Archives of Advances in Biosciences 2021:12(3) doi.org/10.22037/aab.v12i3.35012

Original Article

The Effect of GW9508 on Cytotoxicity and Gene Expression of P53 in C118 Cell Line

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Article Information
Received:2021-04-12
Revised: 2021-06-06
Accepted: 2021-06-26

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Cite this article as:
Molaei H.R., Entezari M. The Effect of GW9508 on Cytotoxicity and Gene Expression of P53 in C118 Cell Line.. Archives of Advances in Biosciences 2021:12(3)

Abstract

Introduction: As one of the most common and invasive brain tumors, glioblastoma which originates in the nervous tissue of the brain has remained a therapeutic challenge given low success of conventional therapies. Small molecules including GW9508, due to their different roles in signaling and intracellular pathways and the production and increase of oxidative stress of mitochondrial origin, can cause cells to progress to apoptosis, also known as a cost-effective pharmacological factor. Therefore, in the present study, the anticancer and cytotoxic effects of GW9508 on A549 class lung cancer cells were investigated.

Materials and Methods: In this experiment, the cell line (C118) was firstly cultured in DMEM culture medium containing 10% FBS and then treated with different concentrations of GW9508. MTT assay was used to determine IC50 and compare the viability of treated cells with different concentrations of GW9508 on days 1, 3, and 5 in the control group. To evaluate the effect, the qRT-PCR test was used with the IC50 concentration on the induction of apoptosis and expression of the P53 gene.

Results: The results showed that GW9508 significantly reduced the viability and proliferation of C118 cells in a dose- and time-dependent manner ($P < .05$). Morphological changes such as reduction of chromatin density and cell rotation were also observed in the cells. Also, molecular results showed that GW9508 was able to increase the expression of the P53 gene.

Conclusions: The GW9508 small molecule induces cell death in glioblastoma cancer cells by reducing cell viability and increasing P53 gene expression. As a result, it has therapeutic potential to induce cell death in cancer cells and to treat cancer.

Keywords: GW9508 Lung cancer, Apoptosis

1. Introduction

Brain tumors are one of the major diseases that have caused many deaths. Eighty percent of primary brain tumors are malignant and thirty percent of all central nervous system tumors are caused by gliomas [1]. Glioblastoma tumors are multiform and one of the most aggressive and most common brain tumors with brain nervous tissue origin. They are in fact produced in the star-shaped cells in brain called astrocytes. These types of tumors are attached to the bloodstream through angiogenesis and therefore grow rapidly. Despite all the advances made in the treatment of this type of tumor, the survival rate with this tumor is so low (about 13-14 months) that only about three percent of patients have a survival rate of more than 4 years [3]. Of the main factors causing cancer are environmental factors such as mechanical damage and electromagnetic radiation. Yet, in addition to the role of environmental factors, disruption of many subcellular pathways such as RAS/PI3K and PI3K/PTYEN/AKT/mTOR can contribute to the development of this cancer [4]. Moreover, due to vascular proliferation of glioblastoma tumors, three pathways including p53 pathway, tyrosine receptor kinase signaling pathway, RAS, phosphoinositide-3 kinase (PiK3), and retinoblastoma pathway are associated with this cancer. Also, different types of primary and secondary glioblastoma are caused by different changes in these pathways [5]. The P53 gene is one of the most important tumor-inhibiting genes mutating in half of all cancers. In fact, when DNA is damaged, the cell enters the S or M stage. In this case, the concentration and activity of P53 protein increases and changes from inactive to active form. P53 encodes a 53 kDa nuclear phosphoprotein whose natural function is to protect the genome from damage. This process leads to genome repair and if not repaired, P53 induces cell death by inducing cell apoptosis, thus eliminating carcinogenic cells [6].

The standard treatment for glioblastoma multiform involves maximal surgical resection of the tumor followed by radiotherapy between 1 and 2 weeks after surgery [7]. Despite extensive clinical and paraclinical research to increase the lifespan of patients with glioblastoma tumor, the disease does not have a good prognosis for patients, remaining a therapeutic challenge for neuro-oncologists and neurosurgeons. Resistance of innate glioblastoma tumor to chemotherapy and radiotherapy, inability to surgically remove the tumor due to the aggressive nature of the tumor, presence of a blood-brain barrier that restricts drug access to the tumor site, and neurotoxicity of therapeutic agents that limit the dosage of drugs are the reasons behind the low success rate of common therapeutic measures [8]. Because of this, nowadays, some effective small molecules in inducing apoptosis (programmed cell death) with few side effects have been identified [9]. In fact, apoptosis is the basis for new therapeutic targets that induce cancer cell death or sensitivity to cytotoxic agents and the effect of radiation therapy. Therefore, this process along with substances that can induce cell death and expression of proapoptic genes has been extensively studied by cancer researchers [10]. GW9508 is one of these small molecules which play different roles in signaling and intracellular pathways, being able to cause cell progression to apoptosis and autophagy by causing and increasing

