**ORIGINAL ARTICLE**

# The Synergistic Effect of Gw9508 and S14161 Small Molecules on Apoptotic Gene Expression and Viability on A549 Cell Line

SIMA OROUEI[1], MALIHEH ENTEZARI[1*], MEHRDAD HASHEMI[1], KIAVASH HUSHMANDI[2], ABOLFAZL MOVAFAGH[3*]
*[1]Department of Genetic Faculty of Advanced Science and Technology, Islamic Azad University,, Tehran Medical Sciences Tehran, Iran.*
*[2]PhD student of epidemiology, Department of food hygiene, Faculty of Veterinary Medicine, Tehran University, Tehran, Iran.*
*[3]Department of Medical Genetics, cancer Research Center, School of Medicine, Shahid Beheshti University of Medical Sciences, Correspondence to Abolfazl Movafagh; E-mail: Movafagh_a@yahoo.com , Telephone: +98 912 130 7881*

## ABSTRACT

**Aim:** The aim of this project was to investigate the effect of GW9508 and S14161 small molecules on their viability and expression of apoptotic genes in the A549 cell line.
**Methods:**In this experiment, the A549 cell line was first cultured in DMEM medium containing 10% FBS and then treated with different concentrations of both compounds. MTT assay was performed on days 1, 3, and 5 to determine IC50 and to compare the viability of cells treated with different concentrations of GW9508 and S14161. The qRT-PCR assay was employed to investigate the effects of S14161 and GW9508 with IC50 concentration on apoptosis induction, expression of genes including p53, Bax, Bad and Bcl2.Both small molecules induced apoptosis by increasing apoptotic Bax, Bad and P53 gene expression and decreasing Bcl2 gene expression.
**Results:**Comparison between the effect of S14161 and GW9508 individually and simultaneously on cell viability using MTT assay results and gene analysis showed that the combined use of both compounds had a stronger effect on inducing cell death and apoptotic gene expression in a dose- and time-dependent manner, the more time passes the more lethal power it gains.
**Conclusion:**Two small molecules of GW9508 and S14161 activate apoptotic gene expression and inhibit the anti-apoptotic gene in two different and parallel pathways, thus being able to act synergistically under concomitant use and have a stronger lethal effect on lung cancer cells.
**Keywords:** S14161, GW9508, Lung cancer, Apoptotic, Cell line

## INTRODUCTION

One of the most deadly diseases of humankind is cancer. According to the Cancer Society of Iran, about 85,000 cases of cancer are diagnosed each year in the country, out of which more than 30,000 die. Cancer can occur at different ages and increases with age [1]. In 2013, the United States estimated 229,000 lung cancer cases with 160,000 deaths, accounting for about 27% of all deaths from all types of cancer [2]. Lung cancer is one of the most common cancers in the world. In fact, it is the third most common cancer after breast and colorectal cancer in Europe [3] and the fifth most common cancer in Iran. The incidence of lung cancer is higher in men and is about 73%, with the highest incidence being in the age group of 55 to 60 years [4]. Causes of cancer generally fall into four categories: environmental factors, hereditary factors, immunological factors, and age. In lung cancer, factors such as smoking are the most important contributing factors, as well as other risk factors such as age, heredity, air pollution, and work environment and materials such as asbestos, radon gas and cadmium are effective in lung cancer [5]. Genes involved in cancer include oncogenic genes, mutant genes, and cancer-suppressor genes (TS). When an environmental stimulus mutates in a somatic cell or when a somatic mutation is mixed with a hereditary predisposing factor, the cells divide more rapidly or frequently than usual and develop cancer [6]. Genes that affect programmed cell or oncogenes deaths regulate programmed cell death and encode proteins that induce or stop apoptosis, including the Bcl-2 family. This gene in the Bcl2 family has two subtypes which are anti-apoptotic (Bcl2 and Bcl-xl) and apoptotic stimulators (Bax, Bak Bad, Bid)[7]. The p53 is a tumor suppressor gene and is activated as a protector of the genome in response to various types of cellular stress including DNA damage, aberrant activation of oncogenes, and oxidative stress. Mutation in this gene has been identified in more than two-third of human tumors[8] . The overall goal in the effective treatment of cancers is to regulate the cell cycle and induce apoptotic cell death. Apoptosis is currently the basis for new therapeutic targets that induce cancer cell death or their susceptibility to cytotoxic agents and increase the efficacy of radiation therapy. Therefore, this process and its resulting compounds have been extensively studied by cancer researchers [9]. Despite the tremendous advances in cancer treatment, millions of cancer deaths occur worldwide each year. Most deaths are associated with increased body resistance to therapeutic drugs, as well as their high cost and their adverse effects in addition to their antitumor properties [10]. Nowadays, compounds known as small molecules have been identified with minor side effects in the treatment of cancer[11].

