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Anticancer Effects of Alpha-lipoic Acid on A172 and U373 Human Glioblastoma Cells

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Highlights:

- The cytotoxicity of ALA on glioblastoma cells was investigated by MTT assay.
- ALA have exhibits antiproliferative, apoptotic, and anti-migrating activity in A172 and U373 cells.
- ALA was investigated by quantitative analysis of mRNA levels of apoptosis-related genes in glioblastoma cells.

ABSTRACT:

The high mortality rate of Glioblastoma multiforme (GBM) patients is partly due to the invasive behavior of the tumor cells. Given the increased resistance to conventional therapies of invasive cells after surgical operations, current treatments are ineffective. Therefore, understanding the mechanisms of GBM cell invasion is critical for the development of successful therapeutic approaches. Natural small molecules and metabolites are widely used as chemotherapeutic and adjuvant agents in cancer treatments because they have strong anticancer properties and minimal side effects. Alpha-lipoic acid (ALA) is an antioxidant that has been found to reduce the level of ROS and increase GPx activity in cancer patients. In this study, we analyzed the *in-vitro* cytotoxic potential and apoptotic effect in A172 and U373 cells in the presence of various concentrations (7.8-500 µM) of ALA. We also investigated scratch assay in both cell lines. The ALA inhibited cell viability of A172 and U373 cells at 48h. In addition, Bax mRNA expression was significantly increased in response to ALA for A172 cells. Furthermore, the BCL-2 level was decreased in A172 cells with ALA after 48h. Caspase 3 and 9 mRNA expressions were increased in ALA treated U373 cell line. In summary, we found that ALA inhibits cell growth and causes apoptosis in A172 and U373 glioblastoma cells.

Keywords:

- Alpha-lipoic acid
- Anticancer
- Glioblastoma
- Wound healing
- RT-PCR

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INTRODUCTION

Glioblastoma multiforme (GBM) is the most common type of malignant brain tumor, and it occurs in the central nervous system. GBM is a disease that is usually seen in adults, has an aggressive character and is also difficult to treat. (Ostrom et al., 2021; Wang and Wang, 2022). Although the incidence of GBM is low, the survival time is also low due to its rapid spread and invasion of vital organs. Even in those receiving chemotherapy and radiotherapy, the average survival time is between 7-15 months and the survival rate is between 0.05% and 4.7% (Li et al., 2021). GBM is a very difficult type of cancer to treat due to its aggressive nature and heterogeneity. Difficulties of treatment in GBM depend on several factors such as rapid cell turnover, limited brain self-repair capacity, and blood-brain barrier (BBB) permeability, resistance to receive desired chemotherapeutics, intratumoral heterogeneity, intrinsic GBM resistance and non-specific toxicity (Lacob and Dinca 2009; Ellis et al., 2015). Today, surgery, radiotherapy and chemotherapy with alkylating agents are still at the center of glioblastoma treatment (Liu et al., 2021; Khasraw et al., 2022). Within the scope of technological and scientific advances for GBM, studies are continuing to develop more effective treatment strategies and to find new treatment agents (Khasraw et al., 2022).

Natural compounds and bioactive molecules derived from herbal plants have been used as ethnopharmacological agents against different diseases for many years (Seçme and Dodurga, 2021). ALA is a short-chain fatty acid that contains sulfur in its structure (Attia et al., 2020). ALA is a natural antioxidant compound and is synthesized in small amounts in animals and plants. Studies showed that ALA can be used in the treatment of diabetes and hypertension and Alzheimer diseases (Barky et al., 2017; Attia et al., 2020). Studies on the antitumor effect of ALA are increasing considerably. Anticancer activities of ALA have been demonstrated in cancer types such as breast (Na et al., 2009), liver (Simbula et al., 2007), lung (Moungjaroen et al., 2006), leukemia (Alpay et al., 2016) and colon cancer (Yoo et al., 2013) by changing cell proliferation, cell cycle, apoptosis, and other cellular mechanisms. In the light of these, this study was aimed to determine the effect of ALA on cell proliferation, cell migration, and apoptosis in A172 and U373 glioblastoma cell lines under *in-vitro* conditions.

MATERIALS AND METHODS

Reagents

Fetal Bovine Serum (FBS), 0.25% Trypsin-EDTA, and DMEM (Dulbecco's Modified Eagle Medium) were purchased from Gibco (USA). 1x phosphate-buffered saline (PBS) and 100x penicillin/streptomycin were obtained from Capricorn (Germany). Alpha-lipoic acid (ALA) was purchased from Sigma (Cat#62320-5G-F). Annexin V-FITC/PI Apoptosis Detection Kit and MTT reagent were purchased from BioVision (USA). Dimethyl sulfoxide (DMSO) was obtained from Carlo Erba (USA).

Cell culture

The A172 and U373 (Human glioblastoma cell lines) were obtained from the European Collection of Authenticated Cell Cultures (ECACC). Both cells were incubated in complete culture medium (1% penicillin/streptomycin mix and 10% FBS) at 37 °C in a 5% CO_2 incubator described previously (Konus et al., 2022). The medium was refreshed 2 times a week, and at 90% confluency cells were passaged.

MTT assay

The cancer cells were seeded on 96-well plates at a concentration of 2×10^3 cells/well in DMEM. Following 24 h, the culture medium was refreshed with culture medium containing different concentrations (7.8, 15.6, 62.5, 125, 250, and 500 µM) of ALA dissolved in 100% DMSO and the plates were cultured for 24 and 48 h. Control groups received DMSO only (not exceed 1%). Cell viability was conducted using the MTT assay (Sahin et al., 2021). The absorbance was detected at 590nm using an Epoch Microplate Spectrophotometer (BioTek Instruments, USA). The ALA was dissolved in DMSO to prepare a stock at a concentration of 50 mM and stored at -20 °C before use. All experiments were performed in triplicate.

Wound stracth assay

For wound stracth assay, the cell suspension was prepared at a concentration of $3x10^4$ cell/well and seeded onto a 6-well plate described previously (Mutlu et al., 2022). After 80-90% confluency, the cells were scratched with a 200 µl tip to make a gap. Afterward, plates were washed for removing cell debris with 1x PBS. Then, EC_{50} of ALA was applied to cancer cells and scratch closure was imaged until the 48h. After incubation, photos of the control and treated wells were taken. For this purpose, 10x microscope magnification was used (Oxion Inverso Inverted Microscope, Euromex). The wound scratch assay was carried out in triplicate and analyzed by ImageJ software 1.53e (USA).

RNA isolation, cDNA synthesis, and qRT-PCR analysis

A172 and U373 cells were treated with ALA for 48 h. innuPREP RNA Mini Kit 2.0 (Analytik Jena, Germany) was used to isolate the total RNAs, and cDNAs were synthesized using OneScript® Plus cDNA Synthesis Kit (abm, USA). Applied Biosystems StepOnePlus™ system was used for qRT-PCR. Changes in mRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method. The mRNA levels of genes involved in apoptosis (Table 1) were normalized to GAPDH. The experiment was performed in triplicate.

Table 1. Sequences of the primers (R, reverse; F, forward).

Gene	Accession code	Primer sequence
GAPDH	NM_002046	**F:**GTCTCCTCTGACTTCAACAGCG **R:**ACCACCCTGTTGCTGTAGCCAA
BAX	NM_004324	**F:**TCAGGATGCGTCCACCAAGAAG **R:**TGTGTCCACGGCGGCAATCATC
BCL-2	NM_000633	**F:**ATCGCCCTGTGGATGACTGAGT **R:**GCCAGGAGAAATCAAACAGAGGC
CASP3	NM_004346	**F:**GGAAGCGAATCAATGGACTCTGG **R:**GCATCGACATCTGTACCAGACC
CASP9	NM_001229	**F:**GTTTGAGGACCTTCGACCAGCT **R:**CAACGTACCAGGAGCCACTCTT

Statistical analysis

Data are given as the mean ± SD by Graphpad Prism 9.0 software. Student's *t*-test followed by Tukey's post-hoc test for multiple comparisons was used to compare the results. $P < 0.05$ was chosen for statistically significant difference.

RESULTS AND DISCUSSION

ALA suppresses A172 and U373 cell viability

The cell viability was performed to evaluate the effects of ALA on cultured A172 and U373 cells by MTT assay as described in methods part. The cell viability was decreased with increasing dose of

ALA in A172 and U373 cells and EC_{50} value was found as 445.90 µM and 440.83 µM, respectively (Figure 1a, b).

