BIRB796 reduces p38MAPK and pHsp27 proteins in U87MG Glioma cells

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Abstract

Background: Hsp27 phosphorylation in glioma cells is closely related to tumor cell proliferation and apoptosis inhibition. The main purpose of this study is to inhibit the phosphorylation of Hsp27 by MAPK inhibitor, BIRB796. Thereby reducing the amount of pHsp27 that inhibits the mechanism of apoptosis in cancer cells.

Methods and Results: The effect of bortezomib and BIRB796 on the U87MG cells was examined. Hsp27 and pHsp27 proteins expression level were exhibited by Western Blot analysis. Besides, we examined BIRB796 effects on p38MAPK (a, b) enzyme inhibition and caspase 3 activity. According to MTT analysis, bortezomib decreased cell proliferation dose dependent manner and BIRB796 did not cause significant reduction. For further analysis, 5nM bortezomib, and 100, 250, 500 nM BIRB796 concentrations that show neither a cytotoxic nor a proliferative effect on the cells were used as combined treatment. It was seen that 5 nM bortezomib treatment significantly induced pHsp27 (76%) and Hsp27 (48%) expression levels in the glioma cells. It was found that BIRB796 treatments significantly decreased the level of Hsp27 and pHsp27. The combined treatments significantly reduced pHsp27 expression level. Our results showed that all the treatments also significantly increased caspase 3 activation. Moreover BIRB796 inhibited p38a and p38b activities depending on time and this agent effect on p38a was stronger than p38b.

Conclusion: These results demonstrate that the cellular MAPK inhibitor BIRB796 agent may be an effective therapeutic option in cancer cells by reducing resistance and decreasing pHsp27 expression in the treatment of brain cancer.

1. Introduction

Glioblastoma (GB) is the most aggressive and recurrent brain-derived cancer caused by mutated genes, viruses, radiation exposure, various chemicals, impairments in the immune system, environmental factors, nutrition in most cases [1]. Because of the high vascularity of GB, it is easy to settle into other tissues by invasion and metastasis. It is very difficult to be treated with surgery, radiotherapy or chemotherapy. Even though this type of treatment was tried, it was not seen that the patients lived for more than 5 years [2]. In brain tumor cells, as in many tumor cells, many mechanisms change, unlike normal cells. Uncontrolled cell division, accumulation of mutations, gaining the ability to invasion, and changes in the expression levels of some proteins can be given as examples. A group of proteins called heat shock proteins (Hsp) or stress proteins are among the proteins whose expression changes in brain tumor cells [3].

There are various heat shock proteins that perform many different functions in the cell. Some Hsps act as protective agents when the cells are exposed to stress (heat shock, oxidative stress, radiation, environmental pollutants, some metabolic products, etc.) due to their chaperone activities. Therefore, they are very important in intracellular metabolism. Thanks to the chaperone activity, it provides the correct 3D structure of the misfolded proteins and destruction of the irreparable proteins [4]. On the other hand, its
increased expression in cancer cells that have a critical importance on tumor development; it inhibits apoptosis pathways of cancer cells, making them more resistant to treatment trials [5]. It is known that Hsp27 expression increases in various cancer types, especially in brain tumors. The increased Hsp27 causes cancer cells to became resistant to therapeutic treatments. Inhibition of Hsp27 expression, results in the release of cytochrome C following exposure of the cell to apoptotic agents and assists in the activation of caspase activation [6].

Mitogen activated protein kinases (MAPK) target serine threonine residues in proteins. These proteins play important roles in a number of biological processes including cell proliferation, differentiation, apoptosis, inflammation and response to environmental stress [7, 8]. The functions of Hsp27 are controlled by MAPKs that mediate phosphorylation of Hsp27 [9]. In addition, pHsp27 activated by phosphorylation shown to be associated with pro-invasive and pro-metastatic activities in the process of ovarian and prostate cancers [10–15].

Protein phosphorylation is an important post-translational modification that affects a most part of the signaling mechanisms. p38MAPK is a well-known enzyme that was extensively studied as a regulator in cell-related pathways such as proliferation, differentiation, viability and apoptosis due to cellular conditions [16, 17]. Some researchers consider, they can help to the treatment of cancer by developing p38 MAPKs inhibitors. BIRB796 that is the most selective and most effective MAPK inhibitor [18], p38α inhibiting phosphorylation by causing structural change in the ATP binding site of MAPK [19]. Inhibition of Hsp27 phosphorylation by BIRB796 in myeloma cancer cells shown to increase cytotoxicity and caspase activation [20, 21].

