Inhibition of endoplasmic reticulum stress-induced autophagy sensitizes melanoma cells to temozolomide treatment

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Abstract. The incidence of malignant melanoma is increasing. The discovery of agents specifically targeting the mutated cascades has provided a good response for patients with oncogenic B-Raf proto-oncogene, serine/threonine kinase (BRAF). However, numerous studies continue to focus on novel methods of treatment to overcome acquired resistance to novel drugs. Recently, it has been revealed that inhibition of endoplasmic reticulum (ER) stress chaperon 78 kDa glucoseregulated protein 78 (GRP78) leads to downregulation of autophagy and increased sensitivity to temozolomide (TMZ) treatment. Melanoma cells have a different sensitivity to TMZ treatment, which corresponds to the basal autophagy level. In the present study, we demonstrated that downregulation of GRP78 mitigated chemoresistance to TMZ in three melanoma cell lines. We found that downregulation of GRP78 led to inhibition of autophagy, cell cycle arrest in the G0/G1 phase, and activation of caspase-7-induced apoptosis, and this was affected by the initial autophagy level. Moreover, inhibition of GRP78 mitigated the combined TMZ and chloroquine effect. Our data revealed that autophagy inhibition through downregulation of ER stress response could overcome resistance to TMZ treatment in melanoma cells with a high basal level of autophagy treatment, which makes this combination a potential potent antitumor treatment for metastatic melanoma.

Introduction

Melanoma incidence has markedly risen, and although targeted therapy with B-Raf proto-oncogene, serine/threonine

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kinase (BRAF) or mitogen-activated protein kinase kinase (MEK) inhibitors is effective for patients with BRAF-mutated melanoma (1), alkylating agents such as temozolomide (TMZ) remain a common therapy for BRAF wild-type patients (2). However, melanomas quickly acquire drug resistance, and recurrence of metastases is observed in almost all cases. To overcome such resistance, numerous studies have focused on identifying the mechanisms involved (3-5).

Several studies have demonstrated that autophagy, a lysosome-dependent degradation process in which cellular organelles are absorbed and degrade for recycling within the cell, may play a significant role in limiting the efficacy of chemotherapy. It is essential that cells undergo autophagy to maintain their vitality and integrity under starvation conditions, infections, and some diseases, such as neurodegenerative diseases, cancers and aging (6,7). Cytoprotective autophagy is often upregulated under anticancer therapy and concurrently with cell death pathways, leading to adaption to therapeutic stress and recurrence. Based on studies that autophagy inhibition can increase the antitumor efficacy of therapies that induce autophagy (8-10), numerous clinical trials have been launched (8,11). It has been demonstrated that high levels of autophagy before treatment predict invasiveness, poor response to cytotoxic chemotherapy, and shortened survival in metastatic melanoma (10,12). Elevated levels of autophagy in primary tumors have also been correlated with fast proliferation and progression (13). Several anticancer drugs, including TMZ and vemurafenib, are known to induce cytoprotective autophagy in cancer cells (9,14).

The endoplasmic reticulum (ER) is a critical cellular organelle for quality control of secretory proteins. Stress ER is the phenomenon of a functional overload of the protein secretion apparatus by misfolded protein chains. For normal protein maturation, a finely tuned correspondence between the biosynthetic load and the functional capacity of the ER is necessary. Disturbances to this balance results in overloading the ER, leading to misfolding and, ultimately, accumulation in the ER of inactive or chemically aggressive proteins (15). Despite the fact that melanoma cells are adapted to a high level of stress, suppression of adaptation mechanisms is a new direction for developing a therapeutic strategy (16).

ER stress leads to activation of two protein degradation pathways, the ubiquitin-proteasome via ER-assisted degradation and

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lysosome-mediated protein degradation via autophagy (17). The unfolded protein response (UPR) is a complex of closely interconnected signal branches, united by a common trigger mechanism. This mechanism is represented by a triad of transmembrane proteins (PERK, IRE1 and ATF6), each of which, under normal physiological conditions, is inactivated by chaperone 78 kDa glucose-regulated protein 78 (GRP78), also referred to as BiP (18).