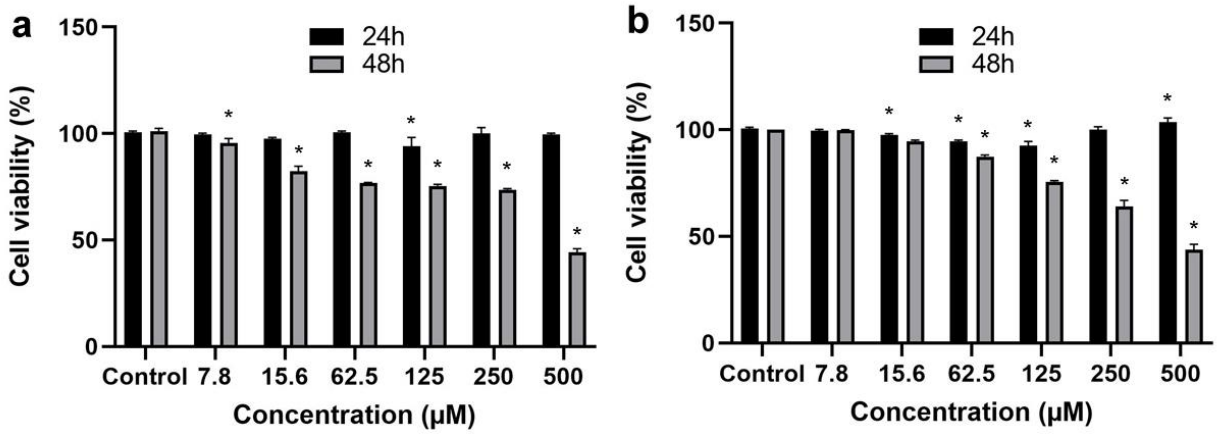

Figure 1. The cytotoxic effects of ALA after treatment for 24 and 48 h on glioblastoma cell lines (a) A172 and (b) U373. Shown are means ±SD from three experiments * $P < 0.05$

Wound stratch assay

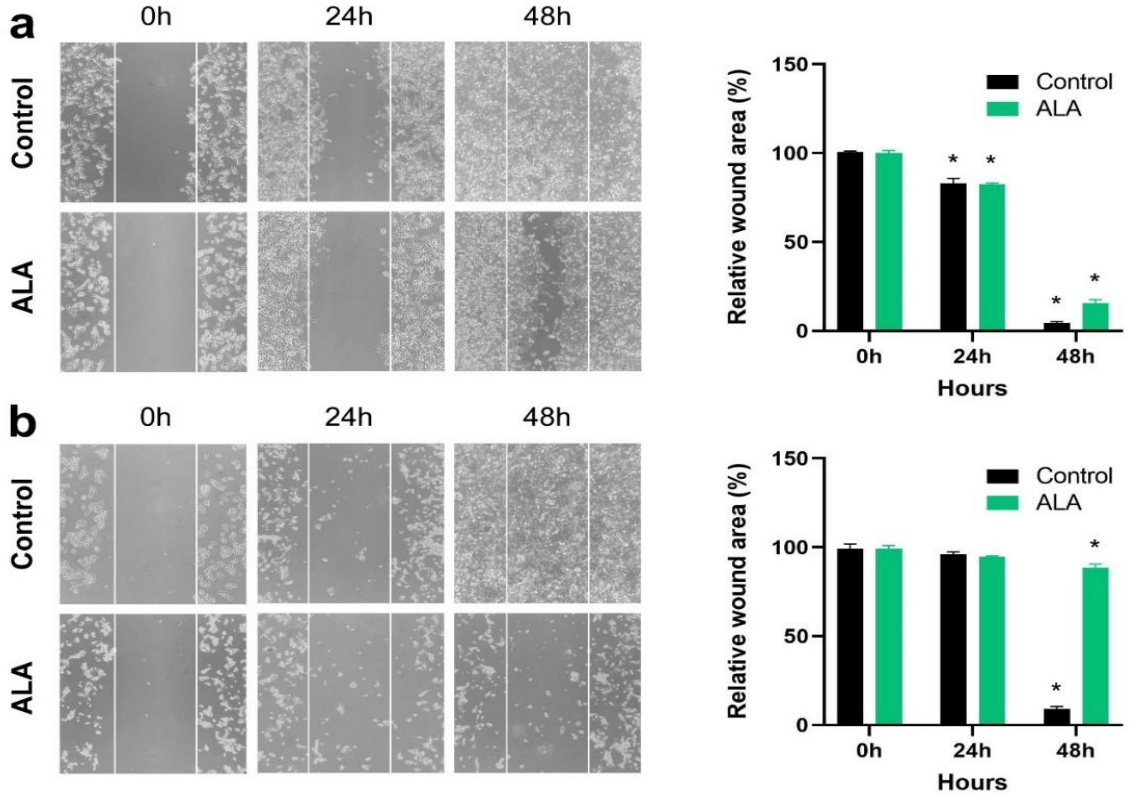

Figure 2. Representative images of *in-vitro* wound healing assays on glioblastoma cell lines (**a.** A172 and **b.** U373) and bar graphs illustrating percentage wound closure at indicated time points during the assay. Shown are means ±SD from three experiments *$P < 0.05$

The *in-vitro* wound stratch assay was conducted to compare the migration rate of A172 and U373 cells of ALA. Figure 2 summarizes the % migration of both cells after 0, 24, and 48 h of incubation with effective concentration (445.90 µM for A172 and 440.83 µM for U373) of ALA. The inverted microscopy (Oxion Inverso, Euromex, Netherlands) images of the scratches after 0 and 48 h

are shown in Figure 2. Compared to treatment, % closure was similar for the control groups until 48 h incubation, the ALA showed the highest migration on 48 h with 86% of wound closure for A172 cells. In addition, the closure of the wound for U373 cells treatment with ALA was also observed (4%), although the cells were disrupted, and the cell morphology changed to more round-shaped (Figure 2).