Small molecules have been known for many years in biology and pharmacology as chemical probes or as mutation agents and refer to groups of molecules with molecular weights less than 900 daltons and this allows the molecule to pass through the cell membrane to reach intracellular target sites. These compounds have different functions in regulating physiological processes [12]. Among these small molecules with anti-cancer properties are 8-Ethoxy-2- (4-fluorophenyl) -3-nitro-2H-1-benzopyran, 8-Ethoxy-2- (4-fluorophenyl) -3-nitro-2H- is S14161, or coumarin, which exerts its anticancer properties against cyclin-D by inhibiting the activity of phosphoinositol 3-kinase (PI3K). This small molecule is a cyclic compound consisting of benzine and pyron rings belonging to a broad

class of compounds found in nature. Both natural and synthetic derivatives of coumarin have a wide range of biological activities including antioxidant, anti-inflammatory, anti-cancer, antiviral and many other activities [13]. The PI3K/AKT pathway is one of the most important pathways involved in cyclin production, which leads to cell division and proliferation. Studies show that PI3K and cyclin D levels are significantly increased in most cancers and increase the proliferation of cancer cells [14]. The other small molecule is GW9508, which is one of the GPR40 agonists and plays different roles in signaling and intracellular pathways. This small molecule can promote apoptosis and autophagy by inducing and enhancing mitochondrial oxidative stress [15]. GPR40 is a receptor for long-chain unsaturated fatty acids, which is a type of G protein-coupled receptor. Recent studies have shown that unsaturated fatty acids such as linoleic acid play an important role in the treatment of cancer and act as extracellular signaling molecules by binding to fatty acid receptors coupled with the G protein[16].

The aim of this project was to investigate the effect of GW9508 and S14161 small molecules on their viability and expression of apoptotic genes in the A549 cell line.

## METHODS

**Preparation of solutions for small molecule:** This was a laboratory study performed in a cell culture research laboratory. Concentrations of2, 5, 10 and 20 and 40 µM were prepared from S14161 (SML-0232-5MG Sigma) and 1, 10, 50 and 100 µM of GW9508 as doses used in cell culture and after filtering with 0.22g/L filter. Micrometers (Bio Fact BSS20-PE2-Korea) were stored at 4°C until use.

**Cell culture and passage:** A549 lung carcinoma cell line was purchased from Pasteur Institute of Tehran and cultured in DMEM medium (Dulbecco's modified Eagle's medium, Gibco, USA) containing 10% FBS (Fetal bovine serum, Gibco, USA) and incubated in an incubator (Sina Co., Inc. Iran), maintained with 5% CO2, 95% humidity at 37°C. When the cell density in the flask reached 80%, cell passage was performed using trypsin (Gibco, USA), which was cultured in 96-well plates after treatment orfroze in liquid nitrogen with DMSO (Dimethylsulfoxide (10%) and FBS (Fetal bovine serum).

**Cell Viability Assessment (MTT):** MTT is a water-soluble yellow tetrazolium salt (3- (4,5 dimethyl-2-thiazolyl) -2,5-diphenyl-2H). The basis of this test is the reduction of tetrazolium salt by mitochondrial succinate dehydrogenase enzyme in living cells. Tetrazolium is a water-soluble yellow salt that is reduced by the active mitochondrial succinate dehydrogenase and converted to an insoluble formazan dye, which is dissolved in an organic solvent such as DMSO. The more active the number is, the more intense the color purple. The color intensity is measured at 570 nm. This method is fast, cheap and suitable in large scales. MTT assay (5Dimethylthiazol-2-5Diphenyltertrazolum Bromide) evaluates the cell viability. Cancerous cells affected by small molecules were considered as the treatment group and untreated cells as the control group. Days 1, 3, and 5 after culturing the cells in 96-well plates, the culture medium was incubated with MTT solution at 0.5 mg/ml for 4 hours and then added to each pit at approximately 100µl DMSO and finally measured using a microplate apparatus reader (Biotek, Germany). The optical absorption of the samples was measured at 570 nm.