It has been demonstrated that induction of GRP78, a major target of UPR, leads to general translation arrest, upregulation of chaperones and folding enzymes, and degradation of misfolded proteins (19). Thus, GRP78 represents a prosurvival arm of the UPR (20). GRP78 maintains ER integrity and assists in autophagosome formation independent of Beclin 1-dependent autophagy. The knockdown of GRP78 causes suppression of the autophagy caused by ER stress (21) and silencing GRP78-dependent autophagy enhances the cytotoxic effects of TMZ on glioma cells (22). In addition, GRP78 induces activation of AMPK and TSC2, which leads to inhibition of mTOR and simultaneous knockdown of GRP78, and Beclin 1 synergistically restores antiestrogen sensitivity in resistant cells (23). Recently, Cerezo et al reported that the HA15 compound (thiazole benzenesulfonamides) displayed antimelanoma activity in vitro and in vivo, both on BRAF resistant and sensitive melanomas, by targeting GRP78, leading to melanoma cell death by concomitant induction of autophagic and apoptotic mechanisms (24). UPR and autophagy occur at the same time and participate in pathological processes, including chemoresistance of tumors (25).

In the present study, we aimed to investigate the role of GRP78-dependent autophagy in inducing sensitivity of melanoma cells to TMZ-treatment regimes.

Materials and methods

Cell lines. Metastatic melanoma cell lines Mel MTP, Mel Z and Mel IL were derived from patients under treatment at N.N. Blokhin National Medical Scientific Center for Oncology (26,27). Cell lines were cultured in RPMI-1640 (Gibco, Paisley, UK) supplemented with 10% fetal bovine serum (FBS) (HyClone; GE Healthcare Life Sciences, Logan, UT, USA), 2 mM L-glutamine (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), 10 U/ml penicillin (Sigma-Aldrich; Merck KGaA), and 0.1 mg/ml streptomycin (Sigma-Aldrich; Merck KGaA) at 37°C under a 5% CO₂ humidified atmosphere.

Transfection. Small interfering (si) RNAs targeting GRP78 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and a control siRNA (siCTRL, sequence, ATAGAGCGATCACATACAGCC) was constructed by Syntol (Moscow, Russia). Melanoma cells ($2x10^5$ cells/well) were seeded onto 6-cm Petri dishes (Nunc, Roskilde, Denmark) and transfected with 10 nM GRP78 or siCTRL using the Lipofectamine RNAiMAX reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. Twenty-four hours after transfection, the cells were treated with 100 μ M TMZ (Sigma-Aldrich; Merck KGaA) and 20 μ M chloroquine (CQ) (Sigma-Aldrich; Merck KGaA) for 24 or 48 h, and cell viability and the percentages of autophagy and apoptosis were determined.

Cell proliferation assay. Melanoma cell lines Mel MTP, Mel Z and Mel IL were plated (8x10³ cells/well) into 96-well plates (Nunc). After 24 h, TMZ (100 μ M) alone or combined with CQ (20 μ M) was added, and the cells were incubated for 48 h. Control cells were treated with an equal amount of dimethyl sulfoxide (DMSO). Cytotoxicity was assessed by incubating cells with 20 μ l of 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) reagent (Sigma-Aldrich; Merck KGaA) for 4 h, and measuring the absorbance at 540 nm with a microplate analyzer (Multiscan FC; Invitrogen; Thermo Fisher Scientific, Inc.) in triplicate.

Colony formation assay. Melanoma cells $(2x10^5)$ were seeded in 6-well plates. Medium containing 100 μ M TMZ, and 20 μ M CQ or an equal amount of DMSO as a vehicle control was added to the appropriate wells and cells were incubated for 24 h. After incubation, cells were reseeded on new 6-well plates $(2x10^3 \text{ cells/well})$ in triplicate and cultivated for 12 days, and the medium was changed every 3-4 days. At the end of the experiment, colonies were fixed in 1% formalin, stained with 0.5% crystal violet, and counted using ImageJ software [National Institutes of Health (NIH) Bethesda, MD, USA]. Three independent experiments were carried out.

