

Original Investigation

The Effect of Ascorbic Acid over the Etoposide- and Temozolomide-Mediated Cytotoxicity in Glioblastoma Cell Culture: A Molecular Study

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ABSTRACT

AIM: Glioblastoma (GBM) is one of the lethal central nervous system tumors. One of the widely used chemical agents for the treatment of glioblastoma is temozolomide. It is an orally administered, deoxyribonucleic acid (DNA) alkylating agent. DNA alkylation triggers the death of tumor cells. However, some tumor cells are able to repair this type of DNA damage and thus lower the therapeutic effect of temozolomide. Laboratory and clinical studies indicate that temozolomide's anticancer effects might be strengthened when combined with other chemotherapeutic agents like etoposide or antioxidant agents like ascorbic acid. In this study, we aimed to evaluate the cytotoxic and oxidative stress effects of ascorbic acid (1000 µM), temozolomide (100 µM) and etoposide (25 µM) agents alone and in dual and triple combinations in a glioblastoma U87 MG cell culture.

MATERIAL and METHODS: The cytotoxic and oxidative stress effects were investigated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and liquid chromatography tandem-mass spectrometry (LC-MS/MS) analysis methods.

RESULTS: Cytotoxicity tests showed that etoposide, temozolomide, "etoposide+ascorbic acid", "temozolomide+ascorbic acid", "temozolomide+etoposide" and "temozolomide+etoposide+ascorbic acid" combinations have anti-proliferative effects. The maximum anti-proliferation response was observed in the "temozolomide+etoposide+ascorbic acid"-added group. Similarly LC-MS/MS analyses showed that minimum oxidative DNA damage occurred in the "temozolomide+etoposide+ascorbic acid"-added group.

CONCLUSION: Ascorbic acid decreases the cytotoxic and genotoxic effect of etoposide and etoposide-temozolomide combination but it has no meaningful effect on temozolomide's toxicity.

KEYWORDS: Glioblastoma, Ascorbic acid, Cytotoxicity, Etoposide, Temozolomide

INTRODUCTION

Any tumor that arises from the glial or supportive tissue of the brain or the spinal cord is called "glioma". One of the types of glioma is astrocytoma. Astrocytomas arise from astrocytes, the star-shaped cells, and are graded to describe their degree of abnormality. The most common grading system uses a scale of I to IV (15,16,18).

Glioblastoma (GBM, WHO grade IV) accounts for 50% of all the intracranial tumors and 70% of the primary malignant brain tumors. Although GBM often occurs in late adulthood (70% at a mean age of 53 years), up to 8.8% occur during childhood. Absence of specific symptoms is the predominant reason for which tumors are often detected at advanced stages, leading to poor prognosis with a mean survival of 1 year for GBM (18).



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Ascorbic acid is a water-soluble antioxidant with the potential to protect biochemical molecules from oxidative damage. More recently, *in vitro* studies on animal models found intravenous ascorbic acid at high doses to be promising in the treatment of cancers. More than three decades ago, benefits of mega-dose ascorbic acid treatment for patients with advanced cancer were claimed. Nonetheless, its effects have not been confirmed by an adequate number of studies (2).

For decades, the basic malignant glioma treatment was radiotherapy followed by chemotherapy with the use of alkylating agents such as nitrosoureas, procarbazine and more recently temozolomide (24). Temozolomide is an orally bioavailable, methylating agent that can pass through the blood-brain barrier and trigger the death of tumor cells. However, some tumor cells can repair this type of deoxyribonucleic acid (DNA) damage, and therefore diminish the efficiency of the therapy. Laboratory and clinical studies indicate that temozolomide's anti-cancer effects might be strengthened when combined with other chemotherapeutic agents like etoposide (5,12).

Etoposide (VP-16,4'-dimethylepipodophyllon-9-(4,6-O-ethylidene-beta-D-glucopyranoside)) is an anti-cancer agent, which is successfully and extensively used in treatments of various types of cancers in children and adults. It forms a ternary complex with the DNA and the topoisomerase II enzyme and so causes DNA strands to break (6,12).

In laboratory studies, the effects of chemotherapeutics can be tested by cytotoxicity and genotoxicity assays in the cell cultures. Cytotoxicity refers to the ability of chemicals to destroy living cells while genotoxicity refers to the ability of chemicals to damage the genomic DNA. Cytotoxicity assays measure cell viability; genotoxicity assays measure the DNA lesions that are the product of the DNA damage.

For cytotoxicity assays, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) tetrazolium reduction assay is generally used. Viable cells with active metabolism convert the MTT into a purple-colored formazan product with an absorbance near 570 nm. When cells die, they do not have the ability to convert the MTT into formazan. This color formation serves as a useful marker of the living cells (17).

For genotoxicity assays, 8-oxo-7,8-dihydroguanine (8-OH-dG), which is an oxidative DNA damage product, can be measured by liquid chromatography tandem-mass spectrometry (LC-MS/MS). Reactive oxygen species (ROS) are generated during the normal cellular metabolism and normally detoxified by anti-oxidant enzymes. Oxidative stress occurs when the endogenous ROS detoxification pathways are inadequate because of some situations, e.g. viral and bacterial infections, inflammation, metabolism of drugs such as etoposide, exposure to some chemicals, or smoking. During oxidative stress, ROS can damage cellular DNA. These endogenous DNA lesions are genotoxic and can induce mutations. The most extensively studied lesion is the formation of 8-OH-dG. As the lesion is relatively easily formed, mutagenic and therefore a potential biomarker of oxidative DNA damages, 8-OH-dG, can be measured by LC-MS/MS to evaluate the oxidative DNA damage effects, in other words the genotoxic effects of chemicals (14,20-22).

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In this study, we aimed to analyze the cytotoxic and genotoxic effects of ascorbic acid over etoposide and temozolomide in a glioblastoma cell culture. For cytotoxicity and genotoxicity analysis, MTT and LC-MS/MS experiments were performed.

■ MATERIAL and METHODS

Cell Culture

Studies were carried out in the human glioblastoma cell line U87MG (HTB-14). They were cultured in Dulbecco's Modified Eagle Medium (DMEM) with stable L-glutamine and sodium pyruvate (Life Technologies, Cat No:22320-030) supplemented with 10% fetal bovine serum (Life Technologies, Cat No:10082-147) without antibiotics in a humidified incubator at 37°C and 5% CO₂ atmosphere. Confluent cells were detached with trypsin-ethylenediaminetetraacetic acid (EDTA) solution (Life Technologies, Cat No:R-001-100) in calcium-free phosphate buffered saline (PBS) and counted in a Neubauer chamber.

Cytotoxicity Assay

The effects of ascorbic acid (1000 µM), temozolomide (100 µM) and etoposide (25 µM) agents alone, in dual and triple combinations on the viability of glioblastoma cell line (U87MG) were studied after 48, 96 and 144 hours of treatment in triplicate. Cells were seeded into 24-well plates in a volume of 500 µl per well at a density of 100,000 cells/well and grown at 37°C in a humidified 5% CO₂ incubator. Cell proliferation was measured by a quantitative colorimetric assay using MTT (3). The data was expressed as a percentage of control.

MTT (Sigma Cat No:M5655) powder was dissolved in PBS at the concentration of 1 mg/ml. Single agent and dual and triple combinations of ascorbic acid (1000 µM), temozolomide (100 µM) and etoposide (25 µM)-added cells were incubated for 48, 96 and 144 hours at 37°C in a humidified 5% CO₂ incubator. After the incubation period, growth medium was removed and 400 µL MTT solution (1 mg/ml) was added to each well. They were incubated for nearly 1 hour until purple precipitates became visible. After the development of visible purple precipitates, each well was filled with 1000 µL dimethyl sulfoxide (DMSO) and isopropanol (1:5) mixture. Plates were incubated for nearly 1 hour until the purple precipitate completely dissolved. Dissolved formazan crystals of each well were measured at 570 nm in the spectrophotometer (Shimadzu UV-VIS, UVmini-1240).