**Morphological and staining studies of Giemsa:** For the morphological study of A549 cells in control groups treated with IC50 concentrations of the small molecules on days 1, 3, and 5 in 96-well plates, digital camera imaging was used while being connected to an inverted microscope with 20X and 40X objective lenses and microscopic images for each cell were compared in control and treatment groups. After preparation of Giemsa staining, the cells were stained and the morphology of the treated cells was compared with the control cells. Giemsa stain is prepared at 4% concentration. The procedure was performed by removing the supernatant and adding 500µl of PBS in each pit for washing and 200 µl of methanol for fixation. The methanol was then removed after 5 minutes and the pits were washed with 200 µl of PBS. Finally, cells were stained with 100 µl of Giemsa for 20 minutes and then washed with PBS. After drying, the cells were examined by invert microscopy.

**RNA Extraction:** RNA extraction was performed according to the extraction kit protocol (CinnaGen-Iran), and finally, a nanodrop device was used to measure RNA and the properties of RNAs at 260/280 nm.

**cDNA Synthesis:** HyperScript (GeneAll-Portugal) kit was used for cDNA synthesis and cDNA was fabricated according to the instructions. For each sample 2µm of synthesized cDNA and 0.5µm forward primer, 0.5µm reverse primer, 0.2µm SYBR Green and 5µl of distilled water will be performed using a Power SYBER Green master mix (Qiagen, Japan) in a final volume of 10µm using Real-Time PCR.

**Real-Time PCR:** Thermal Cycler will be used to measure gene expression. The heat-timetable is performed in three stages as follows. Stage one is denaturation reaction at 95°C for 30 seconds, followed by melting at 95°C for 1 second and finally the third application stage at 60°C for 33 seconds for 40 cycles. Data analysis (CT) of each sample is performed using StepOne software and normalization will be performed using the GAPDH gene (housekeeping control gene) in order to estimate the fold change rate of Bax, Bad, Bcl2, and p53 were compared to control (untreated) samples in comparison with the small molecule-treated sample, and each experiment was repeated three times. Gene sequences are extracted from the NCBI site and forward and reverse primers are designed using Gen runner and primer express software.

Table 1: Applied primers

| Name | Primer sequence (5'3') | Tm (C) |
|---|---|---|
| BAX (F) | GCTGGACATTGGACTTCCTC | 58/5 |
| BAX (R) | ACCACTGTGACCTGCTCCA | |
| BAD (F) | CGGAGGATGAGTGACGAGTT | 58/3 |
| BAD (R) | CCACCAGGACTGGAAGACTC | |
| BCL-2 (F) | GATGGGATCGTTGCCTTATGC | 58/8 |
| BCL-2 (R) | CCTTGGCATGAGATGCAGGA | |
| P53(F) | GGAGGGGCGATAAATACC | 57/25 |
| P53(R) | AACTGTAACTCCTCAGGCAGGC | |
| GAPDH (F) | GCAAGAGCACAAGAGGAAGA | 57 |
| GAPDH (R) | ACTGTGAGGAGGGGAGATTC | |

**Statistical analysis:** The Livak method is used to analyze the data obtained from this reaction. GraphPad Prism, One-way ANOVA and t-test software were also used for statistical analysis. P <0.05 was considered as the significant difference for samples.

## RESULTS

**Evaluation of cell viability in lung cancer cells treated with S14161 small molecule:** The comparison of cell viability was performed using MTT assay and showed the viability rate of A549 cancer cells treated with different concentrations of S14161 tested compared to control. MTT assay showed the viability of cells treated with 2, 5, 10, 20 and 40 µM concentrations of S14161 in 24 hours. Based on the results of the cell viability assay, the IC50 concentration of small molecule was determined to be 10 µM for A549 cells within 24 hours (P <0.05). The cytotoxicity effects of this compound on A54 cell viability were dose-dependent and decreased with increasing concentration of cell viability. It is shown in Fig. 2 that the cytotoxicity effects of S14161 small molecule is dose- and time-dependent, with its cytotoxicity potential increasing on day 3 compared to day 1 and day 5 compared to day 3 and day 1 (P <0.05).

**Evaluation of Cell Viability in Lung Cells Treated with Small Molecule GW9508:** The figure below shows the viability rate of A549 cancer cells treated with different concentrations of GW9508 compared to the control sample. MTT assay was performed after 24 hours to evaluate the viability of cells treated with 1, 10, 25, 50 and 100 µM concentrations of GW9508. Based on the results of the cell viability assay, the IC50 concentration of the GW9508 small molecule was determined to be 25 µM for A549 cells within 24 hours (P <0.05). The cytotoxicity effects of this compound on A54 cell viability were dose-dependent and decreased with increasing concentration of cell viability. According to the results of this test, the cytotoxicity effect of GW9508 small molecule is dose- and time-dependent, with its cytotoxicity increased on the third day compared to the day 1 and on the fifth day compared to days 3 and 1 (P <0.05).

**Evaluation of the viability of A549 cells with small molecules GW9508 and S14161 and their synergism on days 1, 3 and 5:** The cell viability of A549 cells in the treated group with IC50 concentrations of S14161 and GW9508 small molecules and their synergism was compared with the control group (P≤0.05). Cell viability percentage of A549 cells in the treatment group with IC50 concentration of S14161 and GW9508 and their synergism on days 1, 3 and 5 (51.43%, 38.67%, 27.17%), (50.05%, 41.21%, 38.03%), and (32.8%, 22.1%, 9.7%), respectively (Figure 5). Cell viability in the 5-day treated cells was significantly reduced compared to the 3-day and 1-day treated cells and also the 3-day treated cells showed lower cell viability compared to the 1-day treated cells and the time-dependent effect of these small molecules on A549 cells is confirmed. Also, the synergistic effect of these two small molecules was greater than either alone. The viability of A549 cells at the time of synergy was significantly reduced by each alone, which was significantly increased with time.

**Evaluation of morphological changes of A549 cells treated with small molecules S14161 and GW9508 and their synergy:** The Giemsa dye contains two methylene blue dyes inducing blue to violet staining of the cell nucleus and acidic eosin that produces the dark red color of the cytoplasm. Changes in the morphology of the A549 lung cancer cell line were observed with the help of an invert microscope and it was found that different concentrations of small molecules and their synergism, in a dose- and time-dependent manner, caused significant changes in the shape of the treated cells compared to control group. These changes included a significant decrease in cell volume, cell rounding, and granulation. This decrease in cell size, compaction, density, and cell shrinkage, indicated apoptotic cell death in small molecules treated cells compared to the control group. These changes were more pronounced in the synergistic treatment group of S14161 and GW9508.

**Evaluation of gene expression changes in A549 cells treated with IC50 concentration of S14161 and GW9508 small molecule and their synergy:** Gene analysis results showed that the expression of Bax, Bad and P53 apoptotic genes in cells treated with S14161, GW9508 and their synergy increased and the expression of anti-apoptotic Bcl2 gene decreased, indicating the induction of cell death in cancer cells. Compared to the three treatment groups, as shown in the diagram below, cells treated with S14161 had a greater increase in apoptotic gene expression and a greater decrease in Bcl2 gene expression compared to cells treated with GW9508. This means that apoptosis was more induced in cells treated with S14161. In the third group, it was shown that the synergy of these two small molecules performed better than either alone and that A549 cell death occurred more frequently.

Figure 1. Effects of different concentrations of S14161 small molecule on the viability of A549 cells by MTT assay and determination of 10 µM concentration as IC50 of A549 cells (different letters indicate significant differences between groups (P <0.05). The arrow indicates the concentration of IC50).

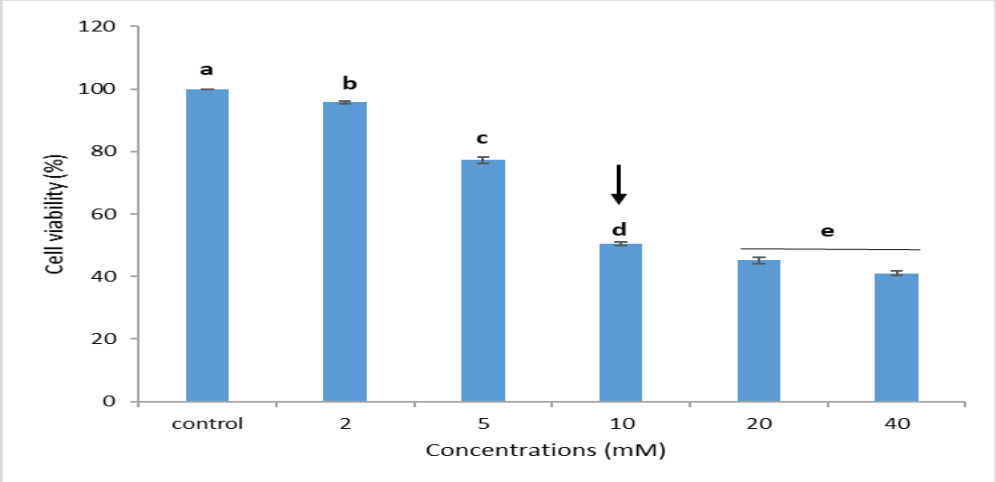



Figure 2.Effects of IC50 concentration of S14161 (G1) on A549 cell viability on days 1, 3 and 5 after treatment - (P <0.05).

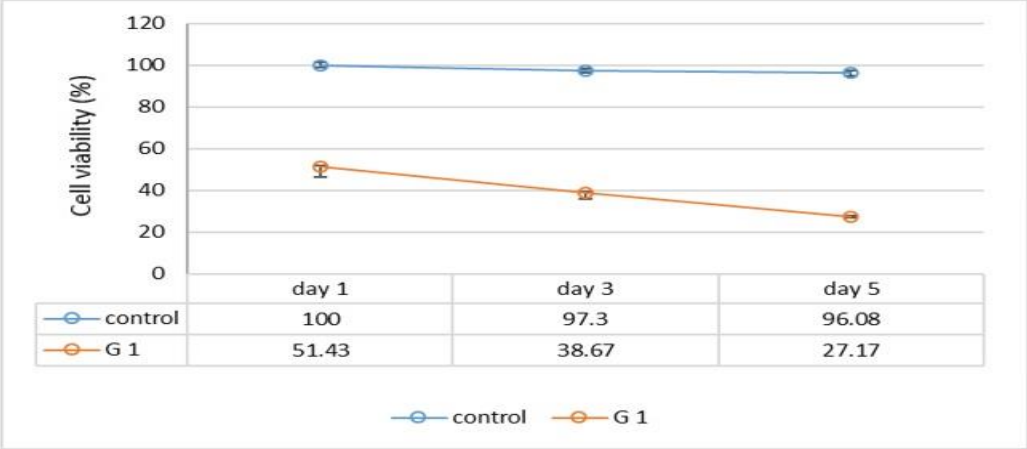



Figure 3.Effects of different concentrations of GW9508 on the viability of A549 cells using MTT assay and determination of 25 µM as the IC50 concentration of A549 cells (different letters indicate significant differences between groups) (P <0.05). The arrow indicates the concentration of IC50).

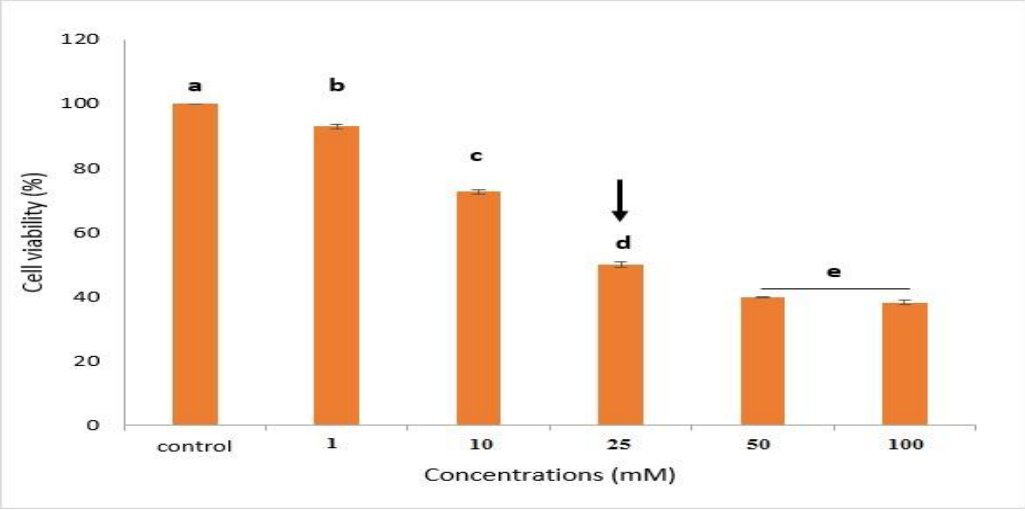


Figure 4.Effects of IC50 concentration of GW9508 (G2) on A549 cell viability on days 1, 3 and 5 after treatment - (P <0.05).

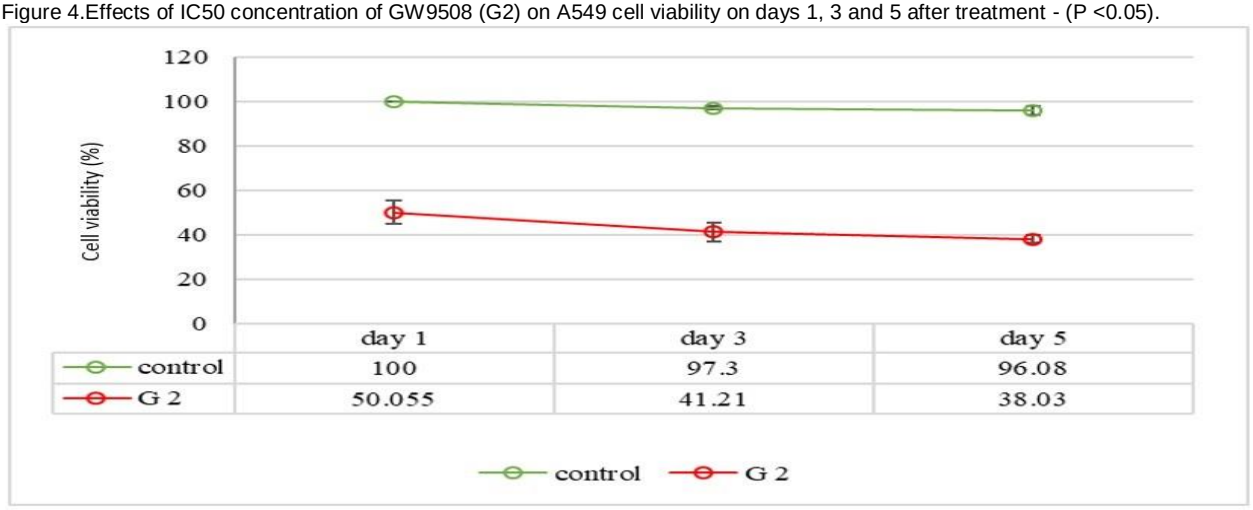



Figure 5.Comparison of IC50 concentration of S14161 (G1) and GW9508 (G2) small molecules and their synergism (G3) on A549 cells on days 1, 3 and 5

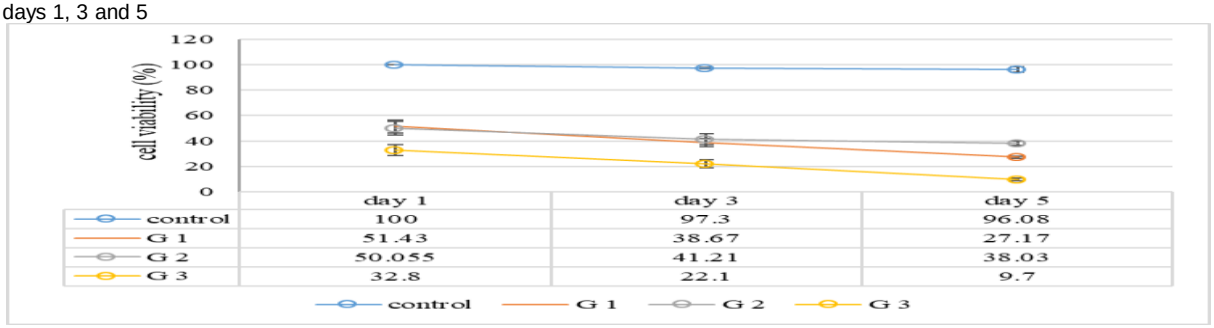



Figure 6: Morphological changes of A549 cells treated with IC50 concentration of S14161 (G1) and GW9508 (G2) molecules and their synergism (G3): in cells treated with a synergistic concentration of these two small molecules compared to control group. Nuclear compaction and density indicate apoptosis.

Gene Expression Analysis Results

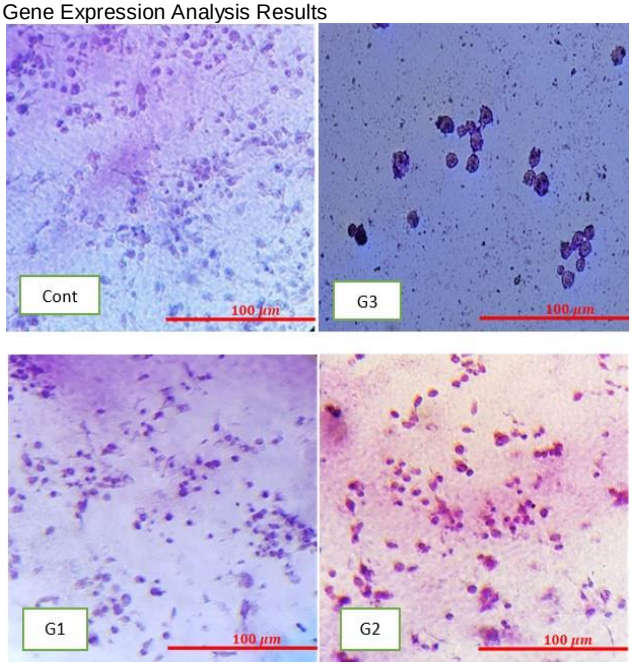
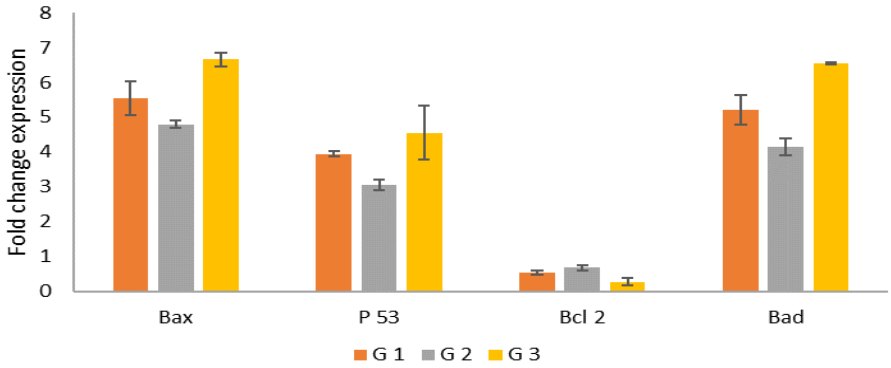


Figure 7.Gene expression changes in A549 cells treated with IC50 concentration of S14161 (G1) and GW9508 (G2) and their synergism (G3) in 24 hours: Increased expression of Bax, Bad and P53 genes and decreased Bcl2 gene expression in A549 cells treated with IC50 concentrations of small molecules and their synergism in the A549 cell line compared to the control group indicates the induction of apoptotic death in cancer cells by this substance.




## DISCUSSION

Cancer is one of the deadly and worrisome diseases worldwide today. Despite the tremendous advances made in the field of cancer treatment, there are millions of cancer deaths worldwide annually, most of which are due to increased body resistance to these drugs. Also, the high cost of these drugs and their many side effects have limited the use of these drugs in addition to their antitumor properties [17]. Almost all of the currently used drugs for treatment are "small molecules" [18,19]. As expected, given the wide range of PI3K/AKT signaling pathway activity in all cellular activities and subsequent involvement of this pathway in a wide range of diseases on the one hand, as well as the widespread effects of current drugs in these diseases, therefore, tend to introduce the less harmful and, of course, more effective small molecules, including the well-known small molecules S14161 and GW9508, is increasing over the time. The findings of this study indicate that A549 cells treated with these small molecules showed cell death and symptoms such as nucleation compaction and cytoplasm shrinkage in morphological studies. In fact, both small molecules induce apoptosis by increasing the expression of apoptotic Bax, Bad and P53 genes and decreasing Bcl2 gene expression. Comparison between the effect of S14161 and GW9508 on cell viability based on MTT results and gene analysis showed that S14161 had a stronger effect on the induction of cell death and apoptotic gene expression than GW9508 and was dose- and time-dependent.

Several studies have investigated the mechanism and function of S14161, and their results suggest that S14161 is a coumarin-derived benzopyrene compound that exerts its anticancer effect by inhibiting PI3K in the PI3K/AKT signaling pathway. In fact, S14161 is known to be a potent PI3K inhibitor that inhibits the PI3K/AKT pathway and induces apoptosis by inhibiting PI3K [20]. Harris's (2003) study showed that the IC50 concentration of S14161 small molecule was 10 micrograms using cell viability assay, which was consistent with the results of the present study, despite being performed on myeloma cell lines. A key factor in the efficacy of the S14161 small molecule on the PI3K pathway and induction of apoptosis by cyclin expression depends on AKT phosphorylation because the U266 myeloma cell line, in which this enzyme was nonphosphorylated, was also resistant to induction of S14161 cell death. It has been shown that S14161 blocks AKT translocation to the plasma membrane in IGF1-stimulated cells [21]. Yin, S.Q. et al. (2013) have shown in a study that the S14161 small molecule inhibits cyclin D through the PI3K/AKT signaling pathway with minimal toxicity to leukemia and myeloma cells [22]. In a study in 2017, Wang et al. investigated the induction of caspase-dependent apoptosis and autophagy by a coumarin hybrid compound on lung cancer cells and found that this combination inhibited the expression of Bcl2 gene in the apoptotic pathway while activating Bax gene and their findings match the results of the present study [23]. In relation to the effect of S14161, this compound has been reported to block AKT translocation to the plasma membrane, and the mechanism of cell death induction by S14161 is activation of caspase-3 and 9, a decrease in the levels of anti-apoptotic proteins including Bcl-2, and an increase in the levels of proapoptotic proteins[24]. In fact, the PI3K/AKT pathway is one of the most important pathways involved in the formation of cyclin D in the cell division and prevents apoptosis. Many protein kinases activate phosphates in the plasma membrane by binding to phosphatidylinositol 3 and affect the activity of many cellular proteins in turn. The most important activated kinase here is AKT protein kinase. One of the important effects of AKT is the phosphorylation and inactivation of the apoptotic initiator Bad protein and consequently the inhibition of apoptosis[25]. Studies of the PI3K/AKT pathway suggest that small molecules capable of inhibiting PI3K can induce apoptosis, for example, BENC-511 has been identified as a potent PI3K inhibitor. Its effect on the induction of apoptosis in A549 lung cancer cells has been investigated. BENC-511, like the S14161small molecule, induces dose- and time-dependent cell death in A549 cells and its mechanism of induction of