oxidative stress of mitochondrial origin. Studies show that GW9508 is an agonist for the G protein-coupled receptor 40 (GPR40). GPR40 is a receptor for long-chain unsaturated fatty acids (FFAR1), which is a type of G protein-coupled receptor, and through this receptor, it induces biological functions such as inducing osteogenic differentiation in osteocyte precursor cells [12, 13].

Taking such findings into account, this study aimed to evaluate the effect of GW9508 small molecule on cytotoxicity and expression of P53 gene in glioblastoma or C118 cancer cell line.

2. Materials and Methods

Preparing the GW9508 solution

GW9508 was purchased as a powder from Sigma Company and in order to prepare the main stock solution, it was dissolved in sterile conditions with DMSO (10ml) and 1, 10, 25, 50, and 100 µM of GW9508 solution was prepared as doses used in cell culture and after the filtration using .22 µm syringe filter (Bio Fact BSS20-PE2, Korea) it was kept at 4°C until use.

Cell culture and passage:

In this study, astrocytoma cell line (C118) was provided from Pasteur Institute of Tehran and then cultured in DMEM culture medium (Gibco, USA) containing 10% fetal bovine serum (Gibco, USA), stored in the incubator (Ependorf, Germany) with 5% CO2, 95% humidity and 37 °C temperature. After cell passage, cells were cultured with approximately 7× 103 cells/cm2 in 96-well plate containing complete culture medium. Twenty-four hours later, cells with different concentrations of small molecules were cultured and cell viability was evaluated at days 1, 3, and 5.

Cell viability assay by MTT colorimetric assay

The viability of cancer cells treated with different concentrations of GW9508 small molecules was measured by MTT method (4,5 3-(Dimethylthiazol-2-yl) -2,5-Diphenyltertrazolium Bromide). Also, using MTT test inhibition concentration of 50% or IC50 of GW9508 small molecule was also determined. The basis of this test is the reduction of tetrazolium salt by the mitochondrial enzyme of succinate dehydrogenase of living cells and becoming insoluble formazan (purple). The absorption of purple dye at a wavelength of 570 nm was measured using ELISA reader (Fax 2100, USA). For this purpose, 1× 104 cells/cm2 were cultured in 96-well plates and incubated for 24 hours. Then, the cells were treated with concentrations of 1, 10, 25, 50, and 100 µM of GW9508 and cell viability was assessed at days 1, 3, and 5. A control group was considered for each cell that remained untreated. Then, MTT test was done, so that at the desired times, the environment was taken out of the wells and to each well about 100 µl of fresh medium containing 10 µM of MTT solution (with a concentration of 5 mg/ml) was added. Cells were kept for 3 hours in the incubator and were incubated at 37°C and then the MTT solution was removed and 100 µl of DMSO (Bioidea, Iran) was added to each well. The optical absorption of samples was measured at 570 nm using a microplate reader (Stat fax 2100, Florida, USA). The following formula was used to calculate the percentage of cell viability:

Cell viability (%) = OD treatment group / OD control group × 100

Morphologic evaluation using imaging

For morphological examination of C118 cells from control and treated groups, with IC50 concentrations of GW9508 small molecule on days 1, 3, and 5 in 96-well plates were taken by a digital camera connected to a reverse microscope with 20 and 40 object lenses, and microscopic images were evaluated and compared for each cell in the control and treated groups.

Morphological study with acridine orange/ethidium bromide staining

This cell staining is a fluorescence technique that uses two fluorescent dyes, namely acridine orange and ethidium bromide (Sigma, USA). For fluorescence microscopy to study cell apoptosis, the cells were first prepared with stock 1 mg/ml acridine orange dye and stock 1 mg/ml ethidium bromide dye and mixed in equal proportions. Then cells were treated with IC50 concentrations of GW9508 small molecule on days 1, 3, and 5 in 96-well plates of cell treatment acridine orange/ethidium bromide dye was added to the cells at a concentration of 100 µg/ml and after 5 minutes they were examined and imaged by a microscope.

RNA extraction and fabrication of cDNA:

RNA extraction was performed according to the kit protocol of extraction of the Cinnagen (an Iranian company). All steps were taken accordingly, using the kit solutions. The nanodrop device was used to measure RNA and the character and size of RNAs at 280/260 nm. By cDNA synthesis Kit (Taq Man Reverse Transcription Kit, GeneAll, Portugal) and according to the kit protocol, for each sample, 2 µl of synthesized cDNA, 0.5 µl of Forward Primer, 0.5 µl of Reverse Primer, 2µl of SYBR Green and 5 µl of distilled water using Power SYBER Green master mix (Qiagen, Japan) in the final volume of 10 µl was performed by Real-Time PCR. Data analysis (CT) of each sample were done using StepOne software and normalization using GAPDH gene (housekeeping control gene) to estimate the percentage of changes (Fold Change) of the expression of P53 gene compared to the difference of GAPDH gene threshold cycle between control samples (without treatment with the drug) relative to the sample treated with small molecules. Each experiment was repeated three times.

Design and synthesis of primers

The primers used in this study are based on the relevant gene sequences obtained from the NCBI site and the direct and inverse primers are designed using Gen runner and primer express software. The relevant sequences are shown in the table below.

Real Time PCR

In order to amplify the desired fragment and synthesize the cDNA of the RT-PCR reaction after reverse transcription reaction, the SYBR Green kit was used. We then ran the thermal cycler program to measure gene expression. Finally, the required temperature and time using the SYBR Green Kit protocol were designed and determined. According to the schedule, in stage one, denaturation reaction was performed at 95 °C for 30 seconds, then melting was performed at 95 °C for 1 second, and finally the third stage, or application at 60 °C for 33 seconds and for 40 cycles.

Statistical analysis

The Livak method was used to analyze the data obtained from this reaction. GraphPad Prism, one-way ANNOVA, and t test were also used for statistical analysis. P <.05 is considered a significant difference for the samples.

3. Results

Evaluation of morphology of C118 cells treated with GW9508 small molecule by inverted microscope

Observation of cell morphology under inverted microscope showed that IC50 concentration of GW9508 small molecule caused the cell rounding and shrinkage of C118 cells treated compared to control group cells. In fact, the treated cells were significantly different from the control group cells. Thus, shrinkage and reduction of the cell volume, condensation of nucleus, reduction of cytoplasm volume, loss of morphology of cell colony as well as cell communication and interaction, and appearance of single cells were seen in the image examinations. This indicates that this concentration has a toxic effect, causing progression that leads to the cell death (Fig. 1).

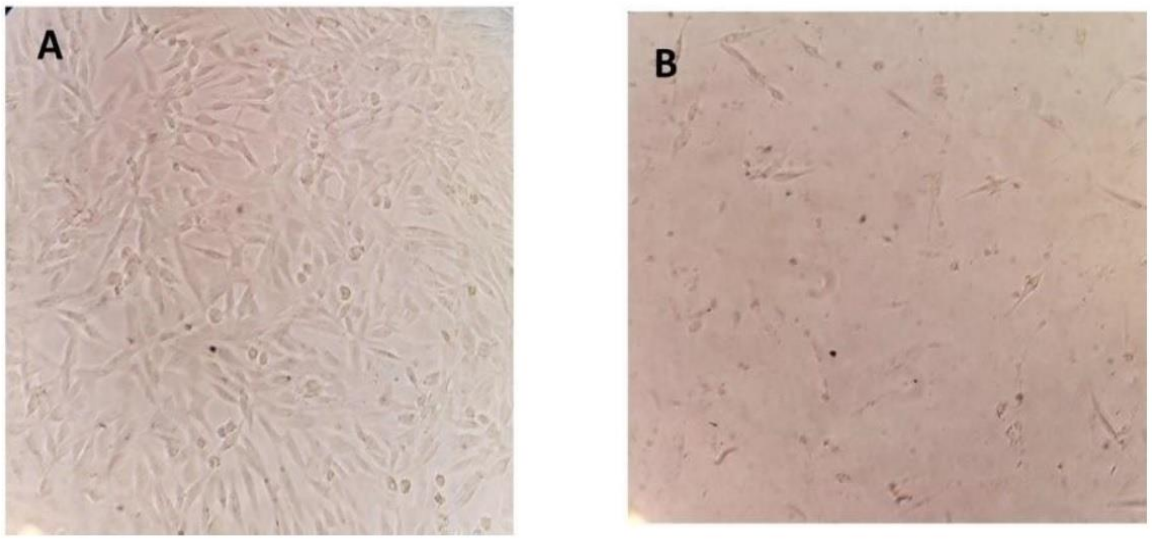


Figure 1. Morphology of class C 118 cells with conventional inverted microscope: A - control group cells (20X magnification) B - cells treated with IC50 concentration of GW9508 small molecule for 24 hours - shrinkage and reduction of cell volume, densification of the nucleus, reduction of cytoplasm volume, and loss of cell colony morphology indicate the toxicity of these concentrations and the progression of cells to apoptosis.