Oxidative DNA Damage Analysis

In this study, the DNA damage product 8-OH-dG was measured by LC-MS/MS to evaluate the oxidative DNA damage effects of chemicals. Firstly, DNA isolations were performed. After that, DNA samples were enzymatically hydrolyzed and analyzed by LC-MS/MS.

DNA isolation

DNA samples from cell cultures were extracted according to the instruction manual of the extraction kit (Zymo Research, Cat No:D3025). Concentrations of the DNA samples were measured by Qubit 2.0 fluorometer (Invitrogen, Cat No:Q32866).

Enzymatic hydrolysis of the DNA

DNA hydrolysis was performed by adding a mixture of DNase I (1U/50 µl) to equal DNA sample solutions, followed by incubation for 8-10 hours at 37°C according to enzyme's instruction manual (MN, Cat No:740963).

Mass Spectrometry

Samples were filtered through a 0.45-µm pore sized membrane before injection. The Agilent 1100 high performance liquid chromatography (HPLC) system (Agilent Technologies, Palo Alto CA, USA) operated by Windows NT based ChemStation software was used. The system consisted of a binary pump, degasser and auto sampler. The column used was a Phenomenex Luna reversed-phase C-18 column (4.6 mm×250 mm, 5 µm) (Torrance, CA, USA). The mobile phase consisted of two solvents: Solvent A, water/formic acid (99:1; v/v) and Solvent B, acetonitrile/solvent A (60:40; v/v). Compounds were eluted in the following environment: 0.5 mL/min flow rate and the temperature was set at 25°C, isocratic conditions from 0 to 10 minutes with 0% B, gradient conditions from 0% to 5% B in 30 minutes, from 5% to 15% B in 18 minutes, from 15% to 25% B in 14 minutes, from 25% to 50% B in 31 minutes, from 50% to 100% B in 3 minutes, followed by washing and reconditioning the column. Triplicate analyses were performed

for each sample. The identification and assignment of each compound was confirmed by LC-MS/MS analysis. Agilent 6430 LC-MS/MS spectrometer equipped with an electrospray ionization source was used. The electrospray ionization mass spectrometry detection was performed with the following optimized parameters: capillary temperature 400°C; drying gas N₂ 12 L/min; nebulizer pressure, 45 psi (10). Data collection was performed with the use of multiple reactions monitoring (MRM) method that monitors specific mass transitions only during the preset retention times. Dynamic MRM also has the ability to simultaneously perform electrospray injection (ESI) in both the positive and the negative mode, allowing for the analysis of different phenolic classes within a single chromatographic run. Transition of m/z 284 - m/z 168 (for 8-oxo-dGuo) was monitored.

RESULTS

Cytotoxicity was determined by the MTT assay. MTT assay data (Figure 1) revealed that ascorbic acid decreased the number of cells within 48 hours. Within 96 and 144 hours, however, it was observed that the ascorbic acid had no cytotoxic effect. In addition, the cell numbers were increased. Temozolomide and etoposide decreased the cell numbers within 48, 96 and 144 hours alone and in dual combinations. It is assumed that the ascorbic acid had an inhibitory impact over temozolomide and etoposide's cytotoxic effects. Interestingly, the best cytotoxic effect was achieved when the ascorbic acid, temozolomide and etoposide triple combination was applied. The results indicate that the utilization of ascorbic acid increases cell proliferations in the temozolomide and etoposide-added cell