BIRB796 can act on both Hsp27 and pHsp27 proteins. We designed such a study to evaluate effects of this inhibitor on U87MG glioma cells. First of all we investigated the cytotoxic effects of BIRB796 on glioma cells. Then we induced Hsp expression in the cells with bortezomib. We designed eight experimental groups and applied the western blot method for immunological analysis. After evaluating the effects of agents on Hsp27 and pHsp27 proteins expression level with immunological analysis, we studied caspase 3 activity. In this way, we were able to investigate the effect on target apoptotic processes. We also investigated the effects of BIRB796 on the enzyme p38 MAPK activity at different times. As a result, we revealed new findings by examining the increase of p38MAPK, the increase of pHsp27 by activating Hsp27 protein phosphorylation of this increased enzyme and its effect on some apoptotic processes. The main purpose of our study is to present new approach that may have therapeutic potential.

2. Materials And Methods

2.1. Chemicals and Reagents

Bortezomib was obtained from Sigma Aldrich (VELCADE®, Millennium Pharmaceuticals Inc. Cambridge, MA, USA), diluted in dimethyl sulfoxide (DMSO), and kept at -20°C until use. The p38 MAPK inhibitor,
BIRB796, was provided by Boehringer Ingelheim Pharmaceuticals Inc. (Ridgefield, CT, USA), dissolved in DMSO, and stored at -80°C until use.

Cell culture reagents were purchased from Gibco and Sigma Aldrich (San Diego, CA, USA and St. Louis, Missouri, USA). Mouse anti-Hsp27, p-Hsp27 monoclonal antibody and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG were obtained from Enzo Life Sciences (Lausen, Switzerland, EU). HRP-conjugated GAPDH Loading Control Monoclonal Antibody and ECL Plus Western Blotting Detection System were purchased from Thermo Fisher Scientific (Rockford, IL, USA). SMART™ BCA Protein Assay Kit was from iNtRON Biotechnology (San Diego, CA, USA). p38MAPK enzyme inhibition kit was obtain from invitrogen (Carlsbad, California, ABD). All the other chemicals were obtained from Sigma.

2.2. Cell culture and conditions

The human glioblastoma cell lines U87MG provided from Istanbul University Culture Collection and were cultured in Dulbecco's Modified Eagle's Medium/High Glucose (DMEM, High Glucose) supplemented with 10% heat inactivated fetal bovine serum (FBS), 1% (v/v) antibiotic-antimycotics solution (100 U/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin B), and 1% (v/v) non-essential amino acids at 37°C in a humidified 5% CO₂ atmosphere. The cells were passaged every 3 days [22].

2.3. Cytotoxic assay

Cytotoxic activities of bortezomib, BIRB796 and bortezomib/BIRB796 combination were determined by cell proliferation analysis using the standard 3- (4,5-dimethylthiazole-2 yl) -2,5-diphenyltetrazolium bromide (MTT) assay [23, 24]. In order to determine the toxic effects of agents and their combined treatments, cells were cultured in 96-well plates of 10⁵ cells per well and 24 h prior to treatment. The agents to be tested were first dissolved in DMSO (less than 1%) and diluted with DMEM / High Glucose medium for use in MTT assay (1–50 nM for bortezomib and 50-2500 nM for BIRB796). After 24 and 48 hours incubation times, the medium on the cells was removed, washed with D-PBS and 30 μl of MTT was added from stock solution (5 mg/ml, prepared in D-PBS). At the end of the 4 hour incubation period, 150 μl DMSO was added and the culture plate was shaken at room temperature for homogeneity of the solution. The absorbance of plate at a wavelength of 540 nm was measured in a microplate reader (EON, BioTek Instruments Inc.). Three independent experiments were performed at least.

2.4. Induction of Hsp expression

It was used bortezomib to increase Hsp expression. We modified the method used by Shah et al. [25] and applied it in our study. U87MG cells were cultured in 6-well plate at 10⁵ cells/mL and allowed to incubate for 24 h (5% CO₂, 37°C). Bortezomib (2.5 nM, 5 nM, 10 nM, 25 nM), an induction agent of the Hsp expression, was applied to the cells at the end of the 24h incubation.