Evaluation of morphology of C118 cells treated with GW9508 small molecule in acridine orange / ethidium bromide staining

Two fluorescent dyes (i.e., acridine orange and ethidium bromide) and a fluorescent microscope were used to evaluate cell apoptosis. Acridine is a cellular dye that naturally crosses cell membranes and turns the nucleus of living cells green. Ethidium bromide passes only through injured membranes and turns the nucleus of apoptotic cells orange, and if the cell is in the late stages of apoptosis, the nucleus becomes compressed, fragmented, and red. C118 cells were treated with IC50 concentration of GW9508 small molecule stained with acridine orange/ethidium bromide dyes and examined by fluorescent microscope. In C118 cells treated with IC50 concentrations of small molecules, the nuclei of the cells were isolated individually compared to the control group where the nuclei of the cells were grouped together. Also, the nuclei of the control group cells are round, large, clear, and green, indicating that they are alive, and the cells of the group treated with this compound, with a single, dense orange nucleus, confirm apoptosis (Fig. 2).

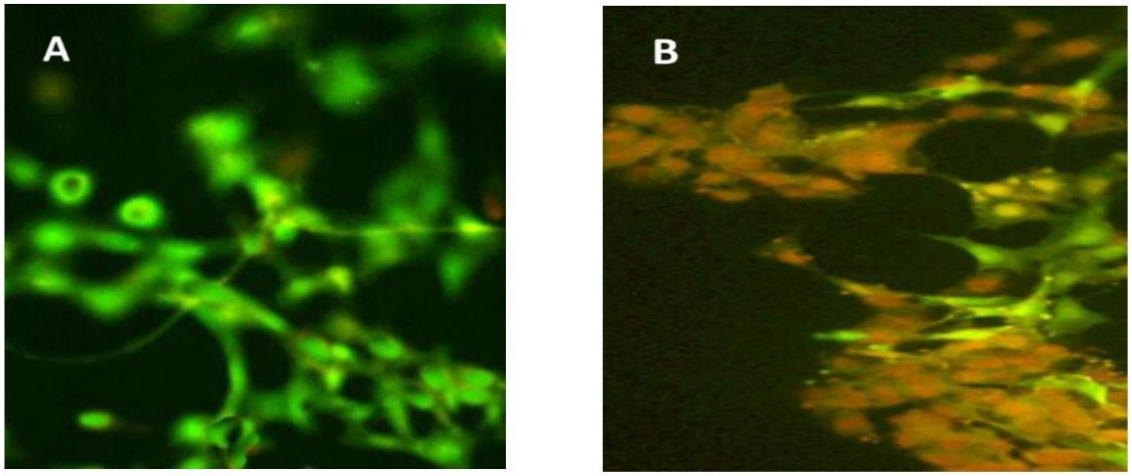
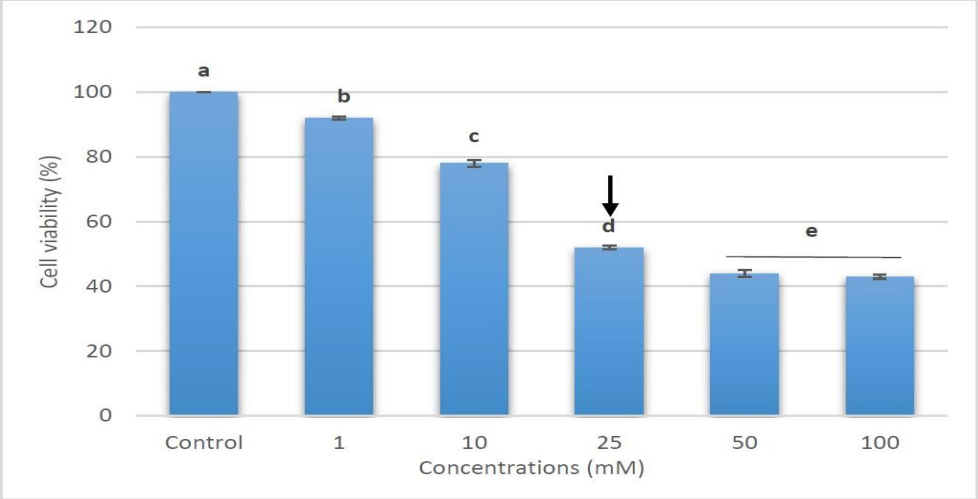


Figure 2 - Evaluation of apoptosis using acridine orange/ethidium bromide staining of C118 cells: A- control group cells (40X magnification) B- cells treated with IC50 concentration of GW9508 small molecule for 24 hours - in orange treatment groups of cells confirms cell apoptosis and green indicates live cells in the control group.

The effect of GW9508 small molecule on C118 cell viability:

Intensity of light absorption obtained from C118 cancer cells which were treated for 21 hours with different concentrations (1, 10, 25, 50, and 100 μM) of GW9508 small molecule was compared to the average light absorption of control cells that were not treated with the drug. The results of evaluating cell viability based on MTT test and growth inhibition percentage in cells treated with different concentrations of GW9508 small molecule are shown in graph 1. Based on MTT test

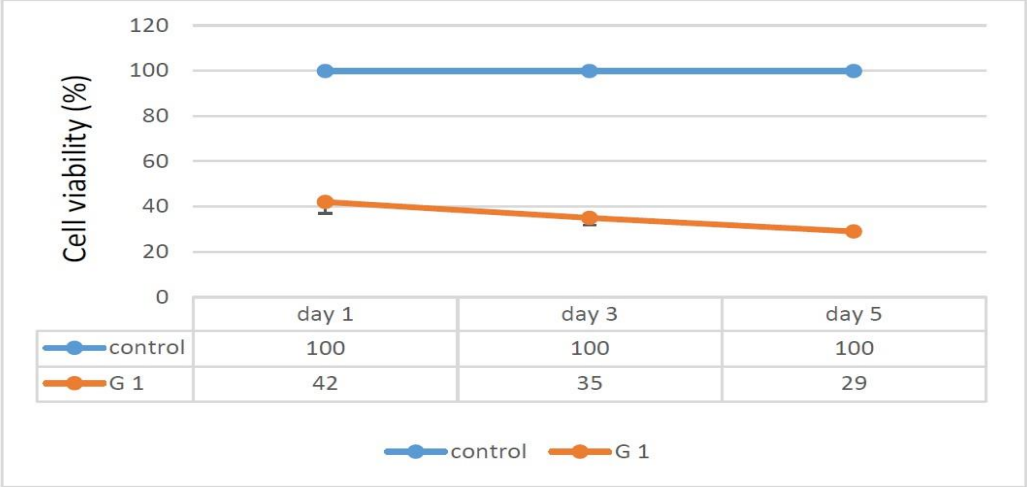
results, IC50 concentration of GW9508 small molecule in 24 hours for C118 cells is 25 μM. The results confirm that the toxicity of this compound is dose-dependent on the viability rate of C118 cells and significantly reduces cell viability by increasing the concentration.



Graph 1- Effects of different concentrations of GW9508 small molecule on the viability of C118 cells using MTT test and determination of 25 μM concentration as the IC50 of C118 cells (Different letters shows significant difference between groups (P <.05). Arrow indicates the IC50 concentration.

Evaluation of C118 treated cell viability with IC50 concentration of GW9508 on days 1, 3, and 5

