancer cells (9,15). Although its molecular mechanisms are still not clearly defined, but several hypotheses have been proposed. Heo *et al.* showed ATRA with the modification of epigenetic changes in promoter of tumor suppressor genes activates p53 dependent pathway and induces cell apoptosis (9). ATRA can bind to related receptors, activates downstream effectors, and induce cell apoptosis and cell differentiation (21). Although vitamin A derivatives recommended by the Food and Drug Administration (FDA) for the treatment of various cancers including lymphoma (22), but recent studies have shown that high doses of these agents can have side effects (22) and even induce angiogenesis in tumor. Maeno *et al* investigated the effect of ATRA on bronchoalveolar carcinoma cells and measured vascular endothelial growth factor (VEGF) mRNA expression and protein. Their results showed that the VEGF increases in groups treated with ATRA (23). Therefore, we used GEN in combination with ATRA, because previous studies have shown that GEN causes down regulation of VEGF gene in cancer cells (24,25). As a result GEN can

attenuate angiogenesis effect of ATRA and enhance its antitumor effects. The results of several studies have shown that GEN can induce cell apoptosis in breast (26), prostate (27), leukemia, lung, and head and neck carcinoma cells (28-34). This isoflavone can also induce autophagocytosis in some cancer cells (29). Therefore, this drug could probably be a good choice in combination with ATRA to inhibit cancer cell growth.

The results of real time PCR in the present study showed that the p14 gene expression in the AG48 group was significantly greater than all other groups. According to these results, long term exposure of cells to combination of ATRA and GEN is needed to significantly increase p14 tumor suppressor gene. Comparison of the effects of ATRA and GEN on p14 gene expression showed that the GEN significantly increased the mRNA level of p14 in the 24 h-treated group in comparison to ATRA, but there was no significant difference in p14 gene expression between ATRA48 and GEN48. These observations may indicate that ATRA need longer time to exhibit its effect on p14 gene expression.

The MTT results indicate that the cell viability in group AG48 is significantly lower than that of all other groups except ATRA48 and GEN48. These results are in contrast with the results of Zhou, *et al* study. They evaluated the apoptotic effects of 50 μ M GEN and 40 μ M ATRA on A549 lung adenocarcinoma cells. They showed that when A549 cells were

exposed to both compounds, their inhibitory effect was stronger than when each compound used individually (18). Furthermore, in our study there were no significant differences between the cell viability in groups ATRA24 and ATRA48 in comparison with GEN24 and GEN48 that indicates the ability of these two drugs in inhibition of the proliferation in OVCAR-3 cells are somewhat similar.

The flow cytometry results showed that there were significant differences in the percentage of apoptotic cells between AG24 and AG48 groups in comparison with all other groups indicating that the combination of ATRA and GEN can significantly increase OVCAR-3 ovarian cancer cell apoptosis. In the current study, we did not observe any significant differences between ATRA24 and GEN24 groups. Nevertheless, significant differences were observed between ATRA48 compared to GEN48 group. Therefore, the apoptotic effects of GEN in comparison with ATRA are uncertain, because the results of studies have been controversial.

The flow cytometry results also showed that the percentage of cell apoptosis in GEN24 group was not significantly higher than that of the control group. These findings contradict the results of Gossner's study. He investigated the effects of GEN on ovarian cancer cells and found that this drug can significantly increase the cell apoptosis in 24 h-treated group compared to the control group (35).

The results of flow cytometry in the study of Zhou on A549 lung adenocarcinoma cells (18) are in line with the results of the present study. Furthermore, their results showed that ATRA mainly arrests the cells in G₀-G₁ while GEN predominantly affects G₂-M checkpoint. Hence synergic effects could be seen when these two drugs are combined.

According to above results we believe that GEN may be an effective drug in combination with ATRA in order to activate ovarian cancer cells to up regulate p14 tumor suppressor gene and it may induce cell apoptosis.

CONCLUSION

Both ATRA and GEN can increase *P14* tumor suppressor gene and induce cell

apoptosis in different times; ATRA and GEN in combination exhibit more effects than each drug alone. Finally we concluded that GEN is an effective drug once used in combination with ATRA to up regulate p14 tumor suppressor gene and it may induce cell apoptosis in OVCAR-3 cell line.

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