apoptosis is inhibition of the PI3K/AKT pathway, downregulation of BCl2 gene expression and ultimately inhibition of cell proliferation [26]. The BYL719 small molecule also acts as an inhibitor of PI3K/AKT signaling pathway on breast cancer cells including MCF-7 in in vivo models of mice and even extracted tumors, and inhibits proliferation [27]. Putting these findings together with the results of the present study suggests that the use of S14161 can be effective in killing cancer cells.

Another studied small molecule is GW9508, which is one of the GPR40 agonists (unsaturated fatty acid receptor). This small molecule can promote cell apoptosis and autophagy by promoting oxidative stress and producing ROS or reactive oxygen radicals, and thus can be effective in the treatment of cancer [15]. Studies have shown that induction of increased reactive oxygen radicals promotes cell death, and in non-surgical treatments such as radiation therapy and chemotherapy increase the sensitivity of tumor tissue to induction and cell death initiation. Therefore, it can be used to kill cancer cells. Due to the role of ROS in triggering cell death, compounds that increase ROS can be used in the treatment of cancer [28]. Many studies have been performed on the effect of unsaturated fatty acids on apoptosis induction; Zhang et al. (2015), for example, stated that trihydroxy trance stilbene (THS) fatty acid induces apoptosis in A549 cancer cells and factors such as Bax gene expression increase and cell viability and Bcl2 gene decrease [29]. Fukushima et al. (2015) found in a study that GPR40 receptor activity inhibits locomotor activity, invasion, and tumor characteristics of pancreatic cancer cells [30]. Since the GW9508 small molecule is an agonist for GPR40, thus due to the anti-cancer characteristic of unsaturated fatty acids after binding to the GPR40 receptor, GW9508 small molecule can induce dose-dependent biological actions by binding to this receptor and is a good option for cancer treatment. In confirmation of the cytotoxic effect of this substance, Philippe et al. (2013) stated in a study that GW9508 specifically and dose-dependently induces oxidative stress in the death of phagocytic precursor cells of bone (osteoclasts) and leads to decreased cell viability [31]. The omega-3 fatty acid is bound by the G protein-coupled receptor (free fatty acid receptor; FFA1). The agonist of this receptor inhibits the proliferation of breast, prostate, and ovarian cancer cells [32]. Evidence suggests that increased signaling of FFAR1 and FFAR4 receptors (free fatty acid receptors 1 and 4) leads to a decrease in diabetes symptoms, inhibiting tumor cell growth and invasion in various cancers, including prostate and melanoma [33]. Putting these findings together with the present findings, GW9508 can be considered a novel combination in the treatment of various cancers[34,35].

As shown, cancer cells treated with the drug combination increased synchronously with cell death, suggesting a better and stronger synergistic effect of S14161 and GW9508 on lung cancer cell cytotoxicity. In general, the simultaneous use of both small molecules has a better effect than using them alone.

## CONCLUSION

Given the pivotal role of the PI3K/AKT signaling pathway and GPR40 receptor in cell viability and proliferation, it is known that GW9508 and S14161 small molecules from two different pathways activate apoptotic genes and inhibit anti-apoptotic genes. They are therefore able to act as synergists under concomitant use and have a stronger lethal effect on lung cancer cells.

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**Ethical approval:** All procedures performed in studies involving human participants were in accordance with the ethical standards of the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

**Disclosure statement:** The authors declare that they have no conflict of interest.

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