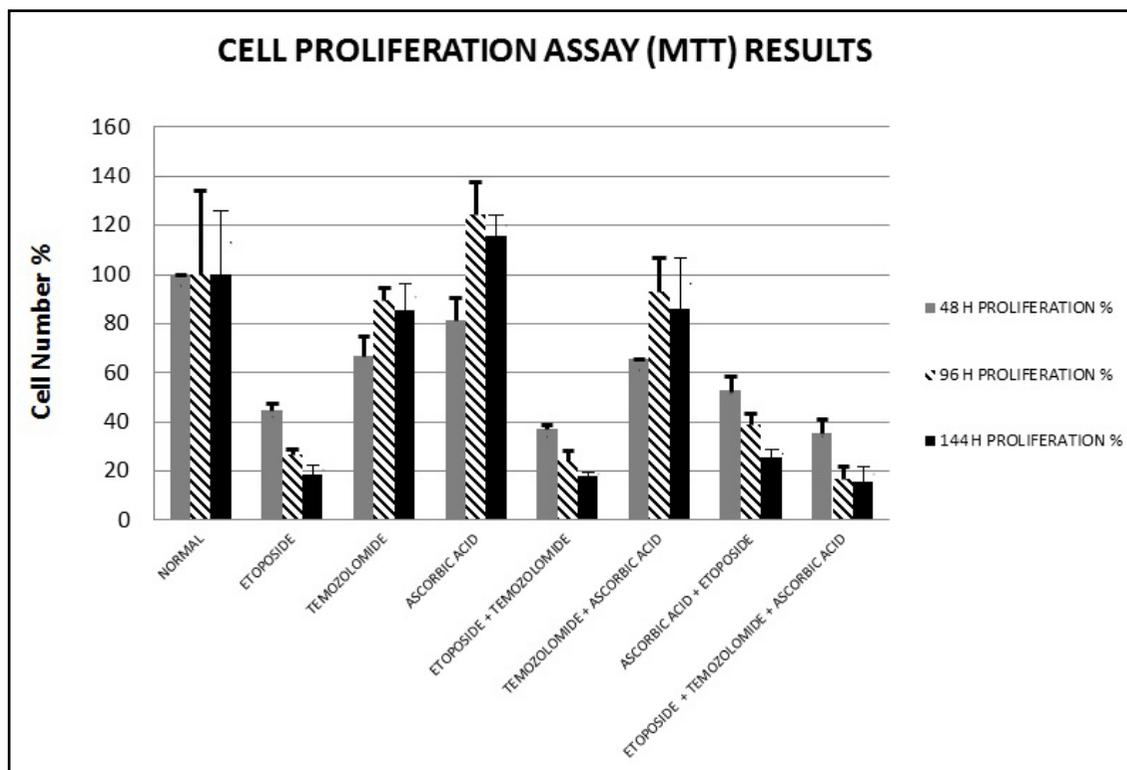


Figure 1: Cell proliferation graphics determined by MTT assay after 48, 96 and 144 hours incubation with Ascorbic acid (1000 µm), Temozolomide (100 µm) and Etoposide (25 µm).

cultures separately. Furthermore, ascorbic acid exhibited cell proliferation inhibitory effect in the etoposide, temozolomide and ascorbic acid-added group and the most cytotoxic effect was noted in the group applied that triple chemotherapeutic combination.

A sensitive method for the detection of 8-oxo-dG in DNA samples has been optimized. It utilizes immunoaffinity column purification of 8-oxo-dG followed by LC-MS/MS MRM mode analysis. The 8-oxo-dGuo base product (an oxidative

DNA damage product) ion at m/z 168 was monitored following cleavage of the glycosidic bond of the 8-oxo-dG (M+H)⁺ ion at m/z 284. DNA damage analysis data that was obtained from LC-MS/MS experiments (Figure 2A, B) showed that ascorbic acid had an impact that increased the 8-oxo-dGuo concentration. It increased genotoxicity when used by itself but it decreased 8-oxo-dGuo concentrations. Therefore, it increased genotoxicity in the etoposide and etoposide-temozolomide combination-added groups. It was also observed that temozolomide had decreasing effect on 8-oxo-dGuo concentration