Immunoblotting. Cells were lysed with lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 1 mM Na₃VO₄, 1 μ g/ml leupeptin, 1 mM phenylmethanesulfonyl fluoride (PMSF), 10 µl/ml inhibition cocktail, and 100 µM DTT for 40 min at 4°C. The cells were then centrifuged at 13,500 x g for 15 min at 4°C. The total protein content was analyzed using a Quant-IT protein assay kit according to manufacturer's protocol (Invitrogen; Thermo Fisher Scientific, Inc.) on a Quibit 2.0 fluorometer (Invitrogen; Thermo Fisher Scientific, Inc.). An equal amount of protein (40-60 μ g) from each group was separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to nitrocellulose membranes. Membranes were incubated with 5% dry milk (Applichem, Darmstadt, Germany) for 90 min, and then incubated with Beclin 1 (1:1,000; cat. no. 3738; Cell Signaling Technology, Inc., Danvers, MA, USA), LC3B (1:500; cat. no. NB100-2220B; Novus Biologicals, Cambridge, UK), p62/SQSTM1 (1:1,000; cat. no. 5114; Cell Signaling Technology, Inc.), caspase-7 (1:100; cat. no. sc-56063; Santa Cruz Biotechnology, Inc.), cleaved PARP (1:1000, cat. no. 44698G; Invitrogen; Thermo Fisher Scientific, Inc.), GRP78 (1:1,000; cat. no. 3183; Cell Signaling Technology, Inc.) and β -actin (1:4,000; cat. no. A5441; Sigma-Aldrich; Merck KGaA) antibodies at 4°C overnight. The membranes were washed in Trisbuffered saline, containing Tween-20 and incubated with appropriate horseradish peroxidase-conjugated secondary anti-mouse antibodies (1:10,000; cat. no. NA991VS) and anti-rabbit (1:10,000; cat. no. NA934VS; both from GE Healthcare, Chicago, IL, USA) for 90 min at room temperature. Immunoreactive proteins were detected using enhanced chemiluminescence reagent Clarity ECL (Bio-Rad Laboratories GmbH, Munich, Germany). The density of bands was determined on a ChemiDoc Touch Imaging



Figure 1. Basal autophagy expression in Mel IL, Mel Z and Mel MTPcell lines. (A) Basal autophagy-related gene expression mRNA level of Beclin 1. (B) Beclin 1 protein expression in melanoma cell lines. (C) Densitometric analysis was performed using ImageJ for each cell line, and protein expression levels of Beclin 1 were normalized to β -actin loading control. The graphs represent the data from one of the three independent experiments.

System (Bio-Rad Laboratories GmbH) and quantified using ImageJ software (NIH).

Cell cycle analysis. After 24 h of treatment with 100 μ M TMZ and 20 μ M CQ, the cells were washed with PBS; the cell pellets were resuspended in 500 μ l of 50 μ g/ml solution of propidium iodide (PI) in buffer (BD Biosciences, Franklin Lakes, NJ, USA) and incubated in the dark at room temperature for 15 min. The PI fluorescence was assessed on a NovoCyte 2000R flow cytometer (ACEA Biosciences, San Diego, CA, USA) and the cell cycle distribution was analyzed using ModFit 3.2 software (Verity Software House, Topsham, ME, USA).

Apoptosis. Apoptosis was determined by caspase-7 activity within the cells 24 h after drug treatment. Cells were treated with 100 μ M TMZ alone or in combination with 20 μ M CQ. Control cells were treated with DMSO. After treatment, cells were trypsinized, centrifuged, permeabilized in 200 μ l 0.1% Triton X-100-citrate buffer, and incubated for 30 min at 4°C with mouse anti-caspase-7 antibodies (1:100; Santa Cruz Biotechnology, Inc.). After incubation, the cells were washed and incubated with anti-mouse antibody AlexaFluor[®] 488 (1:2,000; cat. no. A11001; Life Technologies; Thermo Fisher Scientific, Inc.), washed and fixed in 1% formalin, and followed by analysis on a NovoCyte 2000R flow cytometer (ACEA Biosciences) using NovoExpress v.1.2.4 software. Results are presented as the percent increases relative to the control.