Gene expression analysis

The effects of ALA on caspase 3, caspase 9, BAX and BCL-2 mRNA levels were determined in both cell lines. In our study when the related gene expressions of A172 cells were examined, it was shown that increased BAX expression (10.32-fold) and decreased the expression of BCL-2 (2.26-fold), caspase 3 (1.39-fold), and caspase 9 (1.26-fold) genes. On the other hand, the related gene expressions of U373 cells were examined, and it was shown that increased the expression of BAX, BCL-2, caspase 3, and 9 genes (1.94, 1.73, 2.08, and 2.08-fold), respectively (Table 2). The ratio of Bcl-2/Bax, which was used as the index of apoptotic efficiency, was reduced significantly ALA treated A172 cellss at concentration of EC_{50}. ($P < 0.05$). In conclusion, our study showed that ALA inhibits cell proliferation and induced apoptosis in A172 and U373 glioblastoma cancer cell lines.

Table 2. The changes of mRNA expression levels after treatment with ALA. Negative fold change values indicate an decrease in expression, and positives a increase in expression

	A172		U373	
Gene	**Fold change**	***p-value***	**Fold change**	***p-value***
BCL-2	-2.26	0.003371	+1.73	0.052622
BAX	+10.32	0.000372	+1.94	0.738060
caspase 3	-1.39	0.358729	+2.08	0.062298
caspase 9	-1.26	0.003405	+2.08	0.286318

Although not using a non-cancer cell line is a limitation in this study, it has been reported in the literature that the cytotoxic activity of ALA in non-cancer cells used for control purposes occurs at higher doses than in cancer cells. In a study, it was demonstrated that the ALA dimer did not have cytotoxic activity between 6.25 and 50 μM dose range in HT22 mouse hippocampal neuronal cells (Wang et al., 2019). In another study, it was reported that ALA had no harmful effects on astroglial cells and had no toxic effects (Bramanti et al., 2010).

The cytotoxic effects of ALA were found in several distinct cancer types such as murine melanoma (Packer, 1998), ovarian carcinoma (Vig-Varga et al., 2006), inhibition of cell proliferation, and inducing the apoptosis of colon carcinoma (Wenzel et al., 2005), inhibited cell growth, colony formation ability, and the nontoxic concentrations significantly reduced the migration of breast cancer (Tripathy et al., 2018). Cell migration and proliferation capacity of cells play an important role in metastasis and cancer progression (Yamaguchi et al., 2005). In this study, the wound stracth assay was used to evaluate cell migration. Similar effects were seen in both cell lines, with the ALA reducing the ability of wounds to heal. These findings suggest that ALA inhibits the growth of many types of cancer cells.

Apoptosis is controlled by different genes that are involved in two main apoptotic pathways, extrinsic and intrinsic. There are many mechanisms involved in apoptosis, including antiapoptotic proteins such as the Bcl-2 family and proapoptotic proteins such as Bax and caspases (Yang et al., 1995; Hitoshi et al., 1998; Hongmeii, 2012). It was previously reported that ALA increased the caspase 3 activity and decreased Bcl-XL in human colorectal carcinoma (HT-29) cell line (Wenzel et al., 2005). Choi et al. (2009) reported that ALA treatment inhibited proliferation of the lung cancer cells and caused induction of apoptosis. Similarly, Puchsaka et al. (2016) reported that ALA induced apoptosis in lung cancer cells. These results are consistent with the results of our study.

These results showed that ALA an inhibited cell proliferation and induces apoptosis in glioblastoma cells (A172 and U373). In conclusion, these findings indicate that ALA has the potential as a therapeutic drug to inhibit cancer.

CONCLUSION

Our study has shown for the first time that ALA dose-dependently caused a significant inhibition of A172 and U373 cell proliferation at 48 h. The ALA decreased wound healing ability in both cell lines. Also, the ALA modulated the expression of BAX, BCL-2, caspase 3, and caspase 9. These results highlight the promising anticancer effects of ALA. Although there are various studies in the literature on the anticancer activities of ALA, further studies are needed to clarify these pathways.

Conflict of Interest

The article authors declare that there is no conflict of interest between them.

Author's Contributions

The authors declare that they have contributed equally to the article.

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