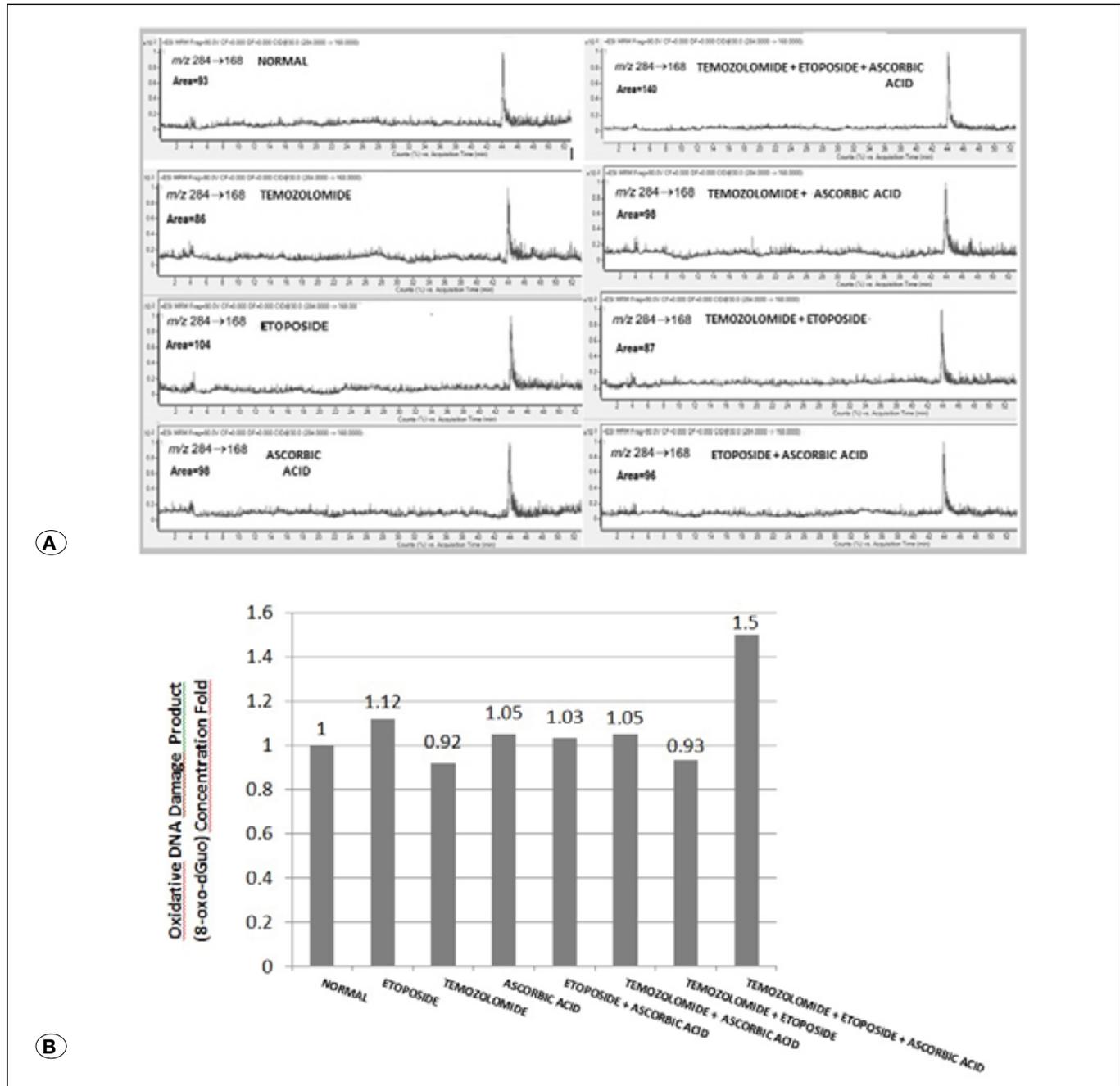


Figure 2: A) Oxidative DNA Damage Product (8-oxo-dGuo) analysis LC-MS/MS Chromatogram after 96 hours incubation with Ascorbic acid (1000 μ m), Temozolomide (100 μ m) and Etoposide (25 μ m). **B)** DNA damage product (8-oxo-dGuo) concentration fold graphics determined by LC/MS analysis after 96 hours incubation with Ascorbic acid (1000 μ m), Temozolomide (100 μ m) and Etoposide (25 μ m).

and no influence was observed on genotoxicity in the ascorbic acid-added group.

