

Apoptosis induction in human glioblastoma multiforme T98G cells upon temozolomide and quercetin treatment

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Abstract Glioblastoma multiforme is the most aggressive primary brain tumour. At the cellular and molecular levels, several mechanisms responsible for apoptosis or autophagy induction are blocked. Identification of molecular targets stimulating cells to initiate programmed cell death should be performed for therapeutic purposes. A promising solution is the combination of temozolomide and quercetin. The aim of our study was to evaluate the effect of both drugs, applied alone and in combinations, on apoptosis and autophagy induction in human glioblastoma multiforme T98G cells. Our results clearly indicate that quercetin and temozolomide induce apoptosis very significantly, having no effect on autophagy induction. At the molecular level, it was correlated with caspase 3 and 9 activation, cytochrome c release from the mitochondrion and a decrease in the mitochondrial membrane potential. Both drugs are also potent Hsp27 and Hsp72 inhibitors. This suggests that the apoptotic signal goes through an internal pathway. Increased expression of caspase 12 and the presence of several granules in the

cytoplasm after temozolomide treatment with or without quercetin preceding appearance of apoptosis may suggest that apoptosis is initiated by ER stress. Additionally, it was accompanied by changes in the nuclear morphology from circular to ‘croissant like’.

Keywords Temozolomide · Quercetin · Apoptosis · Autophagy · ER stress · Glioblastoma multiforme

Introduction

Glioblastoma multiforme (grade IV) is the most common and the most aggressive primary brain tumour. It is ultimately recurrent. Despite the enormous progress in the development of surgical techniques and radio- and chemotherapy, treatment of malignant gliomas is extremely difficult and can extend patients’ lives by only a few months. The average length of survival of a patient with this type of cancer has not improved significantly over the past few decades remaining at 12–15 months. Only 3–5 % of patients survive more than 3 years. The high invasiveness of glioma results from the capability of cancer cells of extensive proliferation and the ‘diffuse infiltration’ between normal brain cells, which makes them elusive targets for effective surgical management [1]. The problem of resistance of gliomas to treatment should also be analysed at the molecular level. In glioblastoma cells, several mechanisms responsible for programmed cell death induction like apoptosis or autophagy are blocked, while molecular chaperones promoting cell survival are overexpressed [2–4]. A better understanding of the complex biology of glioma cells is essential for identification of key molecular targets stimulating signalling pathways that lead to initiation of programmed cell death.

Temozolomide is a new drug in glioma therapy, which alone or in combination with radiotherapy prolongs the

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median survival time. The cytotoxicity of temozolomide is due to formation of O⁶-methylguanine in DNA which mispairs with thymine during the next cell cycle of DNA replication. In consequence, glioma cells respond by undergoing G₂/M arrest and ultimately die by autophagy or apoptosis [5–9]. Our earlier experiments conducted on the anaplastic astrocytoma cell line MOGGCCM revealed that the anticancer properties of temozolomide are potentiated by quercetin (3,3',4',5,7-pentahydroxyflavone), a natural flavonoid found in a broad range of fruits and vegetables, e.g. apples, grapes, lemons, onion, kale or tomatoes, with the daily intake of about 30 mg. It has multiple biological, pharmacological and medical applications. It is one of the most potent antioxidants. Quercetin also facilitates apoptosis of tumour cells by activation of caspase 3 and caspase 9 and cytochrome c release. It is also a well-known inhibitor of heat shock protein expression [10–16].

Therefore, the aim of our study was to investigate the effect of temozolomide and quercetin applied alone or in combination on apoptosis and autophagy induction in the human glioblastoma multiforme T98G cell line. We analysed the morphology typical of programmed cell death as well as the molecular mechanism underlying this process based on Hsp27, Hsp72, cytochrome c, beclin 1, caspase 12 protein expression and caspase 3, caspase 8 and caspase 9 activity. Analyses of the shape of the cell nuclei as a hallmark of glioblastoma were also performed.

Materials and methods

Cells and culture conditions

The human glioblastoma multiforme cells (T98G, European Collection of Cell Cultures) were grown in a 3:1 mixture of DMEM and nutrient mixture F-12 Ham (Ham's F-12) (Sigma) supplemented with 10 % FBS (Sigma), penicillin (100 U/ml) (Sigma) and streptomycin (100 µg/ml) (Sigma). The cultures were kept at 37 °C in humidified atmosphere of 95 % air and 5 % CO₂.

Drug treatment

Temozolomide (TMZ, Schering-Plough) at the final concentrations of 1, 5, 10, 25, 50, 100, 150 and 200 µM and quercetin (3,3',4',5,7-pentahydroxyflavone) (Sigma) at the final concentrations of 1, 5, 10, 25, 50 and 100 µM were used in the experiments. The drugs were dissolved in dimethyl sulfoxide (DMSO, Sigma). The final concentration of DMSO in the culture medium did not exceed 0.01 %, which as indicated in preliminary experiments did not influence cell viability and the expression of the proteins studied. Three variants of drug treatment were performed. In the first

one, the cells were incubated only with quercetin or temozolomide for 24, 48 or 72 h. In the second variant, quercetin and temozolomide were added to the culture medium simultaneously and incubated for 24 or 48 h. In the third one, the cells were pre-incubated with one of the two drugs for 6 or 24 h, followed by treatment with the other drug for the next 18 or 24 h. As a control, the cells were incubated with 0.01 % of DMSO.

Detection of apoptosis and necrosis with fluorochromes

For identification of apoptosis and necrosis, the cells were stained with a mixture of fluorescent dyes Hoechst 33342 (Sