



Article

Effects of Disulfiram and Copper in Combination with Temozolomide on Survival, Tumor Size and Autophagy Markers in an F98 Rat Glioma Model

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Abstract

Glioblastoma (GBM) is the most common and most aggressive malignant primary brain tumor in adults with a median survival of 15 months. One of the main factors responsible for the poor prognosis of GBM is resistance to treatment with temozolomide (TMZ), which has been attributed—among other factors—to autophagy. Preclinical studies have shown that the combination of disulfiram (DSF) with copper (Cu) possesses anti-GBM activity, through various mechanisms, including re-sensitization to TMZ. Herein, we tested for the first time the effects of DSF and Cu in combination with TMZ on the survival of Fischer rats bearing F98 glioma, a model characterized by inherent resistance to TMZ. Tumor size evaluation by Magnetic Resonance Imaging as well as immunofluorescence analysis of two autophagy markers, namely microtubule-associated protein 1 light chain 3 (LC3) and sequestosome-1 (SQSTM1)/p62 (p62), were also performed. According to our results, TMZ-DSF-Cu significantly increased mean survival and induced both LC3 and p62 autophagy markers. Interestingly, these results could not be achieved in the absence of Cu, neither in the presence of TMZ alone, suggesting the importance of combining DSF with Cu in order to sensitize glioma to TMZ, presumably via implication of autophagy modulation.



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1. Introduction

Glioblastoma (GBM), a World Health Organization isocitrate dehydrogenase wild-type grade IV glioma, is the most common and most aggressive malignant primary brain tumor in adults [1,2]. The current standard of care for newly diagnosed GBM includes maximal safe resection to reduce the tumor mass followed by postoperative radiotherapy with concomitant and adjuvant treatment with temozolomide (TMZ). Unfortunately, the effect of this treatment regimen is limited and recurrence occurs within 6–9 months of initial diagnosis, resulting in a median overall survival of 15.6 months [3,4]. There is, therefore, an urgent need to develop new and effective therapeutic strategies for GBM.

The low survival rate of GBM is mainly due to its diffuse nature, making surgical removal challenging, and its resistance to radio- and chemotherapy [5]. GBM resistance to treatment has been attributed—among other factors—to autophagy which allows cellular adaptive survival towards various stress stimuli including drugs and ionizing radiation [6]. Hence, targeting autophagy has been recently proposed as a new potential therapy for GBM [7].

Macroautophagy (hereafter described as “autophagy”) is the process of degradation and reuse of aging or damaged organelles and various macromolecules within cells. During autophagy, a double-membrane vesicle named autophagosome is formed and engulfs cellular content. Subsequently, autophagosome merges with a lysosome to form an autolysosome, leading to degradation of its content by the lysosomal degradative enzymes [8]. Through this process, the cell effectively meets its metabolic requirements and facilitates the regeneration of certain organelles. In GBM, autophagy, which in this case is designated as protective autophagy, is upregulated in response to radio- and chemotherapy, helping to desensitize tumor cells to treatment and giving them an advantage for survival and dissemination [9]. On the other hand, when autophagy is upregulated beyond a certain threshold, it becomes fatal (lethal autophagy), inhibiting cell proliferation and inducing a specific type of non-apoptotic tumor cell death, named autophagic cell death (ACD) [10]. Consequently, inhibition of protective autophagy can make GBM cells more sensitive to chemo- and radiotherapy while induction of lethal autophagy can induce tumor ACD [10]. Thus, the identification of novel therapies that could induce such autophagic alterations might be a viable strategy to overcome chemo- and radioresistance and increase treatment efficacy in GBM patients.

Currently, the clinical application of autophagy in GBM treatment mainly involves inhibiting protective autophagy with the autophagy inhibitors, namely chloroquine (CQ) and hydroxychloroquine (HCQ), and combining them with TMZ in order to sensitize cancer cells to chemotherapy and enhance TMZ’s cytotoxic effects [9,11]. However, based on the results of a phase I and a phase II clinical trial, the use of CQ and HCQ in GBM cannot be recommended due to the high concentrations required for their effectiveness, while they may still elicit significant adverse effects in combination with TMZ, even in low doses [9,11]. On the other hand, there is no strategy to treat the disease by inducing ACD since there are no clinically approved autophagy inducers for GBM. Therefore, we need to develop novel effective and safe autophagy inhibitors and inducers, suitable for clinical use in GBM patients.

Taking into consideration the time-consuming process and the high cost of developing a new medication, as well as the fact that GBM is a rare disease [12], which further

limits financial incentive for drug development, it becomes obvious that there has been growing interest to repurpose older drugs approved for non-oncologic diseases as potential treatments for GBM. One of the most promising drugs to repurpose for treating GBM is disulfiram (DSF), a relatively nontoxic dithiocarbamate disulfide with well-established pharmacokinetics, a safety profile and the ability to readily cross the blood–brain barrier (BBB). DSF has been used for more than seventy years in the treatment of chronic alcoholism, because of the unpleasant symptoms it provokes after ethanol intake [13]. The underlying mechanism is believed to be the accumulation of acetaldehyde in the blood, due to inhibition of the liver aldehyde dehydrogenases (ALDHs) [14].

The original suggestion to use DSF in the treatment of GBM originated from a position paper published in 2009 [15]. Such a role for DSF was later supported by a number of preclinical in vitro and in vivo studies [16–25]. Interestingly, a number of these anti-GBM effects of DSF was shown to be copper (Cu)-dependent through the induction of reactive oxygen species, proteasome inhibition, and inhibition of both ALDH and Nuclear Factor kappa B-related pathways [16,18,21]. In addition, previous studies have shown that DSF and Cu exhibit anti-tumor activity in solid tumors, other than GBM, through either activating or inactivating autophagy [26–28]. However, to date, the impact of DSF and Cu on autophagy, in the context of GBM, has not been evaluated. This study aimed to address for the first time the effects of DSF and Cu in combination with TMZ on survival, tumor size and autophagy markers in an F98 rat glioma animal model.

2. Results

Six groups of ten rats were used for the experiments. The first group served as the control-receiving-only vehicle, while the other five groups received TMZ, DSF, TMZ and DSF, DSF and Cu or TMZ and DSF and Cu.

2.1. Successful Establishment of Glioma Within the Ipsilateral Striatum Following Intrastratial Injections of F98 Rat Glial-like Cells

For histopathological analysis of the brain tissues, we used hematoxylin and eosin (H&E) staining which is the primary tool for diagnosing and grading gliomas, highlighting overall tissue architecture, nuclear atypia, mitotic activity and necrosis. In addition, the Nissl staining was employed to distinguish the tumor from the normal brain tissue.

The presence of hypercellular and infiltrating the adjacent glial tissue neoplasms were documented (Figure 1A). The tumor cells were poorly differentiated and arranged mainly in a diffuse manner, having pleomorphic nuclei and mitotic activity (Figure 1B). Microvascular proliferation and necrosis were also apparent. Necrosis was geographic or pseudopalisading with neoplastic cells surrounding central necrotic areas (Figure 1C). The morphological features were consistent with high-grade diffuse gliomas and specifically with a GBM histological type. Nissl staining further confirmed the presence of tumor cells within the ipsilateral striatum (Figure 1D).

2.2. Survival

In order to estimate survival probabilities after treatment, we generated and analyzed the Kaplan–Meier curve documenting statistically significant differences among the study groups ($p < 0.001$, Figure 2A). However, the only group that demonstrated significantly higher survival probabilities compared to the control was the group of TMZ-DSF-Cu ($p = 0.002$).

Relative to the control, the TMZ-DSF-Cu group significantly increased in mean (95% CI) survival by 3.5 (6.88–0.12) days [Mean (SD): 32.30 (3.43) for TMZ-DSF-Cu group vs. 28.8 (2.3) for control group, $p = 0.04$; Figure 2B], which corresponds to about 12% increase

in life span. No significant differences between the control and the other therapy groups were observed (Figure 2B).

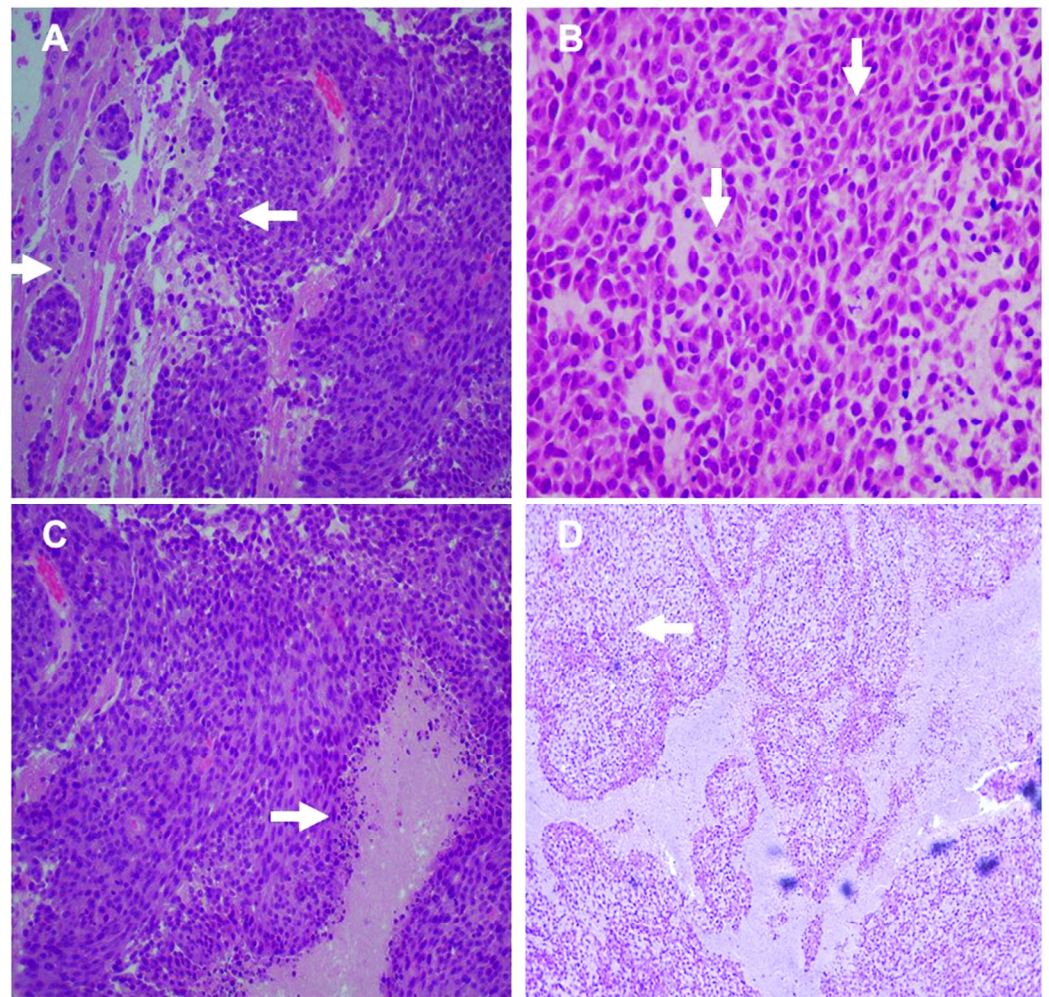


Figure 1. Tumor histopathology. (A) Poorly differentiated tumor cells (right arrow) diffusely infiltrated the adjacent brain tissue (left arrow) (H&E stain, original magnification $\times 100$); (B) tumor cells were of medium to large size with eosinophilic cytoplasm and round to oval hyperchromatic, pleomorphic nuclei. Mitotic figures were apparent (arrows) (H&E stain, original magnification $\times 200$). (C) Pseudopalisading necrosis. Neoplastic cells surrounded central necrotic areas (arrow) (H&E stain, original magnification $\times 100$), (D) Representative image of Nissl-stained section from tumor tissue (arrow). Scale bar: 100 μ M.

2.3. Tumor Size

Magnetic Resonance Imaging (MRI) scans were performed to estimate tumor size in rat brain twenty days after the intracerebral implantation of the F98 cells (Figure 3A). Even though the TMZ-DSF-Cu group displayed a trend towards decreased tumor size as compared to control, this difference was not statistically significant (Figure 3B). Likewise, no statistically significant differences between the control and the other experimental groups were demonstrated pertaining to tumor size (Figure 3B).

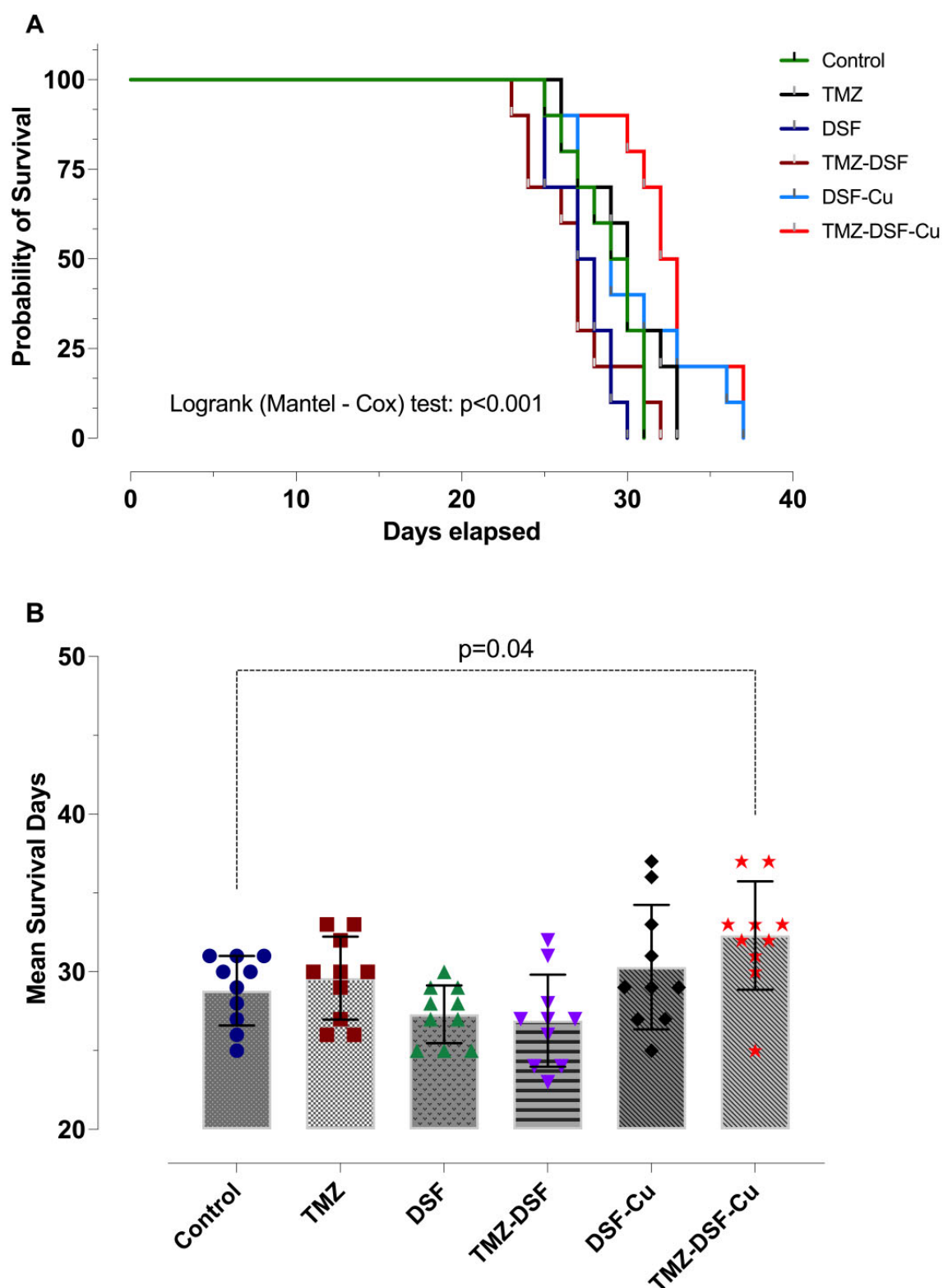


Figure 2. Survival analysis. (A) Kaplan–Meier curves. (B) Survival duration (days). Statistical analysis for survival days was performed using one-way ANOVA with Dunnett’s test for multiple comparisons. Data ($n = 10$ animals per group) are reported as mean $\pm$ standard deviation (SD); individual points represent values for each animal. Abbreviations: TMZ, temozolomide; DSF, disulfiram; Cu, copper.

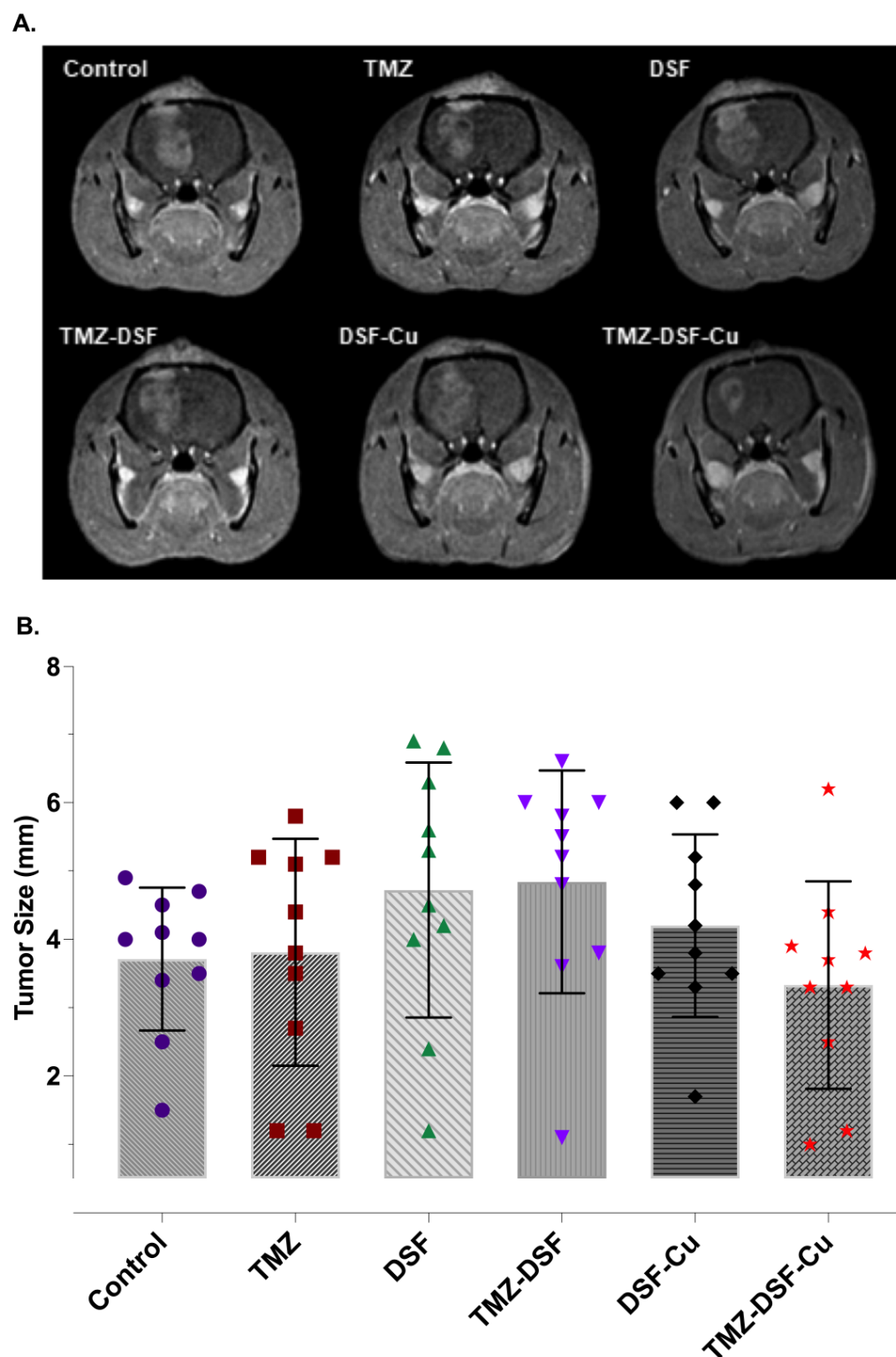


Figure 3. Brain MRIs and tumor size. (A) Representative brain MRIs of the experimental groups (axial postcontrast T1-weighted sequences). (B) Tumor size (mm). Statistical comparisons were performed using one-way ANOVA test. Data ($n = 10$ animals per group) are reported as mean $\pm$ standard deviation (SD); single points represent values for each animal. Abbreviations: TMZ, temozolomide; DSF, disulfiram; Cu, copper.

2.4. Autophagy Markers

In order to evaluate the contribution of autophagy in our experimental set up, we assessed two key autophagy markers, namely microtubule-associated protein 1 light chain

3 (LC3) and sequestosome-1 (SQSTM1)/p62 (p62), whose levels correspond directly to the autophagic flux, within the tumor-positive area (Figure 1D). Our immunofluorescence analysis revealed a statistical significant increase in LC3-positive puncta only in the TMZ-DSF-Cu group, as compared to control ($p = 0.004$, Figure 4), while evaluation of p62 positive signal showed an overall increase in all groups but DSF when compared to the control group ($p = 0.04$ for TMZ-DSF-Cu, $p = 0.03$ for TMZ and DSF-Cu and $p = 0.002$ for TMZ-DSF group; Figure 5).

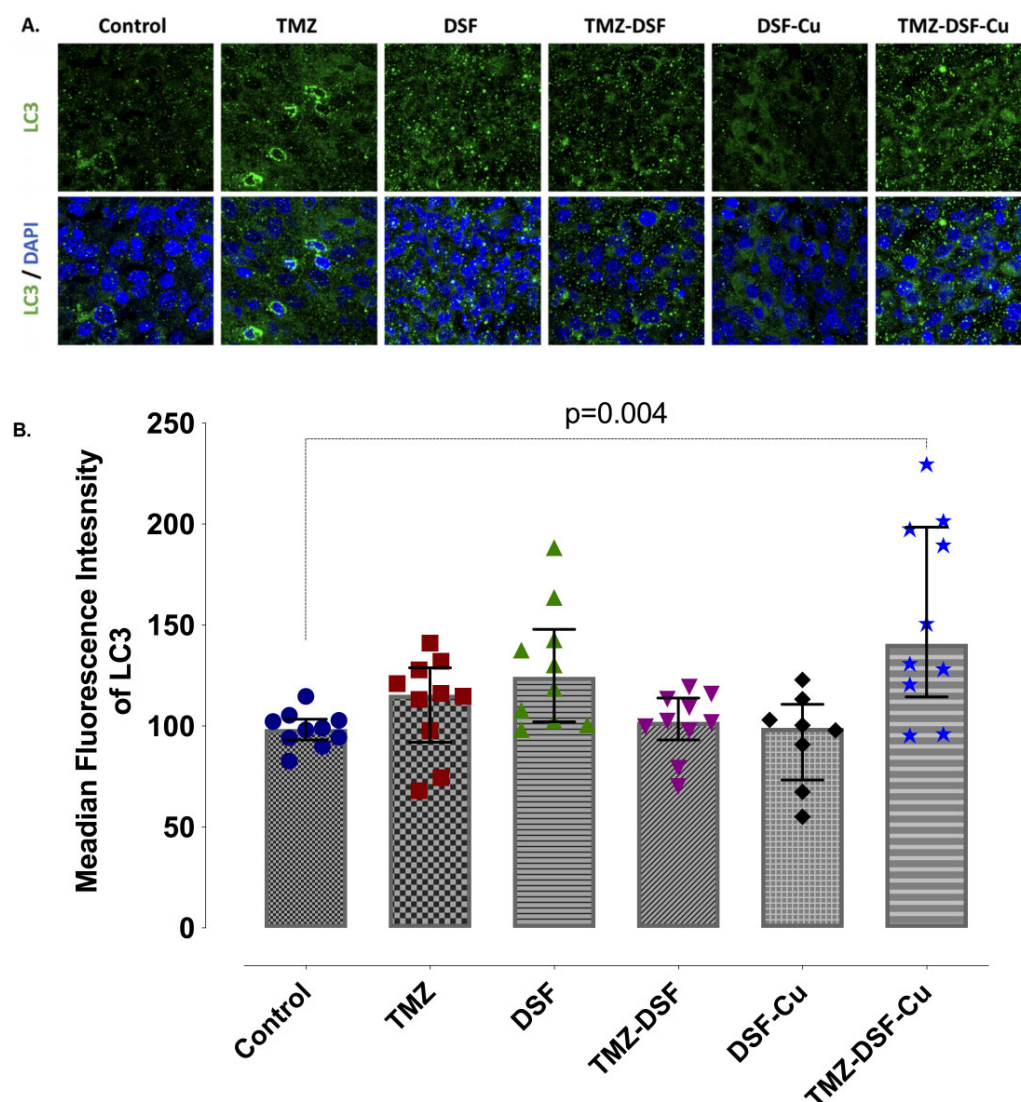


Figure 4. LC3 immunofluorescence. (A) Representative immunofluorescence images of glioma tissue sections stained with the lysosomal marker LC3 from both control and drug-treated animals. Scale bar: 100 μ M. (B) Quantification of LC3 fluorescence intensity in glioma-positive cells ($n = 8$ – 10 animals per group). Error bars indicate median and interquartile range (IQR). Single points show the values for each animal. Statistical comparisons were performed using the Kruskal–Wallis one-way ANOVA test. Abbreviations: LC3, microtubule-associated protein 1 light chain 3; TMZ, temozolomide; DSF, disulfiram; Cu, copper.

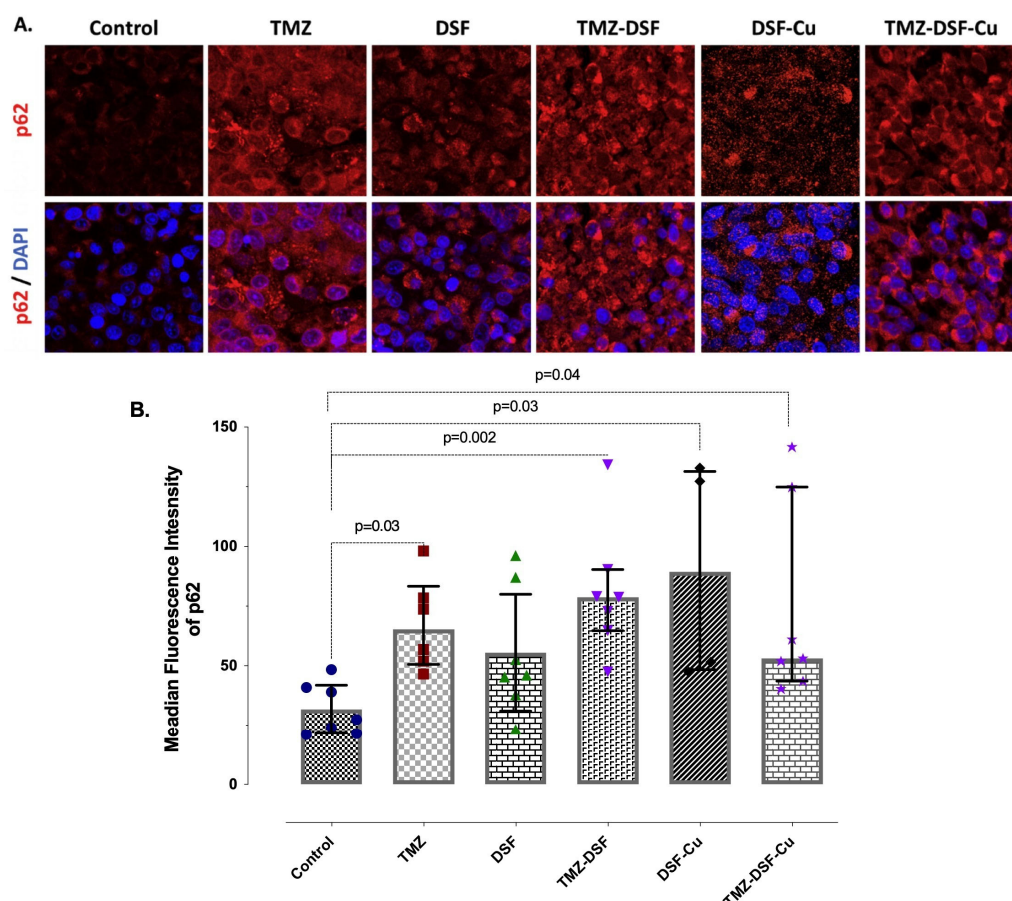


Figure 5. p62 immunofluorescence. **(A)** Representative immunofluorescence images of glioma-containing sections from control and drug-treated animals stained for the lysosomal marker p62 are shown. Scale bar: 100 μ M. **(B)** The fluorescence intensity of p62 in glioma-positive cells was quantified ($n = 4\text{--}7$ animals per group). Error bars indicate median and interquartile range (IQR), with individual points representing each animal's value. Statistical comparisons were performed using the Kruskal–Wallis one-way ANOVA test. Abbreviations: p62, sequestosome-1 (SQSTM1)/p62; TMZ, temozolomide; DSF, disulfiram; Cu, copper.

3. Discussion

In the present study, we tested for the first time the effects of DSF and Cu in combination with TMZ on a syngeneic F98 rat glioma model. We have chosen this model because it exhibits features of the human GBM in terms of tumor microenvironment and growth characteristics, including intratumoral heterogeneity, neovascularization, alterations in the BBB, and an invasive growth pattern. In addition, similarly to the human GBM, F98 gliomas are resistant to alkylating agents, such as TMZ, primarily due to their high expression of methylated-DNA–protein-cysteine methyltransferase (MGMT), an enzyme that deactivates alkylating agents and is the major mechanism of resistance of gliomas [29]. Finally, F98 glioma is poorly immunogenic in syngeneic rats with a predictable and reproducible growth rate, low interval between tumor implantation and tumor confirmation and sufficient length of survival, enabling effective determination of therapeutic efficacy [30].

According to our results, rats treated with TMZ-DSF-Cu demonstrated a limited but statistically significant increase in survival time of about 12%, as compared to the untreated controls, which was also confirmed by the Kaplan–Meier survival curve. In addition, brain MRI measurements revealed a marginal decrease in tumor size of about 10% in comparison to control in the group of TMZ-DSF-Cu, a difference which, however, did not reach statistical significance. In all other experimental groups, which received TMZ,

DSF, TMZ-DSF or DSF-Cu, neither survival nor tumor size were affected by the treatment. Based on these findings, it is obvious that the addition of Cu to the TMZ-DSF combination sensitized the chemoresistant F98 glioma to TMZ, while TMZ alone or in combination with DSF without the presence of Cu failed to show any anti-glioma effects in our model.

As indicated, although the TMZ-DSF-Cu combination significantly extended survival, tumor size reduction was statistically insignificant. This result could stem from the fact that F98 cells invade surrounding brain tissue deeply, possibly preventing the shrinkage of the tumor boundary. In addition, sub-optimal treatment dosages or early study endpoints might not allow for measurable reductions in tumor volume, even if cell death occurs. Finally, in our experimental model, rats were inoculated with 10^3 F98 cells, a number that has been correlated with a wider variation in days until MRI tumor confirmation as compared to large numbers of tumor cells [30].

Our data pertaining to the importance of Cu in DSF's anti-glioma actions have also been documented in a number of previous preclinical studies, suggesting that the combination of DSF with Cu accomplishes a better anti-tumor effect than DSF alone [16,18], while enhancing the therapeutic efficacy of TMZ [21,31]. DSF, which can chelate with Cu ions, binds tumor cellular Cu, reducing its concentration in tumor tissues and preventing its utilization by tumor cells [32], while at the same time acting as a Cu ionophore that facilitates increased Cu uptake into malignant cells [33]. Ultimately, these actions of DSF lead to inhibition of Cu-dependent proliferation of cancer cells [34,35], sensitization of glioma cells to TMZ [21,31], and cancer cell death [36,37].

In order to assess autophagy in our experimental model, we evaluated the expression of LC3 and p62, two widely used autophagy markers, via immunofluorescence staining. We showed that LC3-positive puncta significantly increased only in the TMZ-DSF-Cu group as compared to control, indicative of increased presence of autophagosomes. Interestingly, assessment of p62-positive signal uncovered an overall increase in all groups, as compared to control, even though this increment in the DSF group was not statistically significant. Given that p62 drives ubiquitinated cargo (proteins or organelles) to autophagosomes for lysosomal-dependent degradation, and is itself degraded by the lysosome, the observed increase may suggest an over-activation of the autophagic process in the glioma groups, probably indicating an attempt of the cell to remove pathologic material within the tumor cells.

Perturbation of the autophagy process by DSF or DSF-Cu, with or without co-administration of other chemotherapeutics, has also been demonstrated in previous oncological in vivo and in vitro studies, though not in the context of GBM [26,28,38–41]. Likewise, DSF-Cu inhibited human non-small cell lung cancer, upregulating LC3B-II and decreasing p62 expression [28], while the combination of DSF-Cu with chemotherapeutics suppressed hepatocellular [38] and urinary bladder cancer [39], either increasing LC3B-II and p62 expression [38] or increasing LC3B puncta and decreasing p62 [39]. Concerning the effects of DSF without the addition of Cu, it was shown that it can induce oral squamous cell carcinoma death [40] and increase the cytotoxicity of docetaxel on breast cancer cells [41], leading to increased LC3B and decreased p62 expression. On the other hand, an in vivo model of oesophageal squamous cell carcinoma DSF also revealed anti-tumor activity but in this case, its use increased the expression of both autophagy markers [26].

Under our experimental conditions, and in contrast to the aforementioned studies, DSF, which was administered for a week, did not evoke a statistically significant change in the examined autophagy markers. On the other hand, DSF-Cu, also administered for a week, increased only p62 signal, which was, however, a common finding to all experimental groups with the exception of DSF, possibly related to the tumor per se. Consequently, it is obvious that DSF and DSF-Cu may activate [28,39,40], inhibit [26,38,41] or have no effect on

autophagy, as was the case with our study, depending possibly on factors such as the cell type, the experimental model, the concentration of the drug and the duration of exposure. In addition, autophagy is a dynamic process, and the detection of alterations in autophagic flux at a single time point may not be sufficient to indicate whether it is activated or not [42].

Regarding the effects of TMZ on autophagy, within the ambit of glioma, most previous studies show a consistent induction following the use of this alkylating agent [43–47]. Kanzawa et al. in 2004 [43] were the first to show the induction of autophagy by TMZ in malignant glioma cell lines, even though they did not use both LC3 and p62 autophagy markers. In later studies, it was documented that TMZ increases LC3II and decreases p62 expression in U87 [44,45] and U251 human GBM cells [46,47], confirming its enhancing effect on autophagy. Shi et al. [48], however, could not detect any changes in the expression of LC3II and p62 in surgical specimen-derived SU4 and SU5 glioma stem cells after TMZ treatment.

In our experimental setting, TMZ was administered alone, in combination with DSF or in combination with DSF-Cu for five days. Out of these regimens, only TMZ-DSF-Cu influenced both LC3 puncta and p62 autophagy markers by increasing their levels while TMZ and TMZ-DSF increased only p62, which was, however, a common finding in almost all glioma groups, irrespective of the given pharmaceutical agent. The concurrent increase in both LC3+ and p62+ signals may reflect either an aberrant activation of the pathway upstream of autophagosome formation or a blockade of the autophagosome/lysosome fusion, downstream of autophagosome formation.

Limitations of the Study

From the data presented herein, we cannot draw safe conclusions for either scenario mentioned above, given that we assessed a single regimen dose and a single time point following treatment. Further research is warranted, utilizing cell culture systems and specific inhibitors/inducers of autophagy which may provide further mechanistic insights regarding the underlying pathways.

4. Materials and Methods

4.1. Animals

In total, 60 male Fischer 344 rats, aged 12–14 weeks and weighing 250 to 350 g, were used in this study. The animals were supplied by the Institute of Biosciences and Applications of the National Center for Scientific Research “Demokritos”, Athens, Greece (Facility for Animal Breeding: EL25BIObr019 and Facility for Animal Supply: EL25BIOsup020) and the experiments were carried out at the accredited laboratory of the Experimental, Educational and Research Center of ELPEN (European Reference Number: EL09BIO03) in Athens, Greece.

The animals were brought to the facility two weeks prior to the experiment, to acclimatize to laboratory conditions. They were housed in groups of five in plastic cages with a wood-chip bedding and had free access to tap water and pellet chow. The animal house had a controlled environment of light alternating with dark (lights on at 7 am and off at 7 pm), temperature 21 ± 2 °C, relative humidity 50–70%, and ventilation with 15 air changes/h.

The study protocol was approved by the Project Evaluation Committee of the Experimental Educational Center of ELPEN and by the Greek Regional Veterinary Services (License number: 440/22 January 2016). Our experiments were in accordance with the national legislation on the protection of animals used for scientific purposes (Presidential Decree 56/2013 which adopts the European Directive 2010/63).

4.2. F98 Glioma Model

4.2.1. Cell Culture

Rat F98 glial-like cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with penicillin (100 U/mL), streptomycin (100 mg/mL) (all from PAN Biotech, Aidenbach, Germany) and 10% (*v/v*) fetal bovine serum (FBS, Gibco BRL, Invitrogen, Paisley, UK) in a humidified atmosphere of 5% CO₂ at 37 °C, as previously described [49]. Cells were subcultured when confluent using a trypsin/citrate (0.25%/0.30% *w/v*) solution.

4.2.2. Stereotactic Intracerebral Implantation of F98 Cells

For tumor implantation, the animals were anesthetized by intraperitoneal (*i.p.*) injection of 40 mg/Kg body weight (*b.w.*) of ketamine (Narketan 10, 100 mg/mL, Vetoquinol, Lure, France) and 0.4 mg/Kg *b.w.* of medetomidine (Sedator 1.0 mg/mL, Eurovet Animal Health BV, Bladel, The Netherlands). Additional *i.p.* injections of ketamine were administered as needed to maintain anesthesia throughout the surgical procedures (up to a maximum total dose of 50 mg/Kg *b.w.*). The rats' eyes were lubricated with tobramycin ophthalmic ointment (Tobrex, Alcon Laboratories S.A., Maroussi, Greece) to prevent keratitis while a subcutaneous (*s.c.*) injection of ketoprofen (5 mg/Kg *b.w.*, Ketocan 10%, Candilidis S.A., Maroussi, Greece) to alleviate postoperative pain and *s.c.* injections of 3 mL of sodium chloride 0.9% (saline) to prevent dehydration were given. Animals were then shaved and placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA) with a mouse adapter and lateral ear bars. A midline incision was made in the scalp to expose the skull, after which the bregma was identified and exposed. A 10 µL Hamilton syringe with a 26s gauge needle was attached to the arm of the stereotactic frame and was used for the stereotactic implantation of 10³ F98 cells diluted in 5 µL of DMEM through a burr hole into the right striatum using the following coordinates: +1.0 mm anteroposterior, −3.0 mm mediolateral from the bregma, and −7.2 mm dorsoventral from the dura, according to the rat stereotaxic atlas [50]. The needle was left in place for 5 min and was then slowly withdrawn to reduce backspill and placement of tumor cells into the needle tract and the subarachnoid space. The hole was sealed with bone wax and the operative field was cleaned with betadine before closure of the scalp incision. Anesthesia was reversed with the *s.c.* administration of 0.1 mL atipamezole (Atipam 5 mg/mL, Eurovet Animal Health BV, The Netherlands) at 45 min after injection of ketamine–medetomidine. During the surgical procedures, constant oxygen supply was provided and rats were placed on top of an electric heating pad during recovery from anesthesia; an electric blanket was used until the animal started to move. After recovery, the animals were kept in groups of five in plastic cages with a wood-chip bedding and were given food and water *ad libitum*.

4.3. Treatment of Rats Bearing F98 Brain Tumors

4.3.1. Drugs

DSF, TMZ and Cu gluconate (all from Sigma Aldrich, Taufkirchen, Germany) were dissolved in olive oil, 10% DMSO (Sigma Aldrich, Taufkirchen, Germany) and saline, respectively. DSF and TMZ were administered in a volume of 1 mL/100 g *b.w.*, while Cu in a volume of 0.5 mL/100 g *b.w.*

4.3.2. Treatment Protocol

Sixty animals were divided into six groups of ten. The first group served as control and received only vehicles [1 mL/100 g *b.w.* 10% DMSO intragastrically (*i.g.*) by orogastric gavage at 09:00 a.m. from post-implantation day (PID) 14 to PID 18, 1 mL/100 g *b.w.* olive

oil i.p. at 11:00 a.m. from PID 12 to PID 18, and 0.5 mL/100 g b.w. saline i.g. by oral gavage at 11:10 a.m. from PID 12 to PID 18].

The other five groups were as follows:

Group 2: TMZ (50 mg/Kg b.w.)

Group 3: DSF (50 mg/Kg b.w.)

Group 4: TMZ (50 mg/Kg b.w.) and DSF (50 mg/Kg b.w.)

Group 5: DSF (50 mg/Kg b.w.) and Cu (4 mg/Kg b.w.)

Group 6: TMZ (50 mg/Kg b.w.) and DSF (50 mg/Kg b.w.) and Cu (4 mg/Kg b.w.)

TMZ was given i.g. by orogastric gavage at 09:00 a.m. from PID 14 to PID 18 while DSF and Cu were given i.p. at 11:00 a.m. and i.g. by oral gavage at 11:10 a.m., respectively, from PID 12 to PID 18.

4.4. MRI Tumor Analysis

Tumor formation and tumor size were verified by MRI on the 20th PID using a GE Signa Creator 1.5-T MRI system with a temporomandibular joint coil. Axial T2-weighted, T2 fluid attenuated inversion recovery (FLAIR), and precontrast and postcontrast T1-weighted sequences were obtained (Figure 1A).

For the MRIs, animals were anesthetized by intraperitoneal (i.p.) injection of 17 mg/Kg body weight (b.w.) of ketamine (Narketan 10, 100 mg/mL, Vetoquinol, France) and 0.3 mg/Kg b.w. of medetomidine (Sedator 1.0 mg/mL, Eurovet Animal Health BV, the Netherlands). Then, 0.5 mL/Kg b.w. of a gadolinium-based contrast agent (Magnevist, 10 mL, 469.01 mg/mL, Bayer Hellas S.A., Maroussi, Greece) was intravenously injected into a tail vein followed by fixation of the rat through the nose cone on the restrainer.

The other measures regarding oxygenation, maintenance of body temperature, prevention of keratitis and dehydration, as well as reversal of anesthesia were implemented as described above.

4.5. Monitoring of Clinical Status

All animals were weighed three times per week and their clinical status was monitored daily. Rats with signs of progressively growing tumors, such as sustained weight loss (>20%), ataxia, hemiparesis, reduced activity, lack of grooming, periorbital hemorrhage, tremor, or hunchback posture, were immediately euthanized to minimize their discomfort.

Survival times were determined from the day of tumor implantation to the day of death. For the euthanized rats, 1 day was added to the life span.

Survival analyses of previous studies showed that untreated rats bearing the same F98 glioma with us had a mean survival time of 28 ± 1 days (range 24–37 days) [51,52]. Based on this fact, animals dying before the brain MRI (20th PID) were excluded from further analysis, as were animals that did not show any tumor growth in brain MRI and histological analysis. In case groups were diminished due to excluded animals, additional rats were treated to keep $n = 10$.

4.6. Histopathology

Brains from the animals were excised and fixed in 10% formalin solution for 24–48 h before sectioning. After fixation, each resected cerebrum along with the tumor was sectioned and placed in labeled cassettes. A process with dehydrating and clearing agents was then carried out and subsequently, the tissue samples were embedded in paraffin blocks, cut by a microtome in sections of 3–4 μm thickness, mounted on glass slides and stained with H&E. The microscopic examination was performed under a light microscope (Zeiss, AXIO, Oberkochen, Germany) by an experienced pathologist who recorded the histological features in each experimental rat group.

4.7. Immunofluorescence Analysis of Autophagy Markers

Perfusion, brain fixation, sectioning, and immunohistochemistry were performed according to standardized protocols [53]. Briefly, animals were anesthetized with isoflurane and perfused intracardially with phosphate-buffered saline (PBS; Thermo Fisher Scientific, Waltham, MA, USA, 70011036), followed by an ice-cold fixative of 4% paraformaldehyde (Sigma Aldrich, P6148) under a constant flow rate using a pump. The brains were post-fixed overnight at 4 °C in the same preparation of paraformaldehyde and then transferred to 15% sucrose in PBS overnight, before being incubated in 30% sucrose until freezing. For snap freezing, brains were immersed into frozen 2-methylbutane (Isopentane) at −45 °C for 30–50 s and stored at −80 °C.

Tissues were sectioned at 5 µm and collected on microscope slides (SuperFrost plus, Thermo Scientific). Sections were warmed for 10 min at 60 °C then rehydrated and deparaffinized by immersion in xylene (10 min × 2) followed by immersion in a graded alcohol series (100% ethanol for 3 min × 2, 95% ethanol for 3 min, 70% ethanol for 3 min, 50% ethanol for 3 min), ending with tap water. Heat-induced antigen retrieval was performed in citrate buffer of pH 6.0 (10 mM sodium citrate) for 20 min at 80 °C and left to be cooled on the bench-top for 30 min followed by three washes with PBS. Sections were then incubated in PBS containing 0.3% H₂O₂ for 10 min, followed by three washes in PBS. Unspecific binding was blocked with 5% bovine serum in PBS containing 0.05% Triton X-100, followed by staining with a primary antibody against LC3 (MBL International, Woburn, MA, USA, PM036) or p62 (MBL International, PM045) overnight at 4 °C. The sections were washed in PBS 1× and incubated with secondary antibodies conjugated with fluorophores [CF488A (green) and CF555 (red)] for 1 h at room temperature. Sections were then washed in PBS 1× sections and mounted using a mounting medium. Fluorescence microscopy images were captured with Leica TCS SP5 II on a DM 6000 CFS upright confocal microscope (Leica Microsystems, Wetzlar, Germany) with a 40 × 1.20 objective lens and a z step size of 0.7 µm. Image analysis was performed using the Fiji/ImageJ software (version 10.0) to measure the mean fluorescence intensity for each channel.

4.8. Statistical Analysis

4.8.1. Power Analysis

We used power analysis with the G-Power software (version 3.1) to determine the necessary sample size for our study. The aim was to detect potential statistical differences between six groups of animals regarding our primary outcome (survival days). We used F-tests (ANOVA: Fixed effects, omnibus, one-way) with a significance level (α) of 0.05, power of 90%, and effect size of 0.5. We calculated that 10 animals per group were needed, resulting in a total sample size of 60 animals.

4.8.2. Statistical Methods

Data were analyzed with SPSS (version 28.0; IBM, Armonk, NY, USA) and GraphPad Prism (version 10.6.0; La Jolla, CA, USA).

The normality of the data was assessed using both the Shapiro–Wilk and Kolmogorov–Smirnov tests. For LC3, the Kolmogorov–Smirnov test indicated no statistically significant difference, suggesting the data are parametric, whereas the Shapiro–Wilk test identified a statistically significant difference, indicating non-parametric data characteristics. For p62 mean fluorescent intensity, both tests revealed statistically significant differences (non-parametric test characteristics).

Accordingly, the Kruskal–Wallis test was employed to compare the control and therapy groups. Dunn’s test was used for multiple comparisons using statistical hypothesis testing.

Survival days showed parametric distribution; thus, the one-way ANOVA test was used to compare groups, with Dunnett's test for multiple comparisons. The Kaplan–Meier curves assessed survival probabilities among study groups using the Logrank (Mantel–Cox) test.

Both tests (Shapiro–Wilk and Kolmogorov–Smirnov) demonstrated no statistically significant differences in tumor size. Data analysis was conducted using one-way ANOVA under the assumption of parametric distribution.

5. Conclusions

The development of resistance to TMZ remains the most significant problem in the treatment of GBM and autophagy is one of the main mechanisms implicated in this phenomenon. This study aimed to address the effect of DSF with or without Cu as an add-on therapy to TMZ on the F98 rat glioma model. According to our results, this is the first report of the combination of DSF and Cu with TMZ resulting in increased survival in the F98 rat glioma model. Interestingly, this result could not be achieved in the absence of Cu, and neither in the presence of TMZ alone, suggesting the importance of combining DSF with Cu in order to sensitize glioma to TMZ. In addition, we showed that TMZ-DSF-Cu was the only combination that produced changes in autophagy markers by altering both LC3 and p62 levels, a result that merits further investigation.

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Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors upon request.

Conflicts of Interest: Author Argyro Zacharioudaki was employed by the company BIOEMTECH. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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