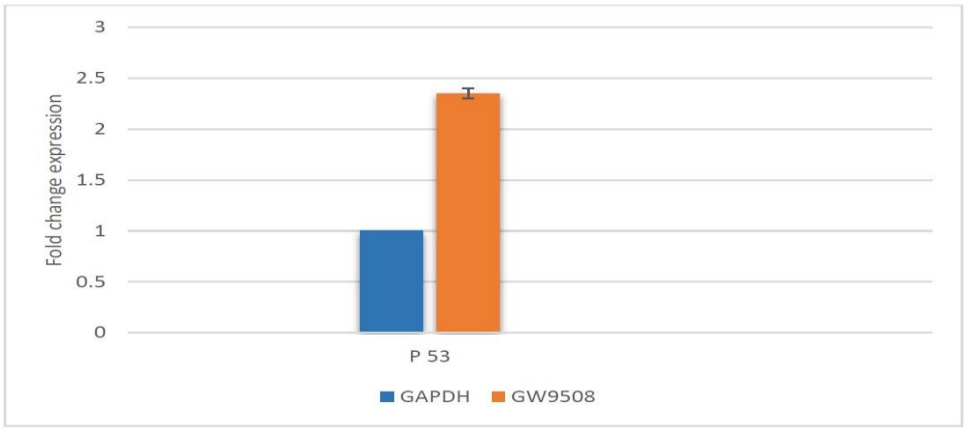
The viability rate of C118 cells on days 1, 3, and 5 in the treated group with IC50 concentration in 25 micromolar of GW9508 small molecule was compared with the control group. The viability percentages of treated cells on days 1, 3, and 5 were 42%, 35% and 29%, respectively. Viability of treated cells in day 5 was significantly reduced compared to treated cells in day 3 and day1. In addition, on the third day, the viability rate of treated cells was lower than the first day, which indicates time and dose dependence of the effect of GW9508 small molecule on C118 cells (P <.05).



Graph 2. The effect of GW9508 small molecule on viability rate of C118 cells on days 1, 3, and 5 after the treatment (P <.05).

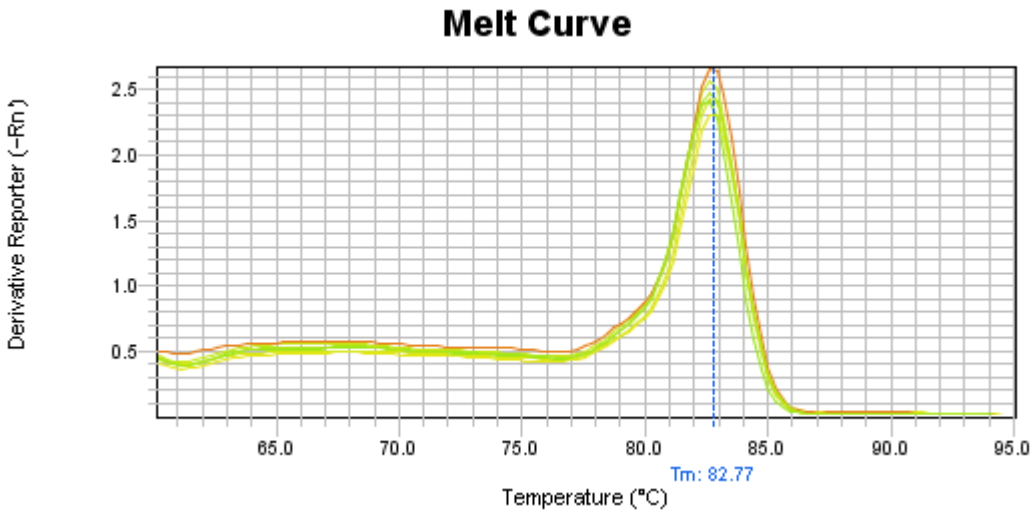
The evaluation of P53 expression change in C118 cells treated with the IC50 concentration of GW9508 small molecule

As you can see in the graph 3, the results of gene analysis in the group treated with GW9508 small molecule show that the expression of P53 gene repressor in 24-hour treated cells with IC50 concentration of the small molecule significantly increased compared to the control group (P <.05), indicating apoptotic cell death in C118 cancer cells.

