Clinical and Experimental Health Sciences

Evaluating the Role of Nrf-2/HO-1 Pathway in Glioblastoma Treatment Efficacy: A Co-Culture Study

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Received: 22.12.2023 **Accepted:** 22.06.2024

ABSTRACT

Objective: Glioblastoma (GB) is a highly lethal form of brain tumor. Although standard therapy appears to be effective, the survival time is quite short, and the recurrence rate is high. Bortezomib (BTZ), is a proteasome inhibitor, used in GB therapies and resulted in serious off-target effects. Carfilzomib (CFZ), is an alternative for BTZ, has known with nonserious off-target effects. This study aimed to examine the potential off-target effects caused by BTZ and CFZ in terms of the therapy related activation of antioxidant mechanisms regarding to Nuclear factor (erythroid-derived 2)-like 2 (Nrf-2)/Heme Oxygenase-1 (HO-1)-dependent response.

Methods: GB cells were co-cultured with heathy astrocyte (HA) cells to mimic the tumor microenvironment in some extent. Cell viability was determined following ionizing radiation (IR), temozolomide (TMZ), BTZ and CFZ alone and in combination. Nrf-2 and HO-1 protein expressions were analyzed by western blotting assay.

Results: Co-culture results showed that the GB cells in the BTZ-treated groups expressed higher levels of Nrf-2 and HO-1 than in the CFZ-treated groups. In the HAs, the group treated with CFZ showed higher Nrf-2 expression than the group treated with BTZ alone, while the same groups in combination with TMZ&IR showed exactly opposite results. HO-1 expression was also not seen in any of the HA groups.

Conclusion: The significant increase in Nrf-2 levels in the CFZ-treated group in the HAs could also be interpreted as CFZ promoting the defence of healthy cells against therapy-induced stress conditions. Although further studies are needed, these preliminary results show that the evaluation of CFZ as a second-line therapy could be a milestone for the treatment of GB.

Keywords: Glioblastoma, proteasome inhibitors, bortezomib, carfilzomib, nuclear factor (erythroid-derived 2)-like 2, heme oxygenase-1

1. INTRODUCTION

Glioblastoma (GB) is the most common and most aggressive grade IV astrocytoma in adults. As seen in clinical studies, GB is a disease with a poor prognosis with a 2-year survival rate of 26-33% and a 5-year survival rate of only 4-5% (1–4). The standard treatment includes the combination of temozolomide (TMZ) and ionizing radiation (IR) after surgical resection of the tumor (if possible) (3,5).

TMZ is an alkylating chemotherapy agent that causes DNA damage in tumor cells, including DNA double strand breaks, which trigger apoptosis and cytotoxicity (6,7). However, development of high rate of resistance to IR and TMZ in tumor cells, results in the recurrence of the disease. Since the exact mechanism and related pathways that underlying the recurrence and resistance of disease is not clear, the presence of GB stem cells (GSCs) is known as leading cause as for now (8,9). As shown in a recently published study (10), one of the struggles about GSCs that must be solved is their higher levels of proteasome activity which causes resistance to therapy. Another cause of therapy resistance is the reversal of the TMZ activity in tumor cells by the help of O-methylguanine-DNA-methyltransferase. It is seen that the proteasome inhibitor, bortezomib, is able to both inhibit proteasome and methyltransferase enzyme and helps to overcome this resistance with its chemo-sensitizing effect (11,12). In the light of these and related other studies, the need of proteasome inhibitors in GB treatment is confirmed.

The ubiquitin proteasome system which is responsible for the degradation of damaged proteins, regulation of signal