■ DISCUSSION

Although there have been many reports of ascorbic acid, etoposide and temozolomide's single and dual effects on many cancers, including GBM (4,5,7,23), no studies have reflected the use of triple combinations in malignant glioblastoma cell lines. In this study, the effect of ascorbic acid, etoposide and temozolomide on glioblastoma cell proliferation was examined by exposing U87 MG cells to ascorbic acid (1000 μ M), temozolomide (100 μ M) and etoposide (25 μ M) for 48, 96, and 144 hours in DMEM medium containing 10% serum.

Genotoxicity data obtained from LC-MS/MS experiments (Figure 2A, B) have shown that ascorbic acid alone has an impact that increases oxidative DNA damage. The findings were confirmed in a number of studies performed on different cell cultures in the literature. For instance, it was reported that incubation of human lymphocytes, neonatal fibroblasts, and leukemia cell line with ascorbic acid for 1 hour resulted in DNA single-strand breaks (20).

Consistent with the cytotoxicity data, genotoxicity was decreased in the etoposide and etoposide-temozolomide combination-added groups. The strongest genotoxic effect was observed when the combination of ascorbic acid, etoposide and temozolomide was applied to the cells. It can be said that cytotoxic and genotoxic effects of temozolomide and etoposide were decreased by ascorbic acid, but the highest cytotoxicity with the slightest genotoxicity was achieved with use of the triple combination. The reason might be that, in the increased genotoxic circumstances, ascorbic acid makes cells stop the cell cycle and go into senescence. However, when relatively less genotoxicity occurs, ascorbic acid can protect cells from DNA damage and cytotoxic effects.

While ascorbic acid can protect DNA against the damage induced by various chemicals (1,8), some reports indicated that it can be genotoxic (9,20). Similar results were obtained from our experiments that aimed to test the effect of ascorbic acid over the chemotherapeutic drugs etoposide and temozolomide in a glioblastoma cell line U87 MG. It was noted that ascorbic acid had a genotoxic potential when used by itself.

In addition, it was observed that temozolomide had a decreasing effect on 8-oxo-dGuo concentration and had no influence over the genotoxic effect in the ascorbic acid-added group. The above may be explained as follows. The therapeutic benefit of temozolomide depends on its ability to alkylate/methylate DNA, which most often occurs at the N (nitrogen) 7 and O (oxygen) 6 positions of guanine residues, causing a base pair mismatch. This methylation damages the DNA, and when mismatch repair (MMR) enzymes attempt to remove the 8-oxo-dGuo adduct, they produce single and double-strand breaks in the DNA and trigger cell death (11,13). Removing the methyl guanine adducts causes a decrease in the guanine residue concentrations. As such, the production of 8-oxo-dGuo adducts diminishes.

■ CONCLUSION

Ascorbic acid decreases the cytotoxic and genotoxic effect of the etoposide and etoposide-temozolomide combination, but it has no meaningful effect on temozolomide's toxicity. To disclose the effects of ascorbic acid on the DNA damages and cytotoxicity, further studies with different chemotherapeutic drugs and cancer cell lines are needed. Cell cycle experiments are also required to clarify the effects of using ascorbic acid alone and in combinations with chemotherapeutic drugs on cell proliferation.

■ ACKNOWLEDGEMENT

This work was supported by Scientific Research Projects Fund of Adana Science and Technology University (Project no: MÜHDBF.BİYO.2014-5). The authors thank Assist. Prof. Tuğba Bağcı Önder from Koç University, Faculty of Medicine, Molecular Biology Department for kindly donating the U 87 MG Glioblastoma cell line.

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