Quantitation of autophagy. Melanoma cells were seeded (3x10⁵ cells/well) in 24-well plates (BD Falcon). Twenty-four hours later, the cells were transfected with 30 viral particles/cell using Premo[®] Autophagy Tandem Sensor RFP-GFP-LC3B and Premo Autophagy Sensor RFP-p62, as described in the manufacturer's manual (Life Technologies; Thermo Fisher Scientific, Inc.) and incubated overnight. The next day, the cells were treated with TMZ and CQ or equal amounts of DMSO as a vehicle control and further incubated for 24 h. Imaging was performed using IN Cell Analyzer 6000 and In Cell Investigator software (GE Healthcare Life Sciences).

Quantitative real-time PCR. Total RNA was extracted from cells using TRIzol reagent (Sigma-Aldrich; Merck KGaA), as previously described (28). For cDNA synthesis, RNA (250 ng) was reverse-transcribed in a final volume of $20 \ \mu$ l

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Table	1.	Primer	sec	uencing

Gene	Sense	bp		
GAPDH	F: 5'-GGGGAGCCAAAAGGGTCATCATCT-3' R: 5'-GACGCCTGCTTCACCACCTTCTTG-3'			
β-actin	F: 5'-GTGGGGCGCCCCAGGCACCA-3' R: 5'-CTCCTTAATGTCACGCACGATTTC-3'	201		
Beclin 1	F: 5'-GAGTTTCAAGATCCTGGACCGTGTCA-3' R: 5'-CTGTTGGCACTTTCTGTGGACATCA-3'	282		
F, forward	; R, reverse.			

using iScript[™] Select cDNA Synthesis kit according to manufacturer's instructions (Bio-Rad Laboratories, Inc., Hercules, CA, USA). No-reverse transcriptase controls were performed by omitting the addition of the reverse transcriptase enzyme, and no-template controls were performed by the addition of nuclease-free water. A relative quantitation of Beclin 1 mRNA expression normalized to two endogenous reference genes (β-actin and GAPDH) was performed using a Bio-Rad CFX96 Real-Time System (Bio-Rad, Laboratories, Inc.) and iTaq® Universal SYBR[®]-Green SuperMix (Bio-Ra, Laboratories, Inc.). Primers are listed in Table I. The PCR reaction mixture (final volume, 10 μ l) contained 5 μ l of 2X SuperMix, 5 pmol of GADPH, β -actin and Beclin 1 and 2 μ l (50 ng) of cDNA. The thermocycling conditions were: 5 min at 95°C, followed by 39 cycles of 5 sec at 95°C, 30 sec at 60°C and 30 sec at 72°C. At the end of the 39 PCR cycles, melting curve analysis was performed by continuously recording the fluorescence during progressive heating up to 95°C with a ramp rate of 0.5°C/sec. All samples were analyzed in duplicate wells of a 96-well plate. The results of real-time RT-PCR were represented by the parameter $\Delta\Delta Cq$ (29).

Statistical analysis. Each treatment condition was set up in triplicate, and each experiment was repeated three times independently. Data are expressed as the mean ± standard deviation (SD), and the concentration-response curves were produced using the GraphPad Prism v.5.0 software (GraphPad, Software, Inc., La Jolla, CA, USA). Statistical analysis was carried out using the Student's t-test. A P-value of <0.05 was considered to indicate a statistically significant difference.



Figure 2. Combined 100 μ M TMZ and 20 μ M CQ results in increased cytotoxicity in melanoma cell lines. Silencing of GRP78 enhanced cytotoxicity of TMZ only in Mel MTP cells and mitigated the cytotoxic effect of combined TMZ and CQ therapy in Mel IL, but not in the Mel MTP and Mel Z cell lines. Cell viability with TMZ and CQ was determined in (A) Mel IL, (B) Mel Z and (C) Mel MTP cell lines by MTT assay. Data are expressed as the mean \pm SD of at least three independent experiments performed in triplicate. Control cells were treated with equal amounts of DMSO as a vehicle control (*P<0.05). TMZ, temozolamide; CQ, chloroquine.



Figure 3. Chloroquine (CQ) enhances the cytotoxic effects of temozolomide (TMZ). (A) Melanoma cells were seeded in 6-well plates and were treated with 100 μ M TMZ alone or in combination with 20 μ M CQ or with DMSO for 24 h, and the number of long-term surviving cells that were able to form colonies was determined 12 days later (CFA). Melanoma cells were transiently transfected with siRNA directed to GRP78 or scrambled siRNA. Twenty-four hours after transfection, the cells were seeded in parallel for either (B) a western blot analysis or (C) a CFA. *P<0.05. CQ, chloroquine; TMZ, temozolamide; CFA, colony forming assay.

Results

Enhanced cytotoxic effect of TMZ under inhibition of ER stress-induced autophagy depends on the initial autophagy level in melanoma cells. All experiments were carried out on three melanoma cell lines with different basal autophagy levels [Mel Z (low basal autophagy), Mel IL (medium basal autophagy), and Mel MTP (high basal autophagy], which were investigated by Beclin 1 mRNA expression (qRT-PCR) (Fig. 1A) and Beclin 1 protein expression (western blotting) (Fig. 1B and C). Previously, we demonstrated that the autophagy inhibitor chloroquine (CQ) enhances the cytotoxic effect of TMZ on both BRAF-mutated and wild-type melanoma cell lines (30).

First, we determined the cytotoxic effect of TMZ on the melanoma cell lines with knockdown of GRP78 by MTT assay. We initially transfected Mel IL, Mel Z, and Mel MTP cells with small interfering (si)RNA to a scrambled sequence (siCTRL) and GRP78. According to the data received, treatment of siCTRL-transfected melanoma cells with 100 μ M TMZ had little to no effect compared to untransfected cells. Knockdown of GRP78 enhanced the cytotoxic effects of TMZ



Figure 4. Downregulation of GRP78 is assessed in cell cycle distribution. Melanoma cells were transfected with siGRP78 or siCTRL and further treated with 100 μ M TMZ alone or combined with 20 μ M CQ for 24 h. Silencing of GRP78 increased the G0/G1 subpopulation under TMZ treatment in Mel IL and Mel Z cell lines. Preincubation with CQ led to an increased G0/G1 phase only in Mel MTP cells. The flow cytometry data and subsequent graphs represent the data obtained from one of three independent experiments (CV <5%). TMZ, temozolamide; CQ, chloroquine.

alone on Mel MTP cells with a high basal level of autophagy but there was no inhibition effect on Mel IL compared to untransfected cells and, notably, silencing of GRP78 mitigated TMZ toxicity on the Mel Z cell line (Fig. 2A and B). Moreover, downregulation of GRP78-dependent autophagy mitigated the cytotoxic effect of CQ on Mel Z and Mel IL cell lines, but induced an antiproliferative effect on Mel MTP (Fig. 2C).

To better understand the mechanism underlying the synergistic cytotoxic effects of TMZ and CQ in our cells, we transfected Mel Z, Mel IL, and Mel MTP cells with siCTRL or siRNA to GRP78. After transfection, cells were cultivated in the presence or absence of TMZ or TMZ in combination with CQ for 24 h in a series of colony-forming assays (CFAs). To confirm the knockdown of the GRP78 protein, we analyzed the protein level of GRP78 by western blot analysis using β -actin as a loading control (Fig. 3B).

TMZ alone did not affect the viability of Mel IL cells; there was a slight increase in the number of colonies relative

to the control. Compared to the DMSO-treated control, the combination of TMZ and CQ reduced the number of colonies by 25% (P=0.05). The number of colonies in the Mel Z cell line decreased by 30% with TMZ, the combination of TMZ with CQ reduced the number of colonies by ~60% compared to the control (P<0.05) (Fig. 3A). Notably, under TMZ treatment alone or combined with CQ, Mel MTP cells did not form viable colonies (data not shown).