DOI: 10.33808/clinexphealthsci.1408641

pathways and immune response, is the major protein degradation mechanism in eukaryotic cells to balance their cellular homeostasis. Since cancer cells maintain their higher proliferation rates mostly depend on this system, proteasome inhibitors have been tried for the treatment of diverse types of cancer including GB, for more than 15 years (13,14). Bortezomib (BTZ), one of the FDA-approved first-generation proteasome inhibitors, is frequently used in combination in the clinic in hematological cancers and increases survival at a high rate (15). BTZ is a proteasome inhibitor that reversibly inhibits both the caspase-like and chymotrypsin-like activities of proteasome with its boronic acid pharmacophore (15–17). The off-target interactions of BTZ causes significant side effects like peripheral neuropathy and limits its use in solid tumor cancers (16,18,19). The extremely aggressive nature of chemotherapy agents used in solid tumor treatment, combined with the effect of BTZ, makes it exceedingly difficult for the patient to overcome all these side effects. Not all patients respond equally to BTZ-centered treatments, and it appears that cancer may recur in some patients who initially respond. Especially solid tumors may develop resistance to BTZ in some cases. Hence, second generation proteasome inhibitors have been studied and new proteasome inhibitors have been developed, like Carfilzomib (CFZ) (18,20,21). CFZ is an irreversible and highly selective proteasome inhibitor, differing from BTZ by its structure and mechanism of action (22). It binds covalently and irreversibly to the 20S proteasome and composes morpholino adducts with its epoxyketone structure, which is not active as boron moiety of BTZ, and inhibits the chymotrypsin-like activity of proteasome (19). Both the minor off-target and superior on-target interactions of CFZ provide it more potential against BTZ (16,19). By causing less nervous system damage and peripheral neuropathy than BTZ, CFZ is a promising proteasome inhibitor to combine in standard therapies (16,21–23).

However, triggering of Nuclear factor (erythroid-derived 2)-like 2 (Nrf-2) dependent antioxidant mechanisms limit the efficiency of proteasome inhibitors, during the treatment (14). Nrf-2 is a key transcription factor involved in the regulation of a wide variety of cytoprotective genes such as inflammation proteins, antioxidant, and detoxification enzymes (8,24,25). Nrf-2 activation is tightly regulated by Kelch-like ECH associated protein (KEAP1), an adapter for Cullin 3 (Cul3)-based ubiquitin E3 ligase. KEAP1 binds to Nrf-2 and enables the ubiquitination process to occur. KEAP1-Nrf-2 system, one of the ubiquitin-proteasome-based regulatory systems, is unique in that it can sense oxidative and electrophilic stresses through reactive cysteine residues in KEAP1 and mediate the expression of cytoprotective enzyme genes through Nrf-2 activity. When cells are exposed to reactive oxygen species (ROS) or electrophilic toxicants, the reactive cysteine residues in KEAP1 are covalently modified by ROS or electrophiles, which stops ubiquitination of Nrf-2. As a result, the amount of Nrf-2 begins to accumulate in the cell. This accumulation induces the transactivation of cytoprotective genes. Nrf-2 regulates the expression of genes encoding detoxifying and antioxidative enzymes like Heme Oxygenase-1 (HO-1). In contrast, Nrf-2 also down-regulates genes encoding proinflammatory factors such as interleukin-6 (IL-6) and interleukin-1β (IL-1β) (26).

Through the proteasomal activation, Nrf-2 levels are kept restricted within the cell under normal physiological conditions and rises very rapidly when cells are exposed to a variety of environmental stresses (24,27). In healthy cells, the overexpression of Nrf-2 is correlated with suppression of tumor formation. However, higher Nrf-2 levels cause dysregulation of autophagy, tumor progression, and chemoresistance in tumor cells along with poor prognosis (8,24,25). In various studies, Nrf-2 has been found to be overexpressed in GSCs, increases their proliferation; and causes tumor recurrence and aggression (25,28). It was also observed that increased Nrf-2 expression in GB cells decreased apoptosis and increased HO-1 expression (29). Nrf-2 and HO-1 upregulation is correlated with the progressed, vascularized, aggressive and therapy resistant GB cells (9,14,24).

Herewith, we aimed to elucidate the mechanisms of second-generation proteasome inhibitor CFZ against first-generation proteasome inhibitor BTZ mediated alterations on the Nrf-2/HO-1 dependent antioxidative response in term of both cancer and healthy cells in GB and healthy astrocyte (HA) cells co-cultivation.

