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Evaluation of Apoptotic and Autophagic Effects of Thioridazine in Monolayer and Spheroid Cell Cultures

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ABSTRACT

Glioblastoma (GBM) is the most frequently seen brain tumor in adults. It is clear that new therapeutic agents are needed in this area. Spheroid cell cultures have advantages on imitating in vivo environment. Therefore, we investigated apoptotic and autophagic effects of Thioridazine (THZ) which is a potential anti-cancer agent on GBM cell lines, T98G and U-87 MG, comparatively in monolayer and spheroid cell cultures.

The cytotoxic effect of THZ on cells was evaluated by MTT method. Besides, apoptotic and autophagic effects of THZ were determined by Annexin V and LC-3 antibody based methods. Validation performed also by assessment of apoptosis and autophagy with specific inhibitors. The analysis has revealed that IC_{50} values at 24 h were 12,67 µM (T98G) and 12.80 µM (U-87 MG) in monolayer cell culture, 29.30 µM (T98G) and 28.68 µM (U-87 MG) in spheroid cell culture. While apoptotic cell rate was determined at 24 h approximate 15% in both cell lines and cell cultures, autophagy induction rate was increased by 6.5- (T98G) and 5.6- (U-87 MG) fold in monolayer cell culture and by 3.3- (T98G) and 4.5- (U-87 MG) fold in spheroid cell culture.

In our study THZ stimulates mostly autophagy rather than apoptosis. In addition, it can be suggested that *in vitro* cytotoxicity studies should perform with not only monolayer cell cultures but also with spheroid cell cultures for obtaining *in vivo*-like data. When all the evaluations are considered, THZ might be thought as a potential chemotherapeutic agent in the treatment of GBM.

Keywords: Monolayer cell culture, Spheroid cell culture, Glioblastoma Multiforme, Thioridazine, Apoptosis, Authophagy

Introduction

Glioma is a cancerous form of astrocytes and accounts for about 80% of brain tumors (1-3). Glioblastoma (GBM), which is the grade IV of glioma, is most frequently seen malignant brain tumor in adults. After diagnosis, average life span is 12-15 months (4, 5). In terms of age, tumor development and molecular properties, GBM divides into two groups as primer and seconder GBM. While primer GBM is mostly seen in individuals aged 55-60 years and over, seconder GBM developed by malignant transformation of grade II or III astrocytoma is seen in individuals under 45 years of age (5-7). The most common difficulties in GBM treatment is resistance to chemotherapy and radiotherapy. The reason for the failure in chemotherapy is that the drugs used in chemotherapy can not pass through the blood-brain barrier or inadequate in inducing cell death mechanism even if it passes. Apart from these, uncontrolled proliferation of cells and metastasis minimise success of surgery (8, 9).

Thioridazine (10-[2-(1-methyl-2-piperidyl)ethyl]-2-(methylthio)-10H-phenothiazine; MW: 407.04 g/mol) included in the phenothiazine drug groups is a powerful anti-psychotic and anti-anxiety agent containing piperidine (10-13). Due to its lipophilic structure, it can be absorbed, diffuse rapidly to the organs and pass though the blood-brain barrier (14, 15). For years, it has been used in the treatament of psychotic disorders like psychos and schizophrenia (16, 17). Besides, recent studies have shown that THZ is also effective in oncologic and haematologic malignancies like breast cancer (18, 19), melanoma (20, 21), monocytic leukemia (22), leukemia (23), ovarian cancer (11, 24, 25), kidney cancer (26), cervical cancer (27), endometrial cancer (12), prostate cancer (28, 29), gastric cancer (30), non-small cell lung cancer (31), pancrease cancer (32), neuroblastoma (33) and Glioblastoma (16, 26).

General approach to the drugs used in cancer therapy studies fosters the interest on cell death mechanisms [34-36]. Nowadays, apoptosis and autophagy are the most studied cell death mechanism types. Apoptosis is triggered *via* two different mechanisms; death receptors mediated external pathway and

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mitochondria associated internal pathway. Constitution of apoptotic signals *via* activating caspases, which is at inactive form within the cell, is the basis of both mechanisms. While Caspase-2, 8, 9, 10 function in initiation of apoptosis, Caspase-3, 6, 7 have role in constitution of apoptotic cell morphology (37). From embryonic development, apoptosis plays role in preservation of tissue homeostasis, elimination of damaged-aged cells and pathogenesis (38-40). On the other hand, autophagy functions in cellular stress conditions, deficiency of nutrition-growh factor, preservation of cell homeostasis, regulation of cell metabolism, morphogenesis, cell differentiation, aging, cell death and destruction of intracellular pathogens (41). In addition, autophagy in multicellular organisms plays role in recovery of proteins or essential cellular components to the cells to provide nutrition and energy balance (42, 43). It is known that there are 3 different types of autophagy (Chaperon-mediated autophagy, micro-autophagy, macro-autophagy) according to the type of signal reaching the cell and the way in which intracellular structures are transported into lysosomes. While chaperon-mediated autophagy takes place via transport of proteins with Lys-Phe-Glu-Arg-Gln (KFERQ) pattern to lysosomes selectively, micro-autophagy ends up with directly destruction of damaged or unnecessary organels and proteins in lysosomes. Besides, macro-autophagy is the most common and known type of autophagy (44).

Although most of pre-clinic research models are used for discovery of potential drug targets, some of datas obtained from monolayer cell culture are inadequate for *in vivo* studies (45). While monolayer cell culture provides fundamental knowledge about cell proliferation, molecular biology, differantiation of stem cells and tissue homeostasis, it ignores the properties of the cell due to its positioning in its natural environment (45-47). Because of adherence of the cells to the culture flasks in monolayer cell culture, cell-cell interaction, polarization and cell migration decrease. As a result, the cells lose the morphology they should have in *in vivo* conditions (48, 49). Therefore, results obtained from monolayer cell culture provide only preliminary datas, but it cannot adapt to the *in vivo* conditions (50, 51). On the other hand, when compared to the monolayer cell culture, in spheroid cell culture, the cells constituted 3D spherical form continue to exhibit similar *in vivo* properties such as cell-cell, cell-matrix interaction, cell proliferation and differentiation.

In light of these facts, due to its ability to pass though blood-brain barrier and its effect in many types of cancer, including glioblastoma, THZ can be thought as a potential agent for treatment of GBM. But its apoptotic and autophagic effects on GBM and differences between monolayer and spheroid cell cultures are still unclear. Thus, the objective of our study was to evaluate the cytotoxic, apoptotic and autophagic effects of THZ on GBM cell lines by comparing monolayer and spheroid cell cultures.

Materials and Methods

Cell lines and culture conditions: The human GBM cell lines, T98G (p53 mutant) and U-87 MG (p53 wild type), were obtained from the Interlab Cell Line Collection (ICLC, Italy) and American Type Culture Collection American Type Culture Collection (ATCC; Wesel, Germany), respectively. T98G cells were cultured in DMEM-F12 (Dulbecco's Modified Eagle's Medium-F12, Capricorn) containing 5% fetal bovine serum (FBS, Capricorn), 1% L-glutamine (Capricorn) and 1% penicillin-streptomycin (Capricorn) at 37°C in humidified air containing 5% CO_2. In addition, U-87 MG cells were cultured in MEM (Minimum Essential Medium Eagle, Capricorn) containing 10% FBS, % 1 L-glutamine and 1% penicillin-streptomycin at 37°C in humidified air containing 5% CO_2.