In Mel IL and Mel Z cells, knockdown of GRP78 with siGRP78 enhanced the cytotoxic effects of TMZ by further reducing the percentage of colonies formed by an additional ~20%. Treatment of siGRP78-transfected melanoma cells with 100 μ M TMZ and 20 μ M CQ reduced the percentage of colonies formed by ~70% in Mel Z cells but did not affect Mel IL. Mel MTP does not form colonies under siGRP78 transfection (Fig. 3C).

It has been reported that GRP78 maintained ER integrity and was involved in autophagosome formation independently



Figure 5. The enhancement of apoptosis by combined TMZ and CQ treatment is dependent on autophagy in melanoma cells. Assessment of autophagy inhibition in activation of apoptosis was evaluated 24 h after transfection with GRP78 or scrambled siRNA. siRNA-transfected melanoma cells were treated with 100 μ M TMZ and 20 μ M CQ or DMSO as a vehicle control for 4 h and caspase activation was performed by flow cytometry (P<0.05). TMZ, temozolamide; CQ, chloroquine.

of Beclin 1-mediated autophagy (22). Thus, inhibition of GRP78 enhanced the cytotoxic effects of TMZ, however the combined effect of TMZ and CQ were detected only in the Mel Z cell line.

Previously, we demonstrated that TMZ (100 μ M) treatment increased the cell population in the G0/G1 phase in melanoma cell lines and TMZ and CQ combination further increased the G0/G1 fraction in BRAF-mutated Mel IL and Mel Z cell lines, but not in BRAF wild-type Mel MTP (29). Thus, we evaluated the cell cycle distribution in the siGRP78 transfected cells.

Transfection with siGRP78 resulted in increased accumulation of cells in the G0/G1 phase compared to the siCTRL cells under TMZ treatment (49 vs. 73% for Mel IL, and 67 vs. 87% for Mel Z). However, CQ did not affect the cycle cell distribution when GRP78 was downregulated as it did in the siCTRL cells (Fig. 4). Notably, the Mel MTP line, characterized by a high level of autophagy, was not sensitive to siGRP78, but the combination with CQ increased in cells in the G0/G1 phase, which was not observed in the siCTRL cells.

Blockade of GRP78-dependent ER stress mitigates autophagy and enhances cell death through caspase-7-mediated apoptosis. Next, we investigated the activation of caspase-7 by flow cytometry in melanoma cell lines. We found that, in the Mel Z cell line, apoptosis was not activated via the caspase pathway. However, activation of caspase-7 occurred under treatment in cells transfected with siGRP78. In cell lines with a medium (Mel IL) and high (Mel MTP) basal level of autophagy, we observed activation of caspase-7 under TMZ treatment, and its combinations with CQ further enhanced activation of apoptosis markers. Silencing of GRP78 led to a more evident activation of caspase-7 in Mel IL and Mel MTP cells compared to the control. However, downregulation of GRP78 enhanced apoptosis only in the Mel MTP cell line (up to 96%). Thus, cells with initially high autophagy were more sensitive to its inhibition (Fig. 5).

To investigate the mechanistic details of GRP78-dependent autophagy, we investigated markers of autophagy and apoptosis. TMZ treatment resulted in a significant increase in the LC3II/LC3I ratio in the siCTRL cells. In contrast, there was no TMZ-associated increase in the LC3B-I/LC3B-II ratio in cells transfected with siGRP78 in either cell line. We found that GRP78 knockdown diminished the ability of TMZ and the TMZ plus CQ combination to convert LC3B-I to LC3B-II, especially in the Mel MTP cells. Moreover, in the GRP78transfected cells, another autophagy marker, p62, which is involved in trafficking cargo to lysosomes, was not degraded in autolysosomes compared to the siCTRL cells. Notably, in the Mel IL cell line, Beclin 1 expression was mitigated in GRP78-transfected cells but was upregulated in Mel MTP cells. Thus, GRP78 plays a significant role in TMZ-dependent autophagy (Fig. 6).

Next, we analyzed the expression of apoptosis markers caspase-7 and cleaved PARP in melanoma cell lines by western blotting. In the single treatment, TMZ increased the levels of apoptosis markers caspase-7 and cleaved PARP in both cell lines. However, there was a slight increase in caspase-7 and cleaved PARP activation under combined TMZ and CQ treatment compared to TMZ alone. Silencing of GRP78 also led to induction of caspase-7 and caused enhanced cleavage of PARP under the TMZ and CQ combined treatment in Mel MTP cells compared to control cells, but there were no differences between Mel IL and Mel Z cells (Fig. 6). Thus, we suggest



Figure 6. Blockade of GRP78-dependent autophagy enhances caspase-7-mediated apoptosis. (A) Western blot analysis of autophagy (Beclin 1, p62 and LC3) and apoptosis (caspase-7 and clPARP) markers after drug treatment in siCTRL- and siGRP78-transfected Mel IL, Mel Z and Mel MTP cells. The graph represents the data from three independent experiments. Densitometric analysis was performed using ImageJ for each cell line, and protein expression levels of (B) Beclin 1, LC3B and p62 and (C) caspase-7 and clPARP were normalized to β -actin loading control, *P<0.05.

that the combined effect of TMZ and CQ was mediated by increased apoptosis.

TMZ-induced autophagy is associated with ER stress. To ensure that TMZ induced autophagy and did not block clearance of autolysosomes, cells were transfected with tandem Premo® RFP-GFP-LC3 and Premo RFP-p62 sensors (Thermo Fisher Scientific, Inc.), as described in the Materials and methods section. Cells were treated with 100 μ M TMZ, and 20 μ M CQ for 24 h and the control cells were treated with equal amounts of DMSO. In the absence of any treatment, the distribution of green fluorescence was diffuse. Treatment with TMZ increased the number of autophagosomes and increased autophagic flux, driven by functional fusion with the lysosome due to the recruitment of GFP-LC3 in autophagy. TMZ combined with CQ enhanced the number of autophagic vacuoles over individual treatment and produced yellow puncta, reflecting lysosomal impairment, as well as distal autophagy blockade producing persistence of green and red fluorescence (31). Under TMZ treatment, p62 puncta were decreased, and blockade of autophagy led to an accumulation of p62-positive vesicles. When Mel Z cells were transfected with GRP78-siRNA for 24 h prior to drug treatment, we detected impaired autophagic flux, and a smaller amount of p62-positive cells and combined therapy had no effect (Fig. 7). The same effect was observed in GRP78-transfected Mel IL and Mel MTP cell lines after 24 h of treatment with TMZ or TMZ plus CQ (data not shown).

Discussion

Targeting mutant BRAF has been the most promising breakthrough for melanoma therapy in recent decades (32). However, BRAFV600-targeted therapy leads to enrichment in resistant cells, resulting in the recurrence of therapy-resistant disease (33). Due to this phenomenon, research has focused on understanding the mechanisms behind the acquired resistance and ways to overcome it. Moreover, there have been no advantages in the treatment of patients without BRAF mutation, except alkylating agents and immunotherapy. There are some drug-resistance mechanisms described for BRAF mutant and non-mutant melanomas, including activation of alternative pathways, such as the MAPK (28) and PI3K signaling (34) pathways, as well as receptor tyrosine kinase activation (35) or activation of cytoprotective autophagy (36).

Autophagy is known to play a complex role in cancer and can both suppress and promote tumorigenesis (37). In general, it is thought that autophagy is used by tumor cells to promote



Figure 7. TMZ and CQ combined treatment is dependent on autophagy in Mel Z melanoma cells. Assessment of autophagy was determined by counting the number of cells expressing the punctate pattern of (A) RFP-GFP-LC3-positive cells [green (cytoplasm stage) and yellow (lysosome stage)] and (B) RFP-p62-positive cells (red) after drug treatments with 100 μ M TMZ and 20 μ M CQ or DMSO as a vehicle control for 24 h. Scale bar, 100 μ M. TMZ, temozolamide; CQ, chloroquine.

survival, with evidence supporting the role of dysregulated autophagy in melanoma (38).

Recently, several research groups demonstrated that targeting autophagy sensitized both BRAF-sensitive and -resistant melanoma cells to PLX4032 and MEK inhibitors. Thus, autophagy blockers may represent a novel treatment regime to increase both cell death and danger-signaling in vemurafenib-resistant metastatic melanoma (13,39).

Previously, we demonstrated that autophagy inhibition could enhance melanoma cell death combined with TMZ therapy on either BRAF-mutated or wild-type cell lines through induction of apoptosis (30). In the present study, we evaluated whether inhibition of GRP78, a Beclin 1-independent activator of autophagy, enhanced cytotoxicity to TMZ treatment and affected combined TMZ and CQ treatment. GRP78 is an ER molecular chaperone that plays an important role in protein folding and assembly, targeting misfolded proteins for degradation, and controlling the activation of transmembrane ER stress sensors (40).

Several studies have revealed that high levels of GRP78 expression were correlated with proliferation in glioma and melanoma cells and that downregulation of GRP78 led to a significant decrease in cell growth (19,24,41).

Our *in vitro* research demonstrated that all tested cell lines with a different basal level of autophagy expressed GRP78. In the present study, we found that TMZ induced an increase in GRP78 protein levels. However, TMZ treatment of cells transfected with siGRP78 led to enhanced cytotoxicity only in the Mel MTP cell line with high basal autophagy; the Mel IL cell line was not sensitive to GRP78 silencing. Moreover, compared to the control, the percent of survived cells was higher in the Mel Z cell line, which has a low autophagy level.

In a series of colony-forming assays, we demonstrated that TMZ resulted in a reduction in the percent of colonies formed under TMZ treatment and that the combination of TMZ with CQ enhanced the cytotoxic effects of TMZ. We discovered that knockdown of GRP78 enhanced the cytotoxic effects of TMZ. Treatment with CQ further enhanced the cytotoxic effects of TMZ on Mel Z and Mel MTP cells but not Mel IL. We demonstrated that GRP78-dependent autophagy limited the cytotoxic effects of TMZ.

An investigation of the mechanistic details of GRP78dependent autophagy revealed that TMZ treatment resulted in a significant increase in the LC3II/LC3I ratio in control cells and that autophagy was mitigated in three melanoma cell lines (with different basal levels of autophagy) transfected with siGRP78. GRP78-knockdown diminished the ability of TMZ and the TMZ plus CQ combination to convert LC3B-I to LC3B-II, particularly in Mel MTP cells, and accumulation of p62 was significantly compromised. Thus, GRP78 plays a significant role in TMZ-dependent autophagy.

Furthermore, we also demonstrated that GFP-RFP-LC3 redistribution to autophagosomes and LC3-II accumulation were decreased after knockdown of GRP78 under TMZ treatment and the addition of CQ impaired autolysosome degradation. These data established the role of ER stress as an important driver of autophagic flux induced by TMZ.

In the single treatment, TMZ increased the levels of apoptosis markers caspase-7 and cleaved PARP in both of the tested cell lines. Suppression of ER stress by silencing of GRP78 reduced TMZ-induced autophagy and cell viability. Activation of caspase-7 demonstrated that TMZ and CQ induced apoptosis in cells with medium (Mel IL) and high (Mel MTP) basal autophagy levels, and resulted in cleavage of PARP. Several studies have revealed that ER stress-mediated autophagy promoted survival in hepatocellular and colorectal carcinoma cells (42), pancreatic cancer and melanoma cells (43), and glioma (19). Therefore, those studies and ours suggest that inhibition of ER stress may be a strategy to enhance the cytotoxicity of TMZ.

In conclusion, GRP78-dependent autophagy limits the cytotoxic effects of TMZ. Our data revealed that CQ improved the cytotoxic effect of TMZ and that autophagy inhibition through downregulation of ER stress response could overcome resistance to TMZ treatment in melanoma cells with a high basal level of autophagy treatment, making this combination applicable as a potent antitumor treatment of metastatic melanoma.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

OR and DK conceived and designed the study. OR, DK, AP and IA performed the experiments. OR and ES wrote the study. OR, DK and AZ reviewed and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All experimental protocols were approved by the N.N. Blokhin National Medical Research Center for oncology ethics committee (Moscow, Russia).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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