2. METHODS

2.1. Cell Culture

In this study, GB and HA cells were used. GB cells were purchased from ATCC (American Type Culture Collection) with the code DBTRG (ATCC® CRL2020™, Manassas, VA United States). HAs were purchased from Innoprot (P10251, Derio, Bizkaia, Spain). The flasks and petri dishes that were used in astrocytes culture, were coated with Poly-L-lysine to provide cell adhesion. Poly-L-lysine (stored at +4 °C) was mixed with dH_2O to obtain 30 µg/mL concentration and then the surfaces of culture dishes were coated with 1 mg/mL Poly-L-lysine. The covers of the dishes were wrapped in parafilm to be isolated, and they were kept at +4 °C for 3 days. The excess Poly-L-lysine was aspirated before use, the dishes were washed once with 1X DPBS (Dulbecco's phosphate-buffered saline) and then appropriate medium was added on the dishes and cells were cultured. As recommended by the ATCC, GB cells were cultured in high DMEM (Dulbecco's Modified Eagle Medium) containing 10% FBS (Fetal Bovine Serum), 1% penicillin/streptomycin, and 0.1% amphotericin; and astrocytes were cultured in DMEM-F12 (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12) containing 2% FBS, 1% penicillin/streptomycin, 0.1% amphotericin, and 1% astrocyte growth supplement in Poly-L-lysine coated flasks in 5% CO_2 and 37 °C incubator conditions.

2.2. Co-cultivation of glioblastoma cells with astrocytes

For co-cultivation of GB cells with HAs, inserts were used in 24-well plates. The used inserts were obtained as being appropriate for 6/24 well plates, having 0.4 µm pore size (PICM01250, Merck Millipore Millicell, Burlington, MA United States). The structures of pores were small enough to prevent cell escape and large enough to permit the medium's ingredients and signal molecules. To perform co-cultivation, the wells were coated with the 450 µL Poly-L-lysin and $15x10^3$ HA cells were cultured onto coated wells in 600 µL medium. Following, inserts were placed into the Poly-L-lysin coated wells and $10x10^3$ GB cells were cultured in inner surface of inserts in 400 µL medium. The cells were incubated in 5% CO_2 and 37 °C conditions for 24h prior to drug exposures.

2.3. Drug and ionizing radiation treatments

The next day following the co-cultivation, TMZ (ALX – 420-044-M025, Enzo Life Sciences, Lausen, Switzerland), BTZ (PS341, Selleck Chemicals, Houston, TX United States) and CFZ (PR-171, Selleck Chemicals, Houston, TX United States) administrations were performed. Stock drug solutions were prepared with DMSO (Dimethyl sulfoxide) in 10 µM concentrations. Formerly obtained IC_{50} drug doses of TMZ, BTZ and CFZ by our group were administered: 300 nM for BTZ, 500 nM for CFZ and 50 µM for TMZ (in 0.1% DMSO) for 48h. Before the IR exposures, medium of cells were refreshed with phenol red free medium due to the detrimental effects of IR to structure of phenol red. Cells were exposed to single dose 2 Gy ionized radiation by using 6 MV photon energy. Following the IR exposure, the mediums of cells were replenished, and drug administrations were performed. The treatment groups were classified as; control (CTRL), TMZ treated, BTZ treated, CFZ treated, IR treated, TMZ&IR treated, TMZ&IR&BTZ treated and TMZ&IR&CFZ treated groups. The IR, TMZ&IR, TMZ&IR&BTZ and TMZ&IR&CFZ groups were IR exposed groups. The mediums of IR-exposed groups were Phenol red-free. Following the IR, cells were incubated 5% CO_2 and 37 °C conditions for 1 hour. After incubation, drug administrations were performed for all groups for 48h.

2.4. Cell viability assay

Following the 48h drug exposures, cell viability was assessed with MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide, Sigma-Aldrich, St. Louis, MO United States) according to manufacturers' protocol. Briefly, 5 mg/mL MTT solution was added into each well and cells were incubated for 3 h at 37 °C. Then, all the media were discarded from wells and 300 µL DMSO was added onto each well to dissolve the formed tetrazolium salts. After the incubation of plate for 10 minutes at 37 °C, the absorbance value was measured at 590 nm using an EnSpire multimode plate reader (PerkinElmer, Waltham, MA United States) to detect cell viability. Experiments were designed as triplicated.

2.5. Western blotting

Isolation of total proteins from cells was performed via Cell Lysis Buffer (Cell Signaling Technology, CST, Danvers, MA United States) containing protease inhibitor cocktail and PMSF (phenylmethylsulphonyl fluoride). Determination of the protein concentrations of the samples were performed with Pierce™ BCA (Bicinchoninic acid) Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA United States) and 20 µg of protein sample was loaded into gels per group. The protein samples were separated with 10–12% SDS-PAGE (Sodium dodecyl-sulphate polyacrylamide gel electrophoresis) and were blotted onto nitrocellulose membranes (Thermo Fisher Scientific, Waltham, MA United States). The membranes then were blocked with 5% non-fat dry milk-TBST (Tris-buffered saline with 0.1% Tween 20 detergent) and incubated with primary antibodies at 4 °C overnight. Following incubation with HRP (Horseradish peroxidase) conjugated secondary antibody for 2 h at room temperature, blots were developed using ECL (enhanced chemiluminescence) reagent (Thermo Fisher Scientific, Waltham, MA United States). The bands were visualized using ChemiDoc™ MP System (Bio-Rad Laboratories, Hercules, CA United States) and band densities were quantified using Image Lab software. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as the loading control.

Antibodies

The following antibodies were used: Nrf-2 (Cell Signaling, 12721S, 1:1000), HO-1 (Cell Signaling, 5061S, 1:1000), GAPDH (Novus, NB300-221, 1:1000), HRP conjugated anti-rabbit (Cell Signaling, 7074P2, 1:10000).

2.6. Statistical analysis

Statistical analyses were performed with GraphPad Prism 9 (GraphPad Prism version 9.0.0 for Windows, GraphPad Software, Boston, MA USA). The data expressed as mean ± SD of three independent experiments and were analysed by one-way ANOVA followed by Tukey's post-tests between groups. A p-value <.05 was selected as statistically significant, and p value < .0001 was considered extremely significant.

3. RESULTS

3.1. BTZ and CFZ significantly decrease the cell viabilities

Cell viability of GB and HA cells decreased significantly after 48 hours of exposures to 50 µM TMZ, 300 nM BTZ, 500 nM CFZ and their combinations along with the IR exposures. The values did not differ between the groups treated with BTZ and CFZ in GB cells. However, the CFZ-treated groups showed higher cell viability than the BTZ-treated groups in HA cells (Fig. 1).

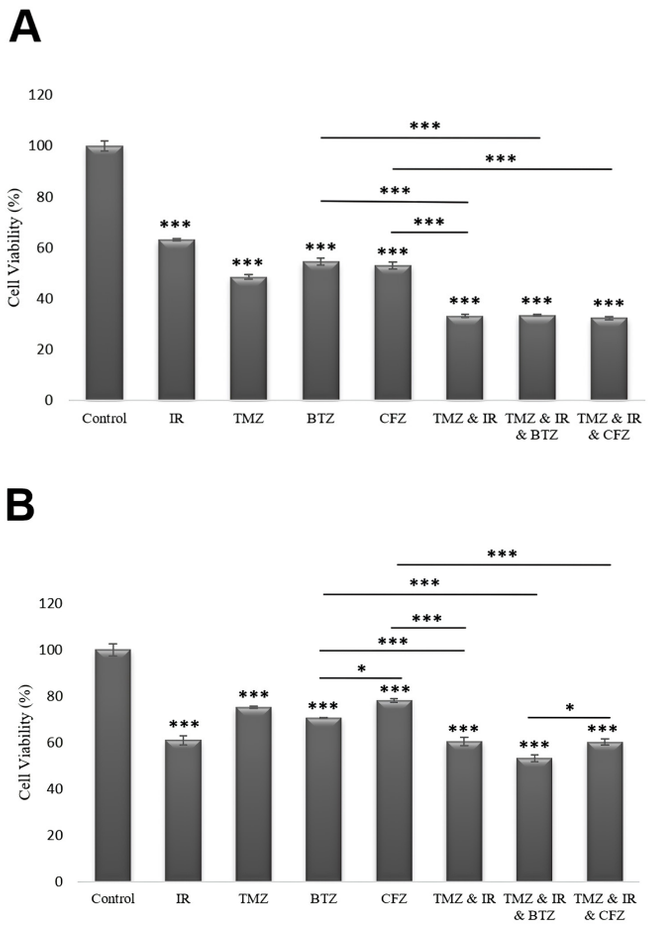

Figure 1. *Percentage viability of* **(A)** *glioblastoma cells and* **(B)** *human astrocytes after IR, 48 h of BTZ, CFZ, TMZ and their combinations treatments with MTT-assay. All the data are presented as means ± SD (n=3). *P < .05, ***P < .001 The P value is < .0001, considered extremely significant for both cell viabilities.*

3.2. BTZ and CFZ treatments effects the Nrf-2 and HO-1 expression levels of cells

According to the western blotting analysis, Nrf-2 and HO-1 protein levels in GB cells showed variable results in response to different therapy combinations (Fig. 2A). The Nrf-2 protein levels of BTZ-treated group was significantly higher compared to the control, CFZ and TMZ & IR & BTZ-treated groups. CFZ treatment alone decreased the Nrf-2 levels compared to both BTZ treatment alone and TMZ & IR & CFZ treated groups (Fig. 2B). HO-1 protein concentrations in the BTZ, CFZ, TMZ & IR & BTZ and TMZ & IR & CFZ treated groups were significantly higher than in the control group, in which HO-1 protein concentrations were strikingly low. BTZ treatment alone increased HO-1 levels compared to the CFZ treatment group (Fig. 2C).

On the other hand, the Nrf-2 levels in the HAs varied in the different treatment groups (Fig. 3A). Compared to the control group, significant increases were observed in the groups treated with BTZ, CFZ and TMZ & IR & BTZ. Nrf-2 levels were lower in the BTZ-treated group than in the CFZ-treated group, the exact opposite of the GB cells. At the same time, Nrf-2 levels were higher in the CFZ-treated group than in the TMZ & IR & CFZ-treated group (Fig. 3B). However, the expression of HO-1 in HAs was not observed as there was no induction factor for this expression.