Reagents: THZ (T9025-5G), MTT (M5655-1G) reagent and chloroquine diphosphate salt solid (CQ) (C6628-25G) were purchased form Sigma. Stock solution of THZ (24.5 μM) was prepared by thawing of 10 mg THZ in 1 ml high grade water. MTT stock solution was prepared by thawing of 5 mg MTT reagent in 1 ml PBS and used during assays by diluted 1:10. Z-IETD-FMK (Caspase-8 Inhibitor, 20μl (10mM), 1064-20C) and Z-LEHD-FMK (Caspase-9 Inhibitor, 20ul (10mM), 1074-20C) were purchased form Biovision and used as diluted to 20 μM during assays. CQ stock solution (1 mM) was prepared by thawing 5 mg CQ in 9690 μl high grade water and used as diluted to 50 μM during assays.

Cytotoxicity Assays: Cytotoxic effect of THZ on cells were determined by using the MTT assay. The cells to be cultured in monolayer culture were cultivated in 96-well plates ($1x10^4$ cells/well). Next day, the cells were treated with increasing concentration of THZ (from 100 nM to100 μM) for 24, 48 and 72 h. In order to measure the living cells percentage 200 μl of MTT solution was added and incubated for 4 h. After incubation, the plates were measured by using a fluorimeter with excitation and emission wavelenght at 550 nm and at 690 nm, respectively.

For spheroid cell culture hanging drop method was preferred. In this method, the cells were cultivated in 96-well hanging drop plates to be 3×10^3 cells per 45 μl drop. Based on the protocol of the hanging drop

method, 1 ml of PBS was added to the wells at the bottom of the plate, 2 ml of PBS was added to the plate's own wells and it was incubated 72 h for spheroid formation.

Due to determination of IC_{50} value at 24 h in monolayer cell culture, after 72 h, the cells were treated with 10, 20, 30, 40, 50 μM of THZ (5 μl of THZ concentration for each well). After incubation, spheroids were transferred from the hanging drop plates to U- bottum plates, MTT reagent was added and then incubated for 8 h. Finally, the plates were measured by using a fluorimeter with excitation and emission wavelenght at 550 nm and at 690 nm, respectively.

In another part of the cytotoxicity assays, cell viability in the presence of apoptosis and autophagy inhibitors was determined by using similar MTT method. In the presence of inhibitors, the cells in monolayer and spheroid cell cultures were treated first with caspase inhibitors (final concentration 20 μM) and CQ (final concentration 50 μM) for 1 and 16 h, respectively. After incubation times, the cells were treated with IC_{50} value of THZ for 24 h. Finally, MTT reagent was added to plates and the plates were measured by using a fluorimeter after 4 and 8 h incubation for monolayer and spheriod cell cultures, respectively.

Evaluation of apoptosis triggered by THZ: To determine apoptotic effect of THZ on T98G and U-87 MG cell lines in monolayer ($1x10^5$ cells/well) and spheroid cell cultures ($3x10^3$ cells/well), cells were treated first absence and/or presence of apoptosis and autophagy spesific inhibitors for 1 and 16 h, respectively. Then, cells were treated with IC_{50} concentration of THZ for 24 h. After incubation, evaluation of apoptosis was performed in Muse cell analysis instrument (Millipore) according to the Muse Annexin V& Dead Cell Kit (Millipore) manufacturer's instructions.

Evaluation of autophagy triggered by THZ: To determine autophagic effect of THZ on T98G and U-87 MG cell lines in monolayer ($4x10^4$ cells/well) and spheroid cell cultures ($3x10^3$ cells/well), cells were treated first absence and/or presence of apoptosis and autophagy spesific inhibitors for 1 and 16 h, respectively. Then, cells were treated with IC_{50} concentration of THZ for 24 h. After incubation, evaluation of autophagy was performed in Muse cell analysis instrument (Millipore) according to the Muse Autophagy LC-3 Antibody Based Kit (Millipore) manufacturer's instructions.

Statistical Analysis: Analyses were performed by using the GraphPad Prism 5 (GraphPad Software, inc.) and expressed as mean values ± standard deviation (SD) or mean values ± S.E.M. Significance among groups was analysed by one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison post-test. $p < 0.05$ was considered statistically significant. *: $p < 0.01$; **: $p < 0.001$.

Results

Cytotoxic Effect of THZ on GBM Cell Lines In Monolayer and Spheroid Cell Culture: In the first phase of the study, to determine cytotoxic effect of THZ on T98G and U-87 MG cell lines in monolayer cell culture, the cells were treated with increasing concentration of THZ for 24, 48, 72 h. As a result, it was shown that THZ effects the cells at each time points in dose- dependent manner. IC_{50} values were calculated from data obtained at 24 h. Besides, results of the 48th and 72nd hours are similar to the 24th hour data. (Figure 1). Therefore, further studies were performed for monolayer and spheroid cell culture for 24 h. Finally, at 24 h, IC_{50} values were determined as 12.67 μM (T98G) and 12.80 μM (U-87 MG) in monolayer cell culture and as 29.30 μM (T98G) and 28.68 μM (U-87 MG) in spheroid cell culture (Figure 2 and Figure 3).

In the second phase of the study, to determine cytotoxic effect of THZ in the presence of apoptosis and autophagy inhibitors in both cell culture methods, the cells were treated with apoptosis and autophagy inhibitors in addition to IC_{50} concentration of THZ. As a result, in monolayer cell culture, in the presence of apoptosis inhibitors, cell viabilities were measured as 70% (Cas-8), 64% (Cas-9) in T98G cell line and as 62% (Cas-8), 66% (Cas-9) in U-87 MG cell line. In addition, in the presence of autophagy inhibitor, cell viabilities were measured as 86% and 83% in T98G and U-87 MG cell lines, respectively as a result of these obtained data, it was observed that THZ was ineffective in the presence of CQ inhibitor, and THZ decreased cell viability in the presence of Cas-8 and Cas-9 inhibitors (Figure 4). On the other hand, in spheroid cell culture, in the presence of apoptosis inhibitors, cell viabilities were measured as 61% (Cas-8), 66% (Cas-9) in T98G cell line and as 64% (Cas-8), 69% (Cas-9) in U-87 MG cell line. Besides, in the presence of autophagy inhibitor, cell viabilities were measured as 88% and 86% in T98G and U-87 MG cell lines, respectively (Figure 5).

Apoptotic Effect of THZ on GBM Cell Lines In Monolayer and Spheroid Cell Culture: To determine apoptotic effect of THZ on T98G and U-87 MG cell lines in monolayer and spheroid cell cultures, the cells were treated first with apoptosis and autophagy inhibitors and than IC_{50} concentration of THZ for 24 h. As a result, cell viability of control groups were measured above 90% both in different

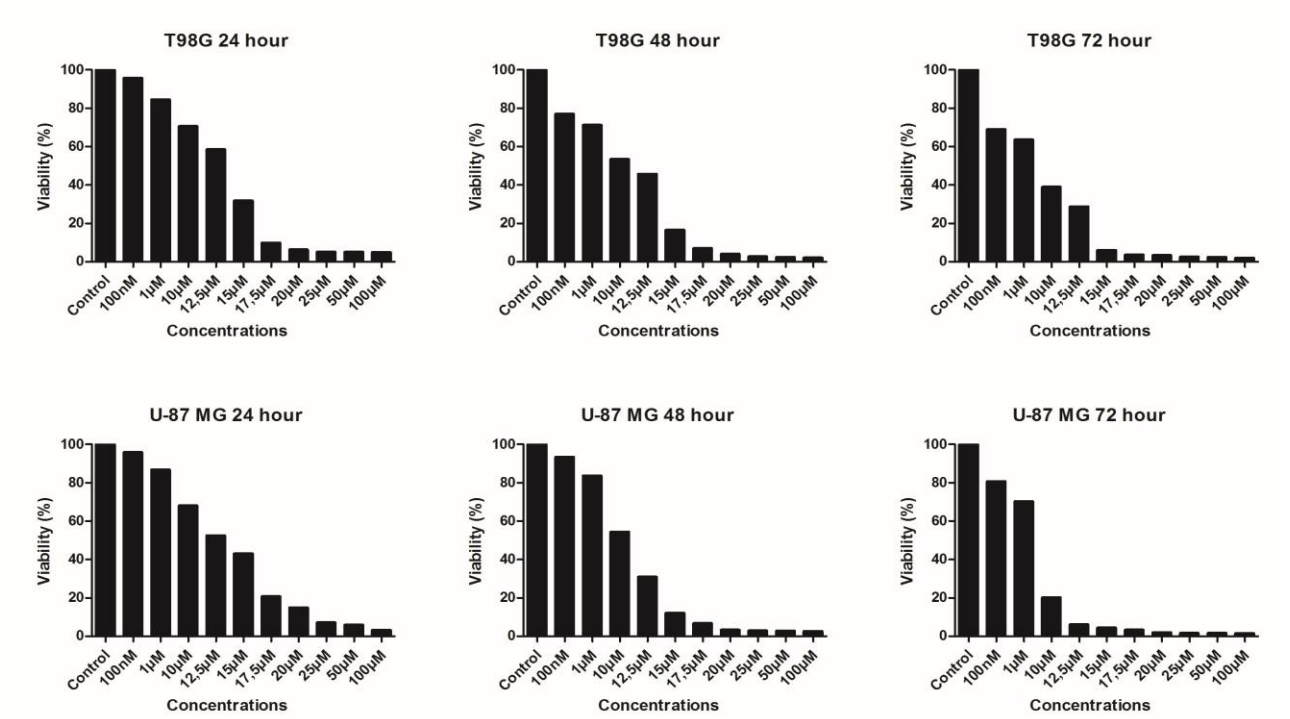

Fig. 1. Determination of cell viabilities of T98G and U-87 MG cell lines at 24, 48 and 72 hours in monolayer cell culture by MTT method (*p<0.01, **p<0.001)

cell culture methods and cell lines. Besides, percentage of apoptotic cells in study groups treated with IC_{50} concentration of THZ was determined as 18.8% (monolayer), 13.8% (spheroid) in T98G cell line and 13.4% (monolayer), 15.26% (spheroid) in U-87 MG cell line. In addition, in the presence of apoptosis inhibitors, decreased apoptosis percentage was determined in both different cell culture methods and cell lines. On the other hand, apoptosis percentage in the presence autophagy inhibitor was determined similar to study group treated with IC_{50} concentration of THZ (Figure 6 and Figure 7) (Table 1 and Table 2).

Autophagic effect of THZ on GBM cell lines in monolayer and spheroid cell culture: To determine the autophagic effect of THZ on both cell lines and in two cell culture methods, the cells were treated first with the apoptosis and autophagy inhibitors and than IC_{50} concentration of THZ for 24 h. As a result, autophagy induction rate was measured as 1.0 in control groups. In THZ treated study groups, it was determined that autophagy induction rate was increased by 6.5- (T98G), 5.6- (U-87 MG) fold in monolayer cell culture and by 3.3- (T98G), 4.5- (U- 87 MG) fold in spheroid cell culture. On the other hand, increased autophagy induction rates were decreased in the presence of apoptosis and autophagy inhibitors in both cell lines and cell culture methods (Figure 8 and Figure 9) (Table 3 and Table 4). The highest decrease occurred in the presence of autophagy inhibitors.

Discussion

Recent studies have shown that THZ, which is used in the treatment of psychotic diseases like psychos and schizophrenia, is also effective in tumor cells. Suppression of proliferation and induction of apoptosis were determined in some *in vitro* studies performed with different cell lines. For instance, a study performed with paclitaxel resistant breast cancer cell lines (MDA-MB-361 ve BT-474) have shown that THZ treatment results suppression of MDR-1 protein, decrease in tumor volume, induction of apoptosis and decrease in proliferation by inhibiting tumor angiogenesis (19, 52). Moreover, another study performed with triple-negative breast cancer cell lines (TNBCL) demonstrated that THZ inhibits self-renewal of several TNBCLs *via* DRD2 inhibition and induce G1 arrest (53). In addition, yet another study performed with cervical (HeLa, Caski and C33A) and endometrial (HEC-1- A and KLE) cancer cell lines has shown that THZ treatment ends up with inhibition of cell proliferation and induction of apoptosis. Arrest of the cell cycle in G1 phase *via* increasing p53 level, inhibition of tumor growth and metastasis via Akt/mTOR/p70s6k signalling pathways have also shown in the same study (12). Besides, one more study performed with hepatoma cell lines (SNU449, LM3, huh7) it was determined that increasing THZ concentrations inhibit cell proliferation by suppressing cell cycle (17). In another

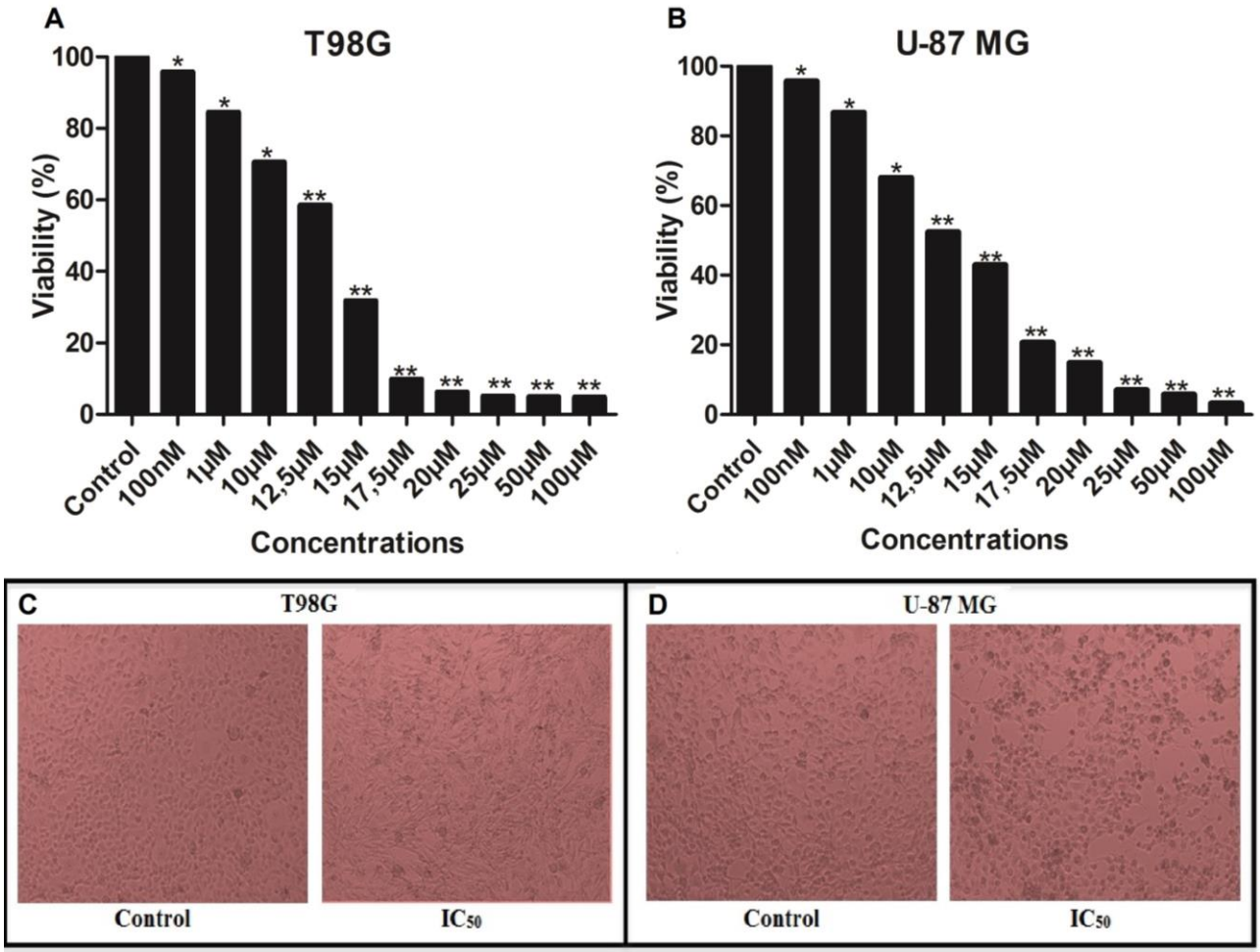

Fig. 2. Cell viabilities of T98G (A) and U-87 MG (B) cell lines following increasing concentration of THZ treatment at 24. h in monolayer cell culture. Phase-Contrast microscope images of T98G (C) and U-87 MG (D) after treatment with IC_{50} concentration of THZ

study performed by Min et al., it was indicated that THZ increase susceptibility to apoptosis in glioma (U251MG), kidney (Caki, ACHN ve A498) and breast cancer (MDA-MB231) cell lines. On the other hand, it was not finding such an effect in healty cells (26). In a study performed with lung cancer cell line (H1975) where THZ is combined with gefitinib, inhibition of cell proliferation and induction of apoptosis was determined (31). Furthermore, a study performed with ovarian cancer cell line (SKOV-3), following THZ treatment, cytotoxic effect and increased caspase-3 activation was determined. At the same time, suppression of AKT phosphorylation and inhibition of cell cycle at G0/G1 phase was also identified. Increased cytotoxicity following combination of THZ, cisplatin and paclitaxel treatment was shown in the same study (11). Moreover, a further study performed with human ovarian cancer cell lines (A2780 and SKOV3) demonstrated that the DR2 blocker THZ induce cell death (apoptosis and autophagy) in a dose-dependent manner and exhibit its anticancer effects *in vitro* and *in vivo* (54). Finally, a study performed with glioblastoma cell lines (GBM8401 and U-87 MG) have shown that increasing concentration of THZ has cytotoxic effect on cell lines. In addition, in the same study, it was also determined that the level of caspase-3 protein did not change and that of LC-II protein increased. Therefore, this situation was interpreted as THZ triggers autophagy in glioblastoma (16). On the other hand, another study regarding to human neuroblastoma (SH-SY5Y) and murine glioblastoma (C6) cell lines, increased caspase-3 level was determined following increasing concentration of THZ treatment (33). Even some studies showed its apoptotic cell proliferation inhibition effect, the view that THZ triggers autophagy rather than apoptosis is more predominant in glioblastoma.

In the light of previous studies, we evaluated cytotoxic, apoptotic and autophagic effects of THZ on U-87 MG and T98G cell lines by comparing monolayer and spheroid cell culture methods in this study. Briefly, the results have indicated that cellular effects of THZ were different in monolayer and

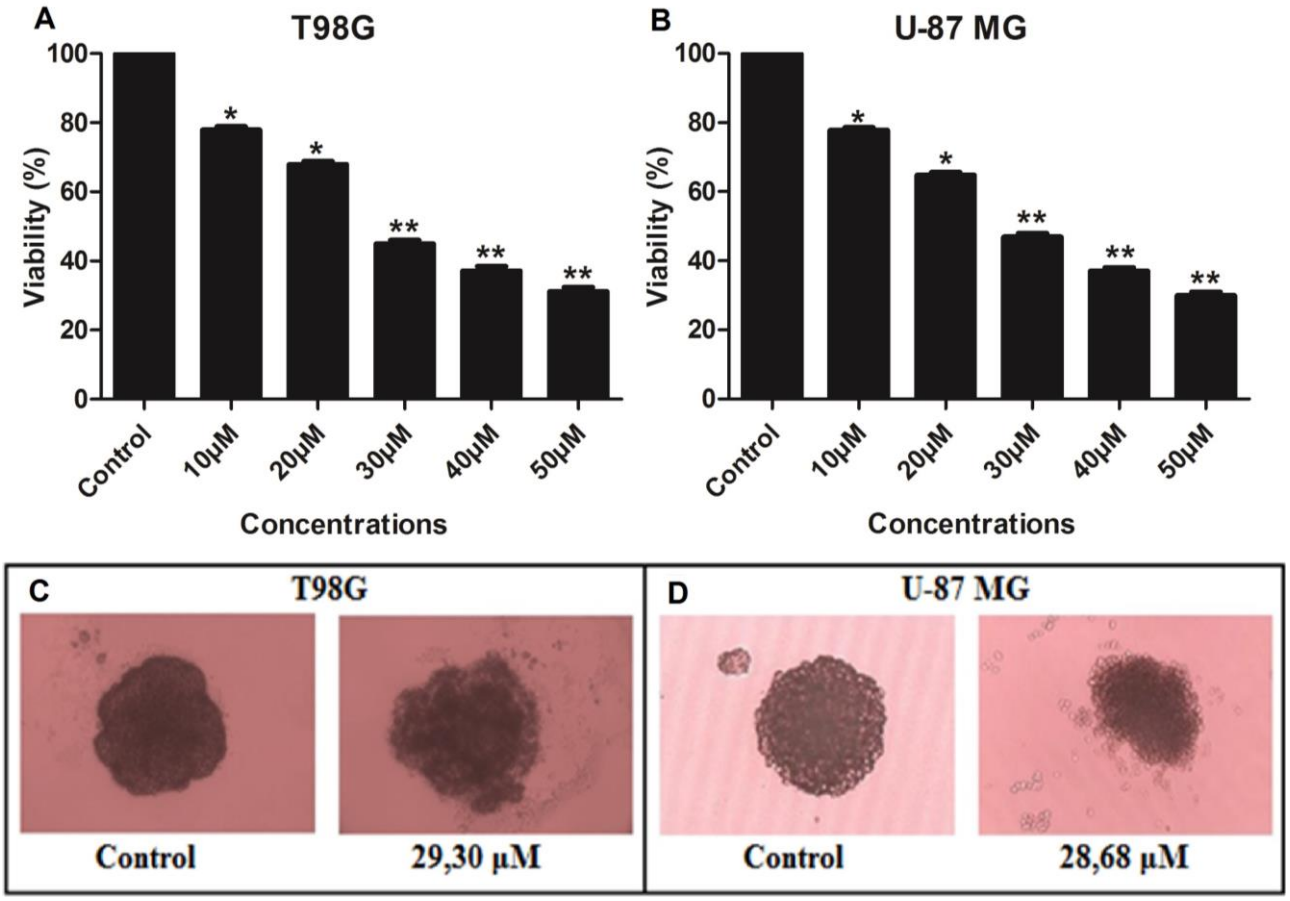

Fig. 3. Cell viabilities of T98G (A) and U-87 MG (B) cell lines following increasing concentration of THZ treatment at 24. h in spheroid cell culture. Phase-Contrast microscope images of T98G (C) and U-87 MG (D) after treatment with IC_{50} concentration of THZ

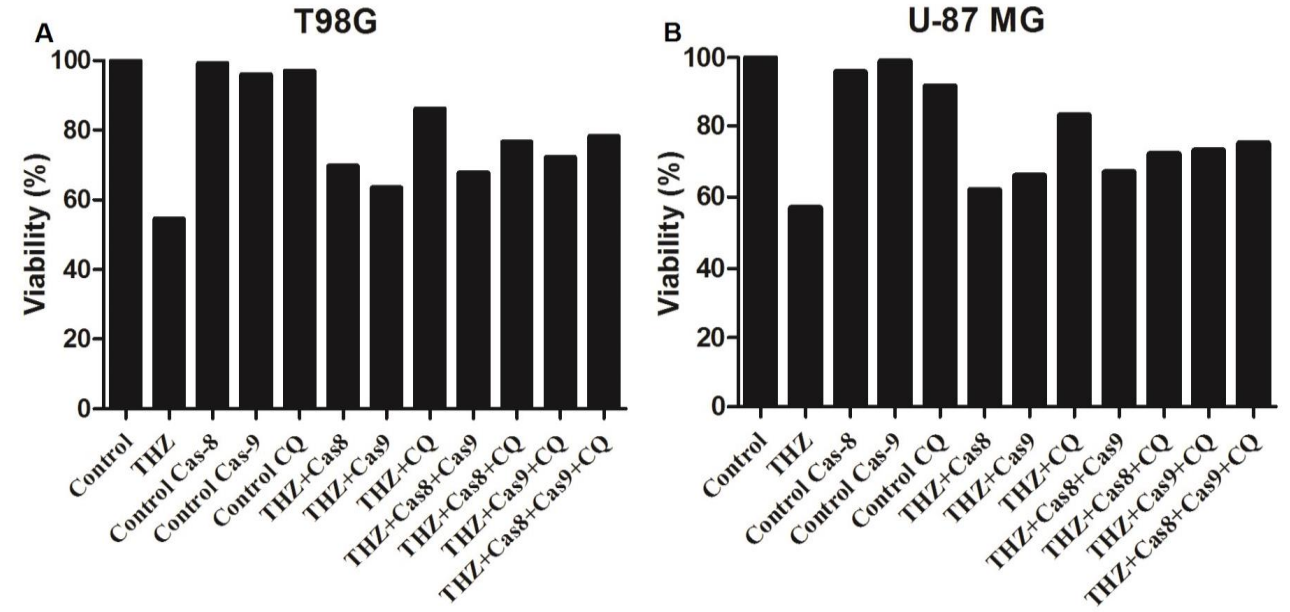

Fig.4. Cell viabilities of T98G (A) and U-87 MG (B) cell lines after treatment with different combination of THZ, Cas8, Cas9 and CQ in monolayer cell culture. Cas8: Cas8 inhibitor, Z-IETD-FMK; Cas9: Cas9 inhibitor, Z-LEHD-FMK; CQ: Autophagy inhibitor, chloroquine diphosphate salt solid

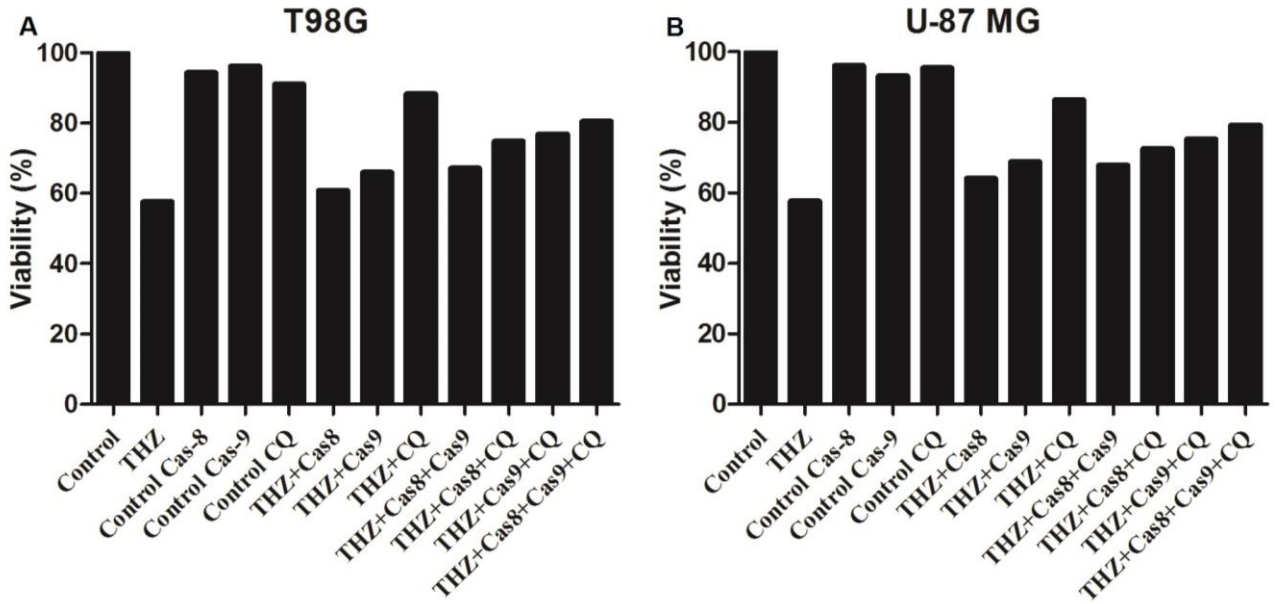

Fig. 5. Cell viabilities of T98G (A) and U-87 MG (B) cell lines after treatment with different combination of THZ, Cas8, Cas9 and CQ in spheroid cell culture. Cas8: Cas8 inhibitor, Z-IETD-FMK; Cas9: Cas9 inhibitor, Z-LEHD-FMK; CQ: Autophagy inhibitor, chloroquine diphosphate salt solid

spheroid cell cultures, and THZ shows its effect mostly *via* autophagic mechanism rather than apoptosis.

In the first phase of this study, cytotoxic effect of THZ on both cell lines in monolayer and spheroid cell culture methods was determined by testing it in different concentrations and times. As a result, IC_{50} values were determined as 12.67 μM (T98G), 12.80 μM (U-87 MG) in monoloayer cell culture and as 29.30 μM (T98G), 28.68 μM (U-87 MG) in spheroid cell culture. In literature, there is limited data on the anti-cancer effect of THZ on GBM, and these studies have been limited to monolayer cell culture. For instance, in a study conducted by Cheng et al., IC_{50} values were calculated below 10 μM in two GBM cell lines (GBM8401 and U-87 MG) treated with THZ for 72 h in monolayer cell culture (16). On the other hand, increased IC_{50} values in spheroid cell culture are in accordance with the results of other studies. Another two studies conducted by Ergüven et al. with Noskapin in T98G cell line and by Lv et al. with Temozolomid, Lomustin ve Cisplatin in U-87 MG cell lines have shown that IC_{50} values are higher in spheroid cell culture than monolayer cell culture. However, in our study both monolayer and spheroid cell cultures and both apoptosis and autophagy mechanisms are investigated (55, 56). Thus, we have confirmed other studies by showing that IC_{50} values are higher in spheroid cell cultures than in monolayer cell culture. In addition to obtained cytotoxicity results, when the cells were treated with apoptosis and autophagy inhibitors in addition to the IC_{50} concentration of THZ, it was shown that cell viability increases in both cell lines and cell culture methods especially following treatment with THZ-autophagy combination. These results suggest that cell death occuring in both cell lines following THZ treatment might be autophagy dependent.

In the second phase of this study, the effect of THZ on cell death mechanism on both cell lines in both cell culture methods was evaluated. As a result, following THZ treatment of GBM cell lines, compared to the control groups, apoptosis rate was increased by 6.8- (T98G), 11- (U-87 MG) fold in monolayer cell culture and by 5.6- (T98G), 9.5- (U-87 MG) fold in spheroid cell culture. In addition, in T98G cell line, in the presence of apoptosis inhibitors, compared to the THZ treated groups, apoptosis rates were decreased by 4.25- (THZ+Cas8), 2.5- (THZ+Cas9) fold in monolayer cell culture and 1.3- (THZ+Cas8), 2.8- (THZ+ Cas9) fold in spheroid cell culture. Besides, in the presence of autophagy inhibitors, apoptosis rates were not decreased significantly compared to the treatment of THZ+apoptosis inhibitors combination (6.3- fold increase in monolayer cell culture and 4.7- fold increase in spheroid cell culture). On the other hand, in U-87 MG cell line, in the presence of apoptosis inhibitors, compared to the THZ treated groups, apoptosis rates were decreased by 2.5- (THZ+Cas8), 3.7- (THZ+Cas9) fold in monolayer cell culture and 4.2- (THZ+Cas8), 4- (THZ+Cas9) fold in spheroid cell culture. Additionally, it was determined that apoptosis rates did not change in the groups treated with the THZ+autophagy inhibitor combination (8.6-fold increase in monolayer and spheroid cell cultures).

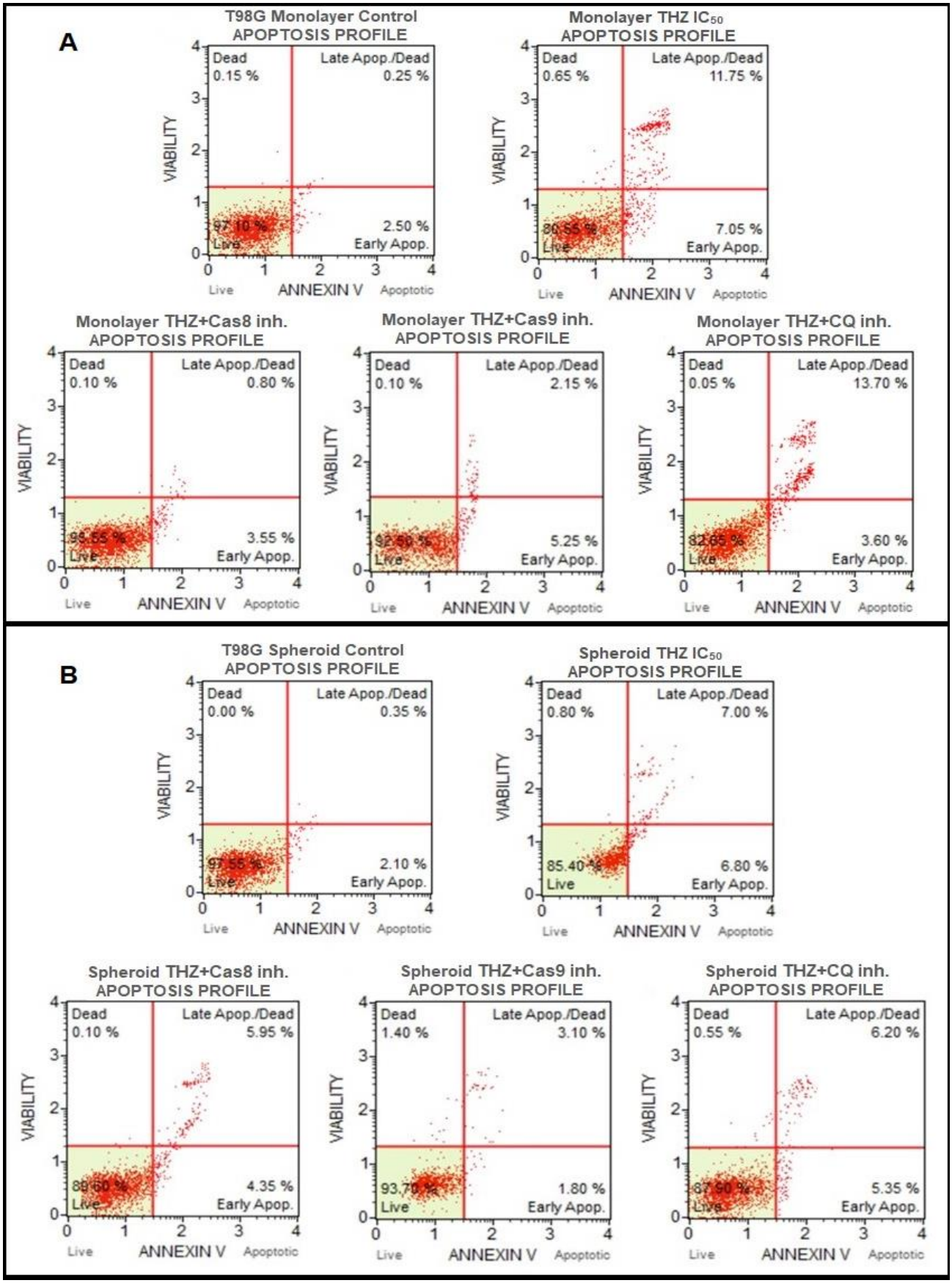

Fig. 6. Apoptosis percentages of T98G cells treated with IC_{50} concentration of THZ and THZ+inhibitor combination in monolayer (A) and spheroid (B) cell cultures

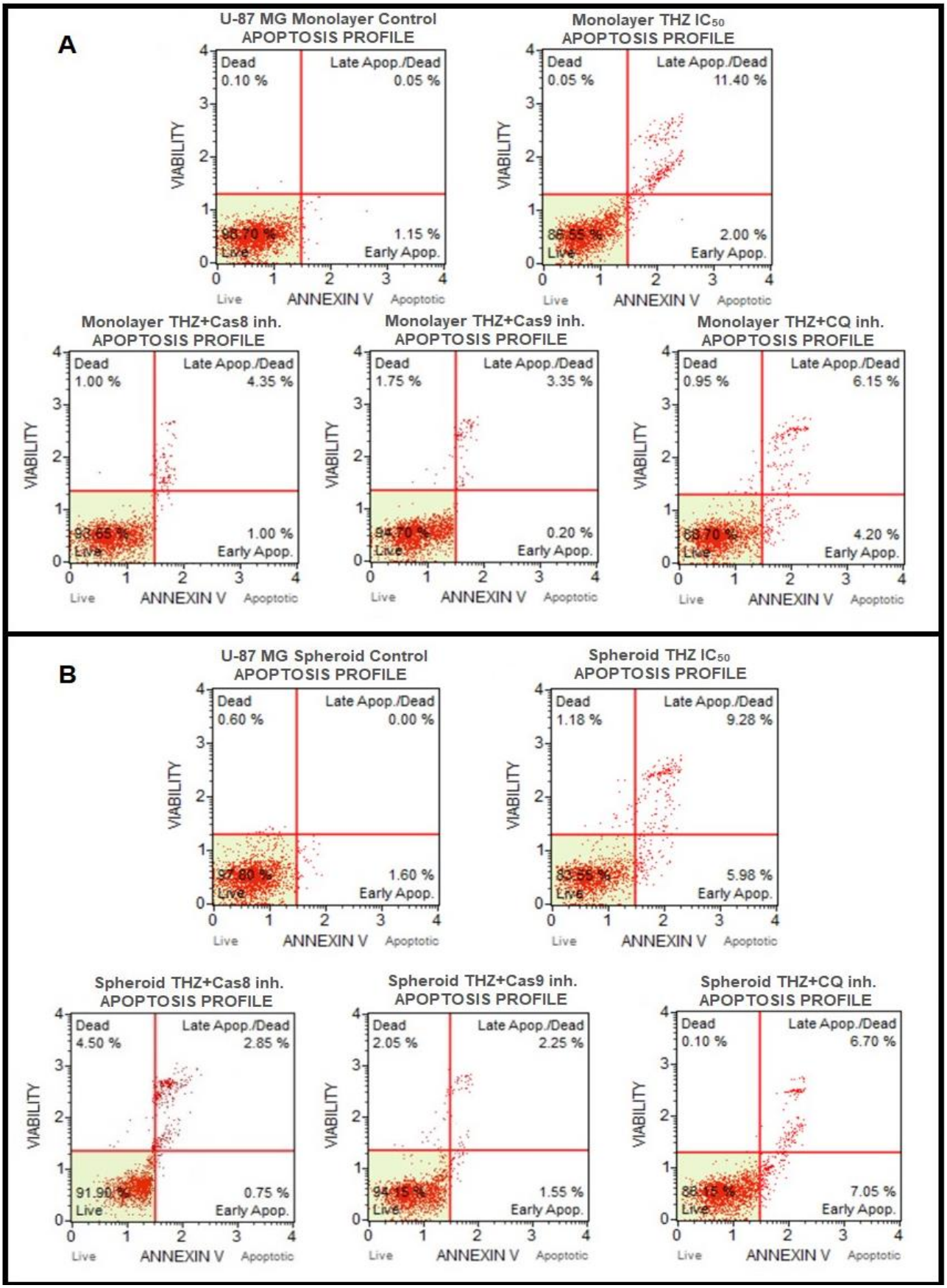

Fig. 7. Apoptosis percentages of U-87 MG cells treated with IC_{50} concentration of THZ and THZ+inhibitor combination in monolayer (A) and spheroid (B) cell cultures

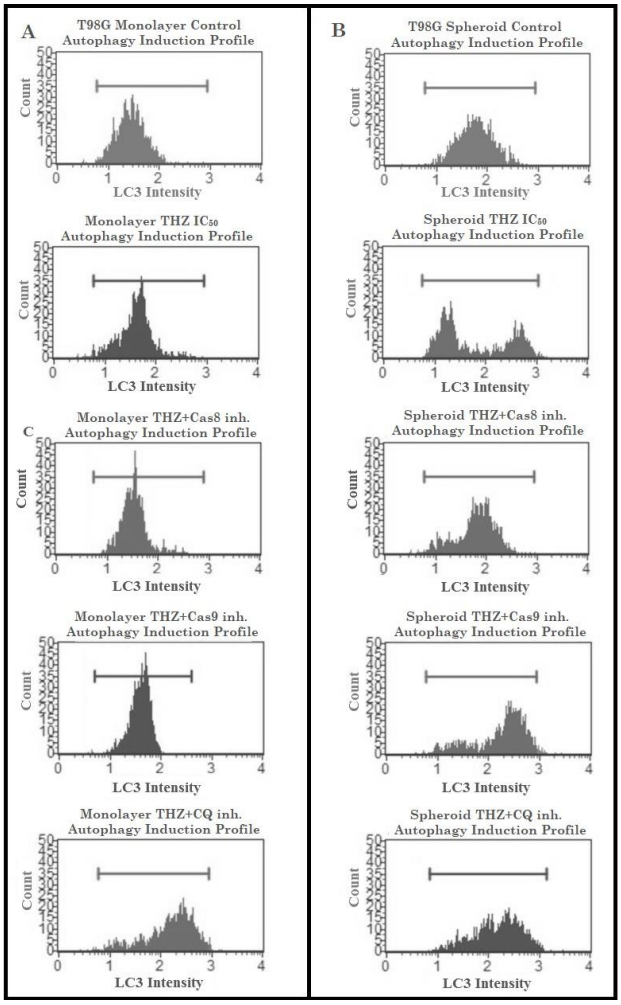

Fig. 8. Autophagy induction rate of T98G cells treated with IC_{50} concentration of THZ and THZ+inhibitor combination in monolayer (A) and spheroid (B) cell cultures

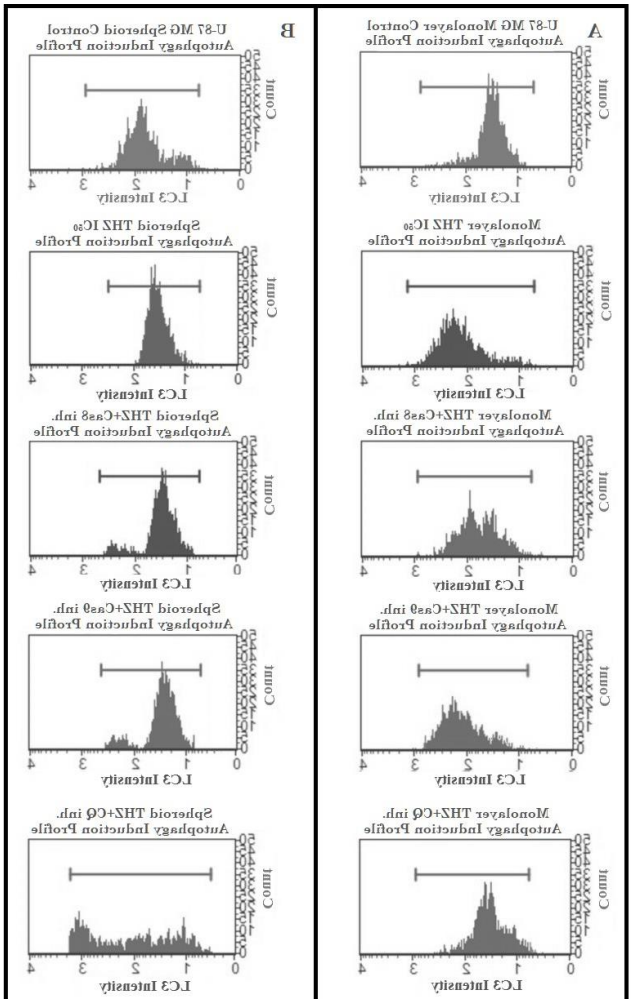

Fig. 9. Apoptosis percentages of U-87 MG cells treated with IC_{50} concentration of THZ and THZ+inhibitor combination in monolayer (A) and spheroid (B) cell cultures

All these findings of the apoptosis studies suggest that THZ-induced cell death may be caused by apoptosis in a small amount due to small increase in apoptosis rate and an increase in cell viability in the presence of apoptosis inhibitors. In another part of this study, when autophagy rates were evaluated, it was determined that compared to the control groups, autophagy induction rate was induced by 6.5- (T98G), 5.6- (U-87 MG) fold in monolayer cell culture and by 3.3- (T98G), 24.5- (U-87 MG) fold in spheroid cell culture. On the other hand, in both cell lines and cell culture methods, it was decreased approximately by 1.5- and 3.5- fold in the in the presence of apoptosis and autophagy inhibitors, respectively. In the light of the datas obtained, it is considered that THZ induced cytotoxicity is mostly caused by autophagy. There are similar studies conducted by different researchers in this field in the literature. As a result of the searches, a general impression is that THZ stimulates different cell death mechanisms in different types of cancer. For instance, in the studies conducted by different researchers, while THZ stimulates apoptosis in murine breast cancer cell line (4T1) and endometrial cancer cells, it induces autophagy in osteosarcoma (U-2 OS) and GBM cell lines (GBM8401 and U-87 MG) (16, 19, 57, 58).

It is known that there is a complex relationship between cancer and autophagy. Studies have shown that autophagy remains ineffective in cancer, supports tumor formation or plays a role as a tumor suppressor (59). These differences are thought to be due to cancer type, tumor stage and oncogenic mutation (60). Although apoptosis is the primary form of cell death types induced by chemotherapy agents, autophagy has also been proposed as an alternative death mechanism that can be induced by anti-cancer drugs (43). Following treatment of GBM *via* chemotherapy and radiotherapy, the effect of treatment is reduced as a result of increased activity of growth factor receptors and Ras stimulation, and consequently the activity of the PI3K-Akt-mTOR pathway. PTEN mutation, which is frequently seen in

Table 1. Comparison of live and apoptotic percentages of T98G cell line treated with IC_{50} concentration of THZ and THZ+inhibitor combination in monolayer and spheroid cultures

T98G Cell Line	Monolayer Cell Culture					Spheroid Cell Culture				
Measured parameters via Muse Annexin V & Dead Cell Assay	Control	THZ (IC50)	THZ (IC50) + Cas8 inh.	THZ (IC50) + Cas9 inh.	THZ (IC50) + CQ	Control	THZ (IC50)	THZ (IC50) + Cas8 inh.	THZ (IC50) + Cas9 inh.	THZ (IC50) + CQ
% Live	97.10	80.55	95.55	92.50	82.65	97.55	85.40	89.60	93.70	87.90
% Early Apoptosis	2.50	7.05	3.55	5.25	3.60	2.10	6.80	4.35	1.80	5.35
% Late Apoptosis	0.25	11.75	0.80	2.15	13.70	0.35	7.00	5.95	3.10	6.20
% Dead	0.15	0.65	0.10	0.10	0.05	0.00	0.80	0.10	1.40	0.55

Table 2. Comparison of live and apoptotic percentages of U-87 MG cell line treated with IC_{50} concentration of THZ and THZ+inhibitor combination in monolayer and spheroid cultures

U-87 MG Cell Line	Monolayer Cell Culture					Spheroid Cell Culture				
Measured parameters via Muse Annexin V & Dead Cell Assay	Control	THZ (IC50)	THZ (IC50) + Cas8 inh.	THZ (IC50) + Cas9 inh.	THZ (IC50) + CQ	Control	THZ (IC50)	THZ (IC50) + Cas8 inh.	THZ (IC50) + Cas9 inh.	THZ (IC50) + CQ
% Live	98.70	86.55	93.65	94.70	88.70	97.80	83.55	91.90	94.15	86.15
% Early Apoptosis	1.15	2.00	1.00	0.20	4.20	1.60	5.98	0.75	1.55	7.05
% Late Apoptosis	0.05	11.40	4.35	3.35	6.15	0.00	9.28	2.85	2.25	6.70
% Dead	0.10	0.05	1.00	1.75	0.95	0.60	1.18	4.50	2.05	0.10

GBM, has been found to cause uncontrolled cell division and suppression of apoptosis by inhibiting PI3K-Akt-mTOR pathway, and it has been suggested that resistance to apoptosis occurs during the treatment for this reason (61, 62). In studies conducted in different types of cancer, it has been determined that THZ activates different cell death mechanisms by inhibiting PI3K-Akt-mTOR pathway (25, 26, 63). There are also some studies suggesting that it has a supportive effect on the treatment for some types of cancer, when anti-cancer agents or compounds which induce autophagy are being used. mTOR inhibitors and these pathway specific agents have been shown to stimulate autophagy and to support anti-cancer effects when combined with chemotherapy and radiotherapy (64).

In conclusion, we have again demonstrated the difference between monolayer and spheroid cell cultures. Based on our results, we concluded that cytotoxicity studies could be performed in spheroid culture to gain more in vivo like results. Furthermore, we think that THZ may be an anti-cancer agent in the treatment of GBM and that the cytotoxic effect is mostly caused by autophagy.

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