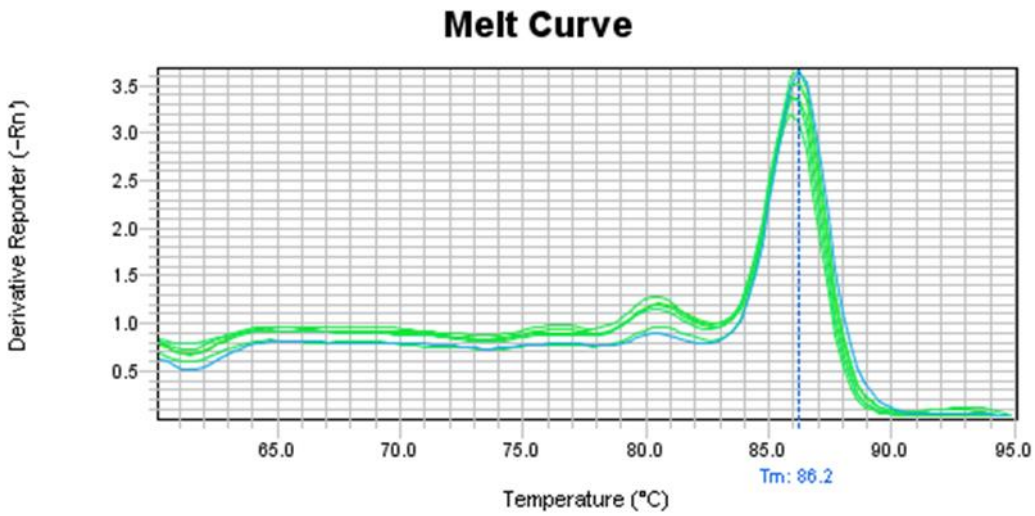


Graph 3- Changes in P53 gene expression in C118 cells under 24-hour treatment with IC50 concentration of GW9508 small molecule (G1): Increased expression of P53 gene in C118 cells treated with IC50 concentration of the small molecule in C118 cell line compared to GAPDH gene indicates induction of apoptotic death in cancer cells by this substance (P<.05, IC50 GW9508= 25mM).

melting curve:



Graph 4- Melting curve of GAPDH Gen



Graph 5 - Melting curve of P53 Gen

4. Discussion

Glioma is a type of tumor engaging the central nervous system (CNS) and one of the most acute malignant tumors in adults caused by the abnormal division of neuroglia cells [14]. Methods for treating the acute glioma based on its type, the incidence place, extent of the tumor, and the patient's general health are determined by surgery and removal of the tumor, radiation therapy, the use of anticonvulsants and corticosteroids, and chemotherapy with DNA alkylation agents such as temozolomide. Due to the side effects, costs, and ineffectiveness of therapies, there is a need to look for new methods and strategies to prevent the disease [15]. Years ago, small molecules in the pharmacology were known as a chemical probe and/or as a mutation factor. Since these molecules weigh less than 900 Daltons, they have a permissible range of molecules through the cell membrane to reach intracellular target positions. These compounds play a regulatory role in the physiological processes of different functions [16]. GW9508 is one of these small molecules that is the GPR40 receptor agonist and plays different roles in messenger pathways and intracellular pathways. This small molecule through generating and increasing oxidative stress of mitochondrial origin can cause the cell to progress to apoptosis and autophagy [15]. Recent studies have shown that unsaturated fatty acids, including linoleic acid, play an important role in the treatment of cancer and act as extracellular signaling molecules by binding to G protein-coupled fatty acid receptors. GPR40 can also induce oxidative stress and apoptosis in pancreatic beta cancer cells [17].

In this study, C118 glioblastoma cancer cells were treated with different concentrations of GW9508 small molecule, then cell viability, cell growth, and proliferation were evaluated on days 1, 3, and 5 after. According to MTT analysis, the concentration of 25 μM was determined as the IC50 concentration for the GW9508 small molecule. The imaging results of this study showed that GW9508 could cause significant changes in the morphology of C118 cells, including lowering the cytoplasm of cells and their shrinkage. These changes were evident in both conventional and acridine orange staining. The mentioned substance significantly reduced the growth and proliferation of C118 cells compared to control cells. The rate of induction of cell death by GW9508 was directly related to the increase in its concentration and duration of treatment. Also, the study of apoptosis and P53 gene analysis using the PCR method showed that the test compound stimulates and increases the expression of P53 gene in C118 cells. Therefore, according to the results, it can be said that GW9508 increases the cytotoxicity and induces apoptosis in cancer cells. So far, many in vitro studies have been conducted, to a lesser extent in animal models, to evaluate the anti-cancer effect of fatty acids as well as agonists of fatty acid receptors on different cell lines. For example, recent clinical studies have shown a potential link between long-chain unsaturated fatty acids and some cancers. Unsaturated free fatty acids exert anticancer and cytotoxic properties through different metabolic pathways (18). Pierre et al. (2013) studied the effects of Trance-10, cis-12 CLA on the induction of cell death in human colorectal cancer, arguing that this substance

causes apoptosis in colon cancer cells by increasing the production of ROS originating in the endoplasmic reticulum. Their studies indicated that CLA-treated tumor cell growth was suppressed by inducing apoptosis and inhibiting cell cycle along with angiogenesis.