2.5. Design of experimental sets

As the experimental set, 8 groups were examined (Control, bortezomib treated, BIRB796 treated, both bortezomib and BIRB796 treated groups) in this study. According to the MTT results, it was determined
that all bortezomib treatments increased Hsp expression in the cell, but only one concentration was chosen as 5nM. However, concentrations of 100 nM, 250 nM, and 500 nM were examined for BIRB796. The combined treatments were carried out using these concentrations. Assays were done at 24 h after treatments.

2.6. Immunological analysis

Control and experimental groups cells were collected for protein isolation after agent treatments. The cells were suspended with cold D-PBS and centrifuged three times at 3000xg for 5 minutes at room temperature (25°C). 200 µl Lysis Buffer [0.02% Tris-HCl (pH 6.8), 0.04% EDTA (ethylenediaminetetra acetic acid), 1% Triton X-100, Complete EDTA and 1 mM PMSF (phenylmethylsulfonyl fluoride)] was added on the samples. After physical, mechanical and chemical disintegration, samples were centrifuged at 20,000xg for 20 minutes at + 4°C. The "Bicinchoninic acid assay" (BCA, SMART™ BCA Protein Assay Kit, iNtRON Biotechnology) kit was used in determining the concentrations of protein extracts in accordance with the manufacturer's instructions. Mini gel electrophoresis system (Mini-PROTEAN® Tetra Cell, Bio-Rad) was used for the separations of water soluble proteins. Samples were mixed with sample buffer (25 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 10% 2-mercaptoethanol, and 0.002% bromophenol blue) and boiled at 100°C for 3 minutes. Proteins (30 µg) were run at 200V in 10% SDS-PAGE for about 40 minutes and transferred to PVDF (polyvinilidene difluoride) membranes by the electroblot method (Trans-Blot® Turbo ™ Transfer System (Bio-Rad)). The membrane was incubated in 5% BSA Fraction V [5% (w/v) BSA Fraction V prepared in PBS containing 0.05% (v/v) Tween-20] blocking buffer for 1.5 hours at room temperature. The membranes were then incubated with mouse anti-Hsp27 (1: 1000) and mouse anti-phsp27 (ser82, 1: 500) monoclonal antibodies overnight at 4°C. At the end of the incubation period, the membrane was washed 4 times for 15 minutes with wash buffer (PBS − 1X containing 0.05% (v/v) Tween-20). At room temperature with secondary antibody (Goat anti-mouse IgG (Fab), HRP labeled, 1:5000) the membrane was incubated for 1 hour. After the membrane was washed again, protein bands were visualized using the “ECL Plus Western Blotting Detection System” kit. The data were normalized relative to GAPDH using the HRP conjugated GAPDH Loading Control Monoclonal Antibody (1:2000). The results were evaluated by making calculations in ImageLab 5.2.1 software. The experiment was repeated at least three times [26].

2.7. p38MAPK enzyme inhibitory assay

The p38 MAPK ELISA Kit (Invitrogen ™; a solid phase sandwich Enzyme Linked Immunosorbent Assay / ELISA) was used in the study. The kit was applied in accordance with the manufacturer's instructions. This assay was performed to detect p38 MAPK (α, β) in BIRB796 treated cell lysates. For this purpose, all experimental groups were created. 10^5 cells/ml were then collected and washed two times with cold PBS. The cells were dissolved in Cell Extraction Buffer (10 mM Tris (pH 7.4), 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM Na₄P₂O₇, 2 mM Na₃VO₄, 1% Triton™ X-100, 10% glycerol, 0.1% SDS, and 0.5% deoxycholate) for 30 minutes on ice and vortexed at 10 minute intervals. It was centrifuged at 13,000 rpm for 10 minutes at 4°C. After that, standard, control and samples were put into the striped wells and incubated at room temperature for 2 hours. The p38 MAPK (α, β) Detection Antibody solution was put
into the wells and incubated at different times (30, 60, 90 min). Then Anti-Rabbit IgG HRP Solution and Stabilized Chromogen were added to the wells at the appropriate temperature and incubation times to turn the wells blue. Finally, stop solution was added to each well and the change of solution from blue to yellow was measured the absorbance at 450 nm [27].

2.8. Measurement of caspase-3 activity

The caspase 3 activity measurements of the substances were performed using the “Caspase 3 Assay Kit, Colorimetric” (Sigma, CASPC3) assay kit and following the manufacturer's instructions. Cells were treated with Cell Lysis Buffer, incubated on ice for 10 min, and centrifuged at 10,000×g for 5 min at 4°C. The reaction mixture (total volume, 100 µl) containing 30 µl of cell lysate and 10 µl of the caspase-3 substrate acetyl-Asp-Glu-Val-Asp-p-nitroanilide (final concentration, 200 µM) in assay buffer was prepared. The experiment was performed in a 96-well plate. The control group reaction mix contained 30 µl of cell lysate and 10 µl (final concentration, 20 µM) of the specific caspase-3 inhibitor acetyl-DEVD-CHO in assay buffer. Both mixtures were incubated at 37°C for 90 minutes and absorbance was read at 405 nm [28, 29].

2.9. Statistical analysis

The data obtained as a result of cytotoxicity and immunological analyzes were evaluated using GraphPad Prism® 7 program. The statistical evaluation was performed with one-way analysis of variance (ANOVA) followed by Dunnett’s or Tukey post hoc tests to multiple comparisons. The limit of significance was accepted as $P < 0.05$.

3. Results

3.1. Cytotoxic analysis of bortezomib and BIRB796

The cytotoxic activity of bortezomib and BIRB796 on the U87MG cells were examined in the presence of different agent concentrations. Bortezomib and BIRB796 were applied to U87MG cells at concentrations of 1–50 nM and 50-2500 nM for 24h and 48h, respectively. Cytotoxicity data are presented as mean percentages of control ± standard deviation (SD). The half-maximal inhibitory concentration (IC$_{50}$) was determined using GraphPad Prism® 7 software. The cytotoxic activity data showed that bortezomib decreased in U87MG cell line in a dose-dependent manner after 24h and 48h incubation (Fig. 1A). For U87MG cells, after 24h the bortezomib treatment IC$_{50}$ value was 11.63 nM and the IC$_{50}$ value after 48h of bortezomib treatment was 8.32 nM. For 24h and 24h BIRB796 treatments, the IC$_{50}$ values could not be determined in U87MG cells. Moreover the cell viabilities were detected about 85% at a concentration of 2500 nM BIRB796 (Fig. 1B).

According to the MTT results, while Bortezomib concentrations were chosen as 5 nM and 10 nM, BIRB796 concentrations were chosen as 50 nM, 100 nM, 250, 500 nM, 1000 nM, 2500 nM. The effects of Bortezomib + BIRB796 agent combination on the viability of U87MG cells after 24 hours of applications
were evaluated (Fig. 2). As a result of these analysis, it was decided to apply 5 nM Bortezomib with 100 nM, 250 nM or 500 nM BIRB796 to the cells for 24h, which does not have a statistically significant toxic effect on cell viability for immunological analysis.

3.2. Bortezomib treatment increases Hsp27 and pHsp27 expression in the glioma cells

Bortezomib is used to increase the expression of stress proteins in myeloma cells [25]. The effects of bortezomib application at different concentrations (2.5, 5, 10 and 25 nM) on the expression of Hsp27 and pHsp27 proteins in U87MG were detected by Western blot analysis and the results were given in Fig. 3. According to the results, it was observed that the concentration of bortezomib 2.5 nM, 5 nM, 10 nM and 25 nM increased Hsp27 expression by approximately 20% (statistically not significant), 48%, 95% and 151%, respectively (Fig. 3A, **** P < 0.0001). Also, bortezomib treatments were significantly increased the expression of pHsp27 by approximately 20% (statistically not significant), 76%, 115% and 186%, respectively (Fig. 3B, **** P < 0.0001). These findings obtained as a result of immunological analyzes are similar to literature information [20, 21].

3.3. BIRB796 effects Hsp27 and pHsp27 expressions in the glioma cells

The effect of BIRB796 on Hsp27 expression was evaluated using immunological analysis (Fig. 4A). It was determined that concentrations of 100 nM, 250 nM and 500 nM of BIRB796 decreased Hsp27 expression by approximately 6%, 11% and 12%, respectively (** P < 0.01, *** P < 0.001). When the results were compared with the control group, the concentrations of 100 nM, 250 nM and 500 nM of BIRB796 were thought to decrease pHsp27 expression by approximately 63%, 76% and 100%, respectively (**** P < 0.0001) (Fig. 5A). BIRB796 treatment was also found to cause a significant decrease in the expression level of pHsp27 protein. In another study, similar BIRB796 concentrations were used to reduce the expression of stress proteins [30].

3.4. BIRB796 and bortezomib combination decreases Hsp27 and pHsp27 expression on the glioma cells

Since these concentrations of BIRB796 had no negative effects on cell viability, all three doses (100 nM, 250 nM, 500 nM) were used in the combined application. Bortezomib (5 nM) and BIRB796 (100 nM, 250 nM, 500 nM) applied to U87MG cells for 24 hours and then the effects of these treatments on Hsp27 and pHsp27 expression levels were examined (Fig. 4B, 5B). It was determined that the combined application did not cause a statistically significant decrease in Hsp27 protein compared to the control group. However, when the 5 nM bortezomib group and the combined groups were compared, approximately 34%, 39%, and 38% reduction was observed in BIRB796 concentrations of 100 nM, 250 nM, and 500 nM, respectively (** P < 0.001, **** P < 0.0001) (Fig. 4B). In pHsp27 expression level, along with 5 nM
bortezomib, it caused a decrease of approximately 45%, 80% and 90% in the applications of 100 nM, 250 nM and 500 nM BIRB76, respectively (**P < 0.001, ****P < 0.0001). It is seen that there is a significant decrease in all the combined applications compared to the 5 nM bortezomib group (****P < 0.0001) (Fig. 5B).

### 3.5. Time dependent p38MAPK enzyme inhibition by BIRB796

In this study, we investigated the effects of BIRB796 on p38α and p38β enzyme activities. First, we observed that BIRB796 inhibits p38 MAPK activities at different concentrations. BIRB796 was found to inhibit p38α and p38β activities depending on time due to its slow binding behavior, and that the effect of this compound on p38α was stronger than p38β (Table 1). Also, the enzyme activity IC$_{50}$ values were also determined and decreased due to the time value. The results are compared to the control group and mean ± standard deviations are given in the Table 1. In a study, it was determined that BIRB796 significantly reduced p38 expression in cervical cancer cells, and this result supports our study [31].

#### Table 1

<table>
<thead>
<tr>
<th>Kinase (IC$_{50}$/µM)</th>
<th>0</th>
<th>30 min.</th>
<th>60 min.</th>
<th>90 min.</th>
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<tr>
<td>p38α</td>
<td>0.085 ± 0.04</td>
<td>0.039 ± 0.02</td>
<td>0.021 ± 0.03</td>
<td>0.019 ± 0.01</td>
</tr>
<tr>
<td>p38β</td>
<td>0.29 ± 0.05</td>
<td>0.187 ± 0.03</td>
<td>0.101 ± 0.02</td>
<td>0.05 ± 0.02</td>
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### 3.6. Caspase 3 activity

In order to measure the caspase 3 activation of the all experimental groups, BIRB796 and bortezomib were applied to U87MG cells. As a result of these treatments, an increase in caspase 3 level was detected in all experimental groups. 45% increase in caspase activation was observed in the 5 nM bortezomib group. In the groups treated with only BIRB796 (100 nM, 250 nM, 500 nM), caspase levels increased by approximately 34%, 90% and 163%, respectively. A dramatic increase in caspase level was observed when bortezomib and BIRB796 agents were combined. Experimental results were compared with each other using the Tukey test. All results were found to be statistically significant (Fig. 6, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 vertical bars indicate standard deviation values).

### 4. Discussion

High expression of Hsp27 in malignant cells was shown to provide resistance to chemotherapeutic agents [32, 33]. A remarkable relationship was found between the concentration of Hsp27 and the malignancy of the tumor. At the same time, it was determined that Hsp27 suppresses the increased apoptosis as a result of cancer treatments [34].
We conducted to increase Hsp expression and investigate the effects of BIRB796 on Hsp27 proteins. For this reason, we used Bortezomib, a proteasome inhibitor. Bortezomib was applied as an agent to increase Hsp expression in the study, considering the literature [25]. The cytotoxic effects of bortezomib were evaluated first, and as a result, 5 nM bortezomib (for 24 hours) treated cells were used for immunological analyzes as a non-toxic dose. According to the results; 2.5 nM, 5 nM, 10 nM and 25 nM concentrations of bortezomib increased Hsp27 expression in U87MG cells (approximately 20%, 48%, 95% and 151%). Since bortezomib at 10 nM and 25 nM concentration have toxic effects on the cells, the dose of 5 nM bortezomib was chosen to increase Hsp expression in the cells. BIRB796 agent was also applied different concentrations to the cells. 100 nM, 250 nM and 500 nM concentrations of BIRB796 without toxic effects were applied to U87MG cells. The expression suppressive effect of MAPK inhibitor BIRB796 on Hsp27 was identified (Fig. 4A) and these data are similar to the literature information [20].

The pHsp27; it is known to prevent apoptosis and help cell survival by protecting cancer cells against heat shock, apoptosis effectors, oxidative stress and ischemia [11]. Similarly, in recent years, phosphorylation levels of Hsp27 were shown to be increased in advanced tumors and it was determined that the increase in pHsp27 level is related to treatment resistance [12, 13, 35, 36]. It was observed that the concentration of 100 nM and 250 nM of BIRB796 significantly reduced pHsp27 expression by approximately 63% and 76%, respectively. At 500 nM BIRB796 concentration, it is deduced that it reduces pHsp27 expression by ~ 100% since no protein band can be seen. In a study similar results were obtained in multiple myeloma cells [20]. With this study, it was gained from literature that the BIRB796 treatment to the U87MG cell line inhibits pHsp27.

Compared to the control group, there was no significant reduction for Hsp27 protein in the combined administration, but approximately 34%, 39% and 38% reductions were observed when compared with 5 nM bortezomib (Fig. 4B). The pHsp27 protein was significantly reduced when compared to both the control group and 5 nM bortezomib (approximately 45%, 80% and 90%, Fig. 5B).

In a study, it was found that the combined administration of bortezomib and BIRB796 to MM.1S cells significantly reduced the level of pHsp27 expression [20]. It was shown that pHsp27 expression was decreased when BIRB796 was applied to cells. In the literature, there are studies evaluating the combined application in myeloma cell lines. However, there is no information regarding the U87MG cell line. With this study, the results of the combined application in the U87MG cell line are presented to the literature.

MAPKs play a role in many intracellular metabolic pathways and act as tumor suppressors or oncogenes. For this reason, it is investigated what effect they have on many type of cancer cells. Various treatment strategies are tried to be developed by using MAPK inhibitors to examine their effects. In such studies, cancer cells with MAPK inhibitors are expected to return to the normal cell cycle and/or cell deaths by the apoptosis mechanism. BIRB796, the most selective and most effective MAPK inhibitor ever known, is an agent that binds to the ATP binding site of p38α, causing inactivation of the enzyme [19]. Preventing phosphorylation of Hsp27 in the cell with the effect of BIRB796 is an important step in breaking the resistance of cancer cells and ensuring that they enter normal cell cycles.
BIRB796 was shown in this study to significantly reduce Hsp27 phosphorylation. The kit was used for direct measurement of the inhibition of MAPK enzyme involved in this phosphorylation, and the results are shown in Table 1. BIRB796 was treated on U87MG cells and cells were collected. Enzyme inhibitions were tested at different times (30, 60, 90 min) using p38α and p38β antibodies with p38 MAPK ELISA kit. As a result of this experiment, % inhibition values were found compared to the control group and IC₅₀ values were calculated. According to the results of the analysis, it was observed that BIRB796 was more effective on the p38α enzyme than p38β. At the same time, the inhibition values increased when the application time was extended. The slow binding capacity of the inhibitor to the enzyme has also been supported in other literatures [19].

In order to test the activation of caspase-3, an enzyme involved in apoptosis pathways, “Caspase 3 Assay Kit, Colorimetric” (Sigma, CASPC3) was used in accordance with the manufacturer’s instructions. For this purpose, 8 different experimental groups were designed (Bortezomib, BIRB796 and combined treatment). The caspase 3 levels of the cells were analyzed by applying 5nM Bortezomib, 100, 250, 500 nM BIRB796 and the combination of these compounds on the U87MG cells. BIRB796 treatment to U87MG cells at concentrations of 100 nM, 250nM, and 500 nM increased caspase 3 levels by 1.3, 1.9, and 2.6 fold, respectively, compared to the control group. As a result of only use of bortezomib, it was observed that the caspase 3 level increased by 1.4 times compared to the control group. In addition, 5nM Bortezomib and different BIRB796 concentrations were applied to U87MG cells, and the result of this experiment significantly increased the caspase 3 level by 1.7, 2.2, 2.8 times, respectively, compared to the control group. Experimental results were compared with the control group and all groups were compared with each other using the Tukey test. These data are consistent with the literature [31, 37].

In conclusion, it was shown that applying the BIRB796 agent to suppress pHsp27 expression in the treatment of brain tumors is an effective therapeutic option. BIRB796 suppresses HSF phosphorylation by providing MAPK inhibition and consequently indirectly reduces pHsp27 expression. BIRB796 is also a molecule responsible for MAPK phosphorylation. In addition to all studies to reduce the expression level of pHsp27 and Hsp27 proteins that provide resistance in U87MG cells, the use of BIRB796 can also be considered an appropriate therapeutic agent.

### Abbreviations

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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>Hsp27</td>
<td>Heat shock protein 27</td>
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<tr>
<td>pHsp27</td>
<td>phosphorylated- Heat shock protein 27</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
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<tr>
<td>GBM</td>
<td>Glioblastoma multiforme</td>
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<tr>
<td>IC₅₀</td>
<td>The half-maximal inhibitory concentration</td>
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Declarations

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Author contributions

SNB made the conception, design and performed the experiments. EÖU participated in the development of methodology. SNB wrote the manuscript. EÖU revised the manuscript. All authors read and approved the final manuscript.

Conflict of interest

The authors declare no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

References


Figures

![Figure 1](image-url)
Effect of Bortezomib and BIRB796 at different concentration/times on the viability of U87MG cells. A Bortezomib dose-response for control (0 nM) U87MG cell viability. Bortezomib was applied on the cells at different concentrations (0 – 50 nM) and different incubation times (24 and 48 h). For U87MG cells, the 24h and 48h IC\textsubscript{50} values of bortezomib are 11.63 and 8.32 nM, respectively. Graph represents the mean ± SD (n = 16). *P < 0.05 **P < 0.01 ***P < 0.001 ****P < 0.0001 determined by one-way ANOVA using Dunnet's multiple comparison test. B BIRB796 dose-response for control (0 nM) U87MG cell viability. BIRB796 was applied on the cells at different concentrations (0 – 2500 nM) and different incubation times (24 and 48 h). Graph represents the mean ± SD (n = 16). *P < 0.05 **P < 0.01 ***P < 0.001 ****P < 0.0001 determined by one-way ANOVA using Dunnet’s multiple comparison test.

Figure 2

Effects of combined therapy of Bortezomib (5 nM and 10 nM) + BIRB796 (50 – 2500 nM) agents on the viability of U87MG cells for 24 hours (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 vertical bars indicate standard deviation values ).
Figure 3

Hsp27 and pHsp27 expression levels after bortezomib treatments in U87MG cells. A,B Graphical representation of the effect of bortezomib administration at different concentrations (2.5 – 25 nM) on expression of Hsp27 (A) and pHsp27 (B) proteins in U87MG human glioma cells by Western blot analysis results (mean ± SD, n = 5, ****P<0.0001, vertical bars indicate standard deviation values). Data demonstrates that bortezomib dose dependently increased Hsp27 and pHsp27 expression. Bortezomib. All data were normalized to GAPDH.
Figure 4

Hsp27 expression levels after BIRB796 and combine treatments in U87MG cells. A, B Graphical representation of the effect of bortezomib administration at different concentrations (100 – 500 nM), effect of Bortezomib (5 nM) and BIRB796 MAPK inhibitor at different concentrations (100, 250, 500 nM) on expression of Hsp27 proteins in U87MG human glioma cells by Western blot analysis results (mean ± SD, n = 7, **P<0.01, ***P<0.001, ****P<0.0001, vertical bars indicate standard deviation values). Bort. Bortezomib. BIRB. BIRB796. All data were normalized to GAPDH.
**Figure 5**

pHsp27 levels after BIRB796 and combine treatments in U87MG cells. **A,B** Graphical representation of the effect of BIRB796 administration at different concentrations (100 – 500 nM), effect of Bortezomib (5 nM) and BIRB796 at different concentrations (100, 250, 500 nM) on expression of pHsp27 proteins in U87MG human glioma cells by Western blot analysis results (mean ± SD, n = 7, ***P<0.001, ****P<0.0001, vertical bars indicate standard deviation values). Bort. Bortezomib. BIRB. BIRB796. All data were normalized to GAPDH.
Figure 6

Bortezomib, BIRB796 increased caspase 3 activity in U87MG. All groups were normalized to the control group (100%). Data represents the mean ± SD. (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, vertical bars indicate standard deviation values). Bort. Bortezomib. BIRB. BIRB796.