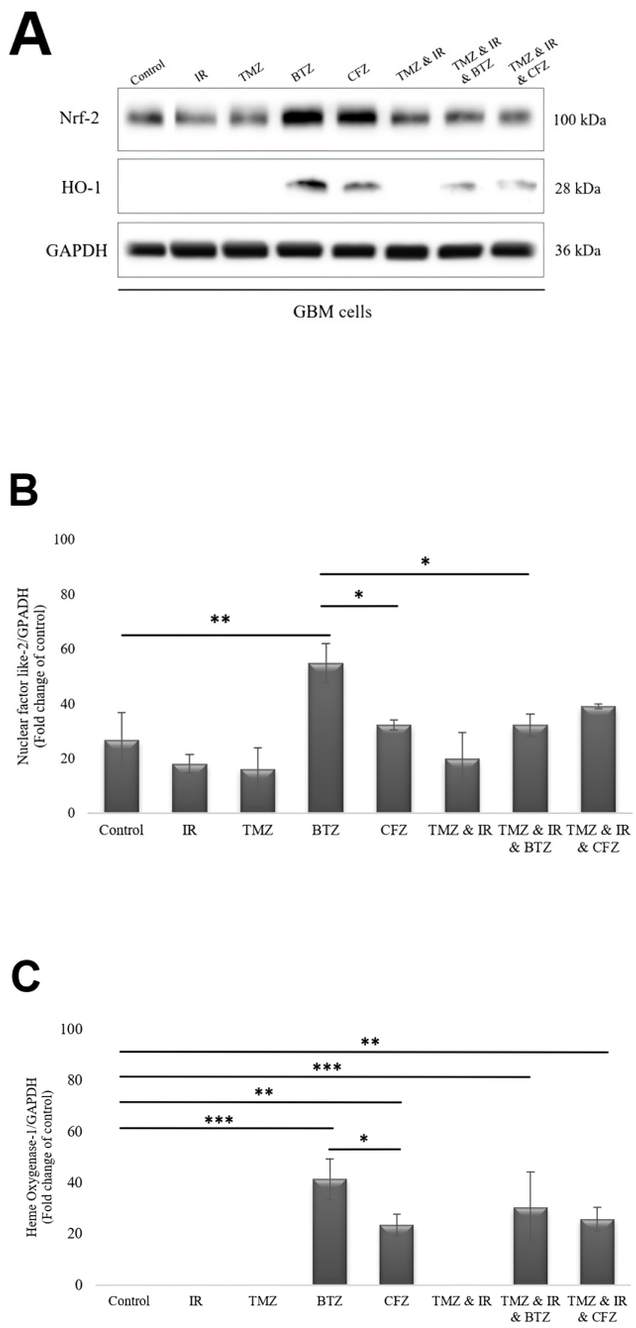

Figure 2. *IR, TMZ, BTZ, CFZ and their combination treatments affect the Nrf-2 and HO-1 levels in glioblastoma cells A Western blotting analysis of protein levels of GB cells treated with IR, 50 µM TMZ, 300 nM BTZ, 500 nM CFZ and their combinations with indicated antibodies. B – C Quantification of Nrf-2 and HO-1 protein levels after IR, TMZ, BTZ, CFZ and their combination treatments for 48 h in GB cells. All the data are presented as means ± SD (n=3). *P < .05, **P < .001, ***P < .001 The P value is < .0001, considered extremely significant for protein expressions.*

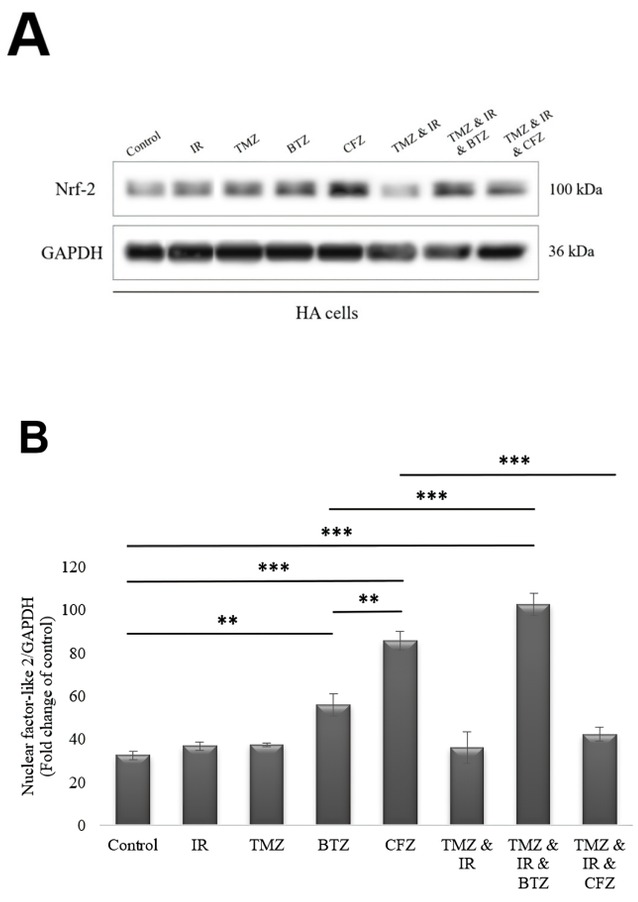

Figure 3. *IR, TMZ, BTZ, CFZ and their combination treatments affect the Nrf-2 levels in human astrocytes* **A** *Western blotting analysis of protein levels of HA cells treated with IR, 50 µM TMZ, 300 nM BTZ, 500 nM CFZ and their combinations with indicated antibody.* **B** *Quantification of Nrf-2 protein levels after IR, TMZ, BTZ, CFZ and their combination treatments for 48 h in HA cells. All the data are presented as means ± SD (n=3). **P < .01, ***P < .001 The P value is < .0001, considered extremely significant.*

4. DISCUSSION

In this study, we tested proteasome inhibitors BTZ and CFZ in co-cultivation of GB-HA cells. Since diverse types of antioxidant mechanisms get activated as a response to applied treatments, we aimed to observe the given Nrf-2/HO-1 dependent antioxidant response in combined BTZ and CFZ treatments from GB and HA cells by co-culturing and analyzing the relevant protein expressions of the cells.

The aim of the therapies is to eradicate the malignant tumor without affecting the surrounding healthy cells when it comes to overcoming tumors. However, the healthy cells responsible for maintaining the microenvironment and the development of the tumor will most likely be affected by potential off-target effects of the administered chemotherapies (30). Herein, we focused on the off-target effects of applied GB therapies on both GB and HA cells in terms of unintentionally activated antioxidant signalling pathways.

Nrf-2 is an essential transcription factor that regulates the mechanisms of cellular protection against a wide variety of stress conditions (13). Their levels are kept low within the cells in the absence of stress conditions by way of KEAP-1 proteins. KEAP-1 regulates Nrf-2 levels by inclining the ubiquitination of Nrf-2 proteins and hence causing degradation in proteasomal systems (13,31,32). When we considered the GB cells underwent the cellular stress by applied treatments and through the exposed proteasome inhibitors, the degradation of Nrf-2 proteins through proteasomal pathways halted and consequently, Nrf-2 levels and accordingly HO-1 levels increased within the cells. Not surprisingly, the GB cells are more prone to be affected by proteasomal inhibition when compared to HA cells. Together with the remarkable decrement in proteasomal activity, Nrf-2 and HO-1 levels reached higher levels in proteasome inhibitor treated groups, especially with BTZ treatment.

The following groups of GB cells showed increased Nrf-2 expressions; BTZ, CFZ, TMZ & IR & BTZ and TMZ & IR & CFZ; and in parallel with this finding, HO-1 levels in these groups showed significant increases. Thus, in GB cells, with the increased stress conditions by applied treatments, cells resisted to survive by increasing cytoprotective Nrf-2 and HO-1 proteins to prevent apoptosis which is not intended by an efficient treatment.

On the other hand, while BTZ, CFZ and TMZ & IR & BTZ treated groups showed increased Nrf-2 levels in HA cells, we did not observe HO-1 expression in HA cells with western blot analysis even with increased level of protein concentrations loaded to gel. HO-1 is a downstream target of Nrf-2 and serves as an essential indirect antioxidant enzyme within the cell (33). Unlike the tumor cells, the induction of HO-1 expression by Nrf-2 with the drug exposures did not occur. HO-1 as being the downstream protein of Nrf-2, is expressed in highly activated intracellular antioxidant response. Since the drug exposures were not sufficient to generate excessive oxidative stress and there were any known inducers, like heavy metals, UV light, hydrogen peroxide, and lipopolysaccharide, to induce HO-1 expression in healthy cells (31,34), HO-1 expression was not seen in HA cells. The elevation of Nrf-2 levels in HA cells indicates the attempt of the cells to defense themselves both to applied treatments and culminated side effects.

The proteasomal activity increment is one of the upregulation mechanisms of cancer cells to enhance their survival rates. Thus, proteasomal system inhibitors are highly promising approaches to overcome cancer cell survival that results from proteasomal system activity. However, as seen in this study, inhibiting proteasomal activity may result in the activation of other cytoprotective mechanisms within the cells, like increased Nrf-2 and HO-1 levels. Accordingly, as an alternative promising therapy approach beyond the proteasomal inhibitors may be targeting the downregulation of the Nrf-2 levels within the cells (24).

For GB cells, both the Nrf-2 and HO-1 levels of BTZ treated group are over the levels of CFZ treatment except for combination groups and vice versa for HA cells. As

hypothesized, the increment in antioxidant protein levels in tumor cells and the decline in healthy cells may be the reason for the inefficaciousness and off-target outcomes of the treatment like neuropathy. Most of the side effects of BTZ eventuates through inflammation and oxidative stress because of its multiple intracellular actions caused by its boron moiety (19). In this regard, the lower Nrf-2 and HO-1 levels of CFZ present intended therapy results as having morpholino adducts with its epoxyketone structure, which is not active as boron moiety of BTZ and ends up with lower off-target results. In another study with healthy mouse NSC cells (21), the fact that stress proteins like stress-70 protein, superoxide dismutase [Mn], Protein disulfide-isomerase A3, heat shock protein (HSP) 90-alpha, protein disulfide-isomerase A6, catalase, HSP32, HSP47 and HSP70 expressions that can induce neuropathy were lower in the CFZ group compared to BTZ or were expressed at the same levels as the control group confirms the results of our current study.

The groups with combined treatment have the opposite results to the groups treated with BTZ and CFZ alone. While the combined treatment with BTZ leads to a higher induction of the antioxidant signalling pathways in HA cells, the treatment with BTZ alone shows a lower induction, but in GB cells these results are exactly opposite and not consistent with the fate of cell survival. Furthermore, the combined treatment with CFZ is shown to lead to a decrease in the relevant proteins, while treatment with CFZ alone increases the levels in HA cells as intended, whereas the combined treatment groups maintain relatively higher levels in GB cells. However, the effects of the combined treatment on antioxidant metabolic pathways are not maintained by cellular viabilities. It can be concluded that as the complexity of the applied therapy increases, the number of affected metabolic pathways increases and it becomes more difficult to control all metabolic pathways.

Besides, as Fig.2 shows TMZ&IR treated group has lower Nrf-2 and HO-1 levels when compared to BTZ and CFZ groups. However, this reduction does not state an increment in the cellular viabilities of same groups. In Fig.1, it is clearly seen that BTZ and CFZ treated groups show higher levels than TMZ&IR treated group.

If we consider all these results together, we concluded that neither the treatment with TMZ&IR nor the combination treatment with BTZ and CFZ guarantees effective treatment. On the other hand, CFZ treatment alone outperforms BTZ treatment alone and outperforms all treatment groups.

In summary, we present that Nrf-2 levels increased within the cells in response to proteasomal inhibition. Besides, in GB cells, increased Nrf-2 levels induced the elevation of HO-1 expression levels. Through the activation of cytoprotective mechanisms, cancer cells take advantage of Nrf-2 to withstand to undergoing apoptosis with all its strength. This effect especially is seen in the administration of BTZ rather than CFZ. While the activation of Nrf-2 causes tumor progression and resistance to therapy, silencing of Nrf-2 in tumor cells shows diminishment of cell migration and metastasis (14,24). Targeting the Nrf-2 may seem to be an efficient way to overcome the challenges resulting from its overexpression. However, since the physiological roles of Nrf-2 is highly vital for healthy cells, on-target therapy may result in deleterious side effects in patients (35). Alternatively, targeting indirectly by the way of related cellular pathways may be more prone to get constructive results. In this regard, prioritizing the second-generation proteasome inhibitor CFZ as a replaced therapy, may provide the intended Nrf-2 levels which are lower for GB cells and higher for HA cells. Further studies that will replace the treatments with CFZ may provide an outstanding insight for the GB treatment.

5. CONCLUSION

Besides the known roles of Nrf-2 and HO-1 in cancer development and progress, there are still lots of mysteries. In consideration of our results, it may be hypothesized that the inefficacy of current treatments that includes BTZ, may be the result of increased levels of devastating Nrf-2 and HO-1 in GB cells. Replacing the standard treatments with CFZ and investigating the Nrf-2 originated tumor progression and resistance in GB may likely focus on directly the basis of tumor bulk and give more consistent results in future.

Acknowledgement: *We would like to thank all participants involved in the study.*
Funding: *This study was supported by The Scientific and Technological Research Council of Türkiye-TÜBİTAK 118S522 and TÜBİTAK 2209A, and by Marmara University Research Fund (BAPKO) with the Project Number TYL-2023-10894*
Conflicts of interest: *The authors declare that they have no conflict of interest.*
Ethics Committee Approval: *This study was not required any approval by Ethics Committee.*
Peer-review: *Externally peer-reviewed.*
Author Contributions:
Research idea: BY, SAY, MFB
Design of the study: BY, AŞ, BMA, OK
Acquisition of data for the study: ZG, SAY, MFB
Analysis of data for the study: ZG, SAY, AŞ
Interpretation of data for the study: ZG, AŞ, BY
Drafting the manuscript: ZG, AŞ, BY
Revising it critically for important intellectual content: ZG, AŞ, BY
Final approval of the version to be published: ZG, AŞ, BY, SAY, MFB, BMA, OK

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How to cite this article: Güneş Z, Başkent MF, Arslan Eseryel S, Atasoy BM, Köstek O, Şahin A, Yılmaz B. Evaluating the Role of Nrf-2/HO-1 Pathway in Glioblastoma Treatment Efficacy: A Co-Culture Study. Clin Exp Health Sci 2024; 14: 760-767. DOI: 10.33808/clinexphealthsci.1408641