Their studies also showed that apoptosis occurs in the mitochondrial membrane [19]. There's strong evidence of the relative inhibitory effect of omega-3 fatty acids on many human cancers [20] including breast cancer [21]. In breast cancer, inhibitory and stimulatory effects of tumors with various GPR40 receptor agonists demonstrate that the use of omega-3 fatty acids or synthetic GPR40 agonists such as GW9508 and TUG-891 also inhibit tumor growth [22]. In another study, the agonistic effect of free fatty acid receptors (FFAR), including the GW9508 small molecule, on the proliferation of MDA-MB-231 breast cancer cells and MCF-7 cell line has been investigated. The results of this study indicate that this compound inhibits the proliferation of cancer cells. GW9508 is a specific agonist of FFA1, has very high cytotoxicity, and prevents the migration and metastasis of MCF-7 breast cancer cells. The PCR results of this study indicate that the gene and protein of FFAR, including GPR40, are expressed in breast cancer. Additionally, activation of FFAR receptors by GW9508 agonist proliferation and cell migration inhibits MDA-MB-231 and MC7-7 cancer [23]. The results of the present study are in line with those of recent studies, as it was observed in this study that GW9508 small molecule increases the cytotoxicity and induces apoptosis in glioblastoma cancer cells. This might be because GW9508 is an agonist with GPR40, which leads to the induction of oxidative stress of mitochondrial origin and cell death in cells [24]. Since the apoptotic process has two intrinsic pathways with the mitochondrial origin and an extrinsic pathway induced by death receptors, the rate of death induced by superficial death receptors is higher than that of mitochondrial pathway [25]. Thus, GW9508 with triggering this pathway causes cell death, DNA fragmentation, chromatin condensation, and cell shrinkage, all of which were found in this study. Another study conducted in 2018 by Behnoosh Rafieinia et al. on the HT29 cell line showed that the viability of cells treated with specific doses of GW9508 was reduced. Bcl-2 expression levels decreased while Bax and Bad expression decreased compared to the control group; as a result, GW9508 could have anticancer and cytotoxic effects in which case it could be used for the treatment of cancers [26]. In this study, it was found that the GW9508 small molecule has the power to significantly increase the expression of the tumor suppressor gene or P53. Because the P53 molecule is one of the most important inhibitors of the cell cycle, this molecule inhibits the cell cycle by interrupting the G1/S phase and prevents tumor formation by inducing apoptosis [27].

Given that, GW9508 inhibited cell cycle in C118 cells by inducing P53 gene expression, and induced cell death. Also, due to vascular proliferation of glioblastoma tumors, three pathways related to this cancer have been identified, one of which is the p53 pathway. Different types of primary and secondary glioblastoma are caused by various changes in these pathways [5]. P53 is one of the most important tumor-inhibiting genes, mutating

in half of the cancers. In general, the anti-cancer effects of P53 occur in three different ways: 1-stimulation of DNA repair proteins, 2- stimulation of programmed cell death, and 3- stopping the cell cycle at the G1/S stage [28]. When DNA is damaged, the cell entry into the S or M stage is inhibited. In this case, concentration, and activity of P53 increases and gets activated from a nonactive form. The normal function of P53 is to protect the genome from damage. This process leads to the repair of the genome, and if not repaired, P53 causes cell apoptosis via cell death and deletes the carcinogenic cell [6]. Inhibition of P53 protein breakdown is therefore an appropriate strategy for the treatment of cancer [29]. GW9508 small molecules activate the mitochondrial pathway of apoptosis by binding to the GPR40 receptor; thus increasing the expression of the P53 gene and inhibiting its degradation. The P53 gene, in turn, inhibits the cell cycle in the G1 phase and causes cell death by inducing cell death.

5. Conclusion

Since the GW9508 small molecule is an agonist for GPR40, it can cause dose-dependent anti-cancer effects by binding to this receptor and, therefore, it is a good option for cancer treatment given the anti-cancer properties of unsaturated fatty acids after binding to the GPR40 receptor. Overall, the findings of this study coupled with those of previous ones show that GW9508 small molecule can be considered a new combination in the treatment of various cancers.

Acknowledgement

We would like to extend our appreciation and thanks to all participants, staff, and managers who made this study possible.

Conflict of interest

The authors declare no conflict of interest.

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Name	Primer Sequence	Tm (C)
GAPDH (F)	5´ACCTTGGAAATAAATGGGAAG-3´	56.9
GAPDH (R)	5´- CTTCTGTGTTGCTGTAGTTGC3´	57.1
P53 (F)	5′-GTTTCCGTCTGGGCTTCTTG-3′	64
P53 (R)	5′-CCTGGGCATCCTTGAGTTCC-3′	64

Table 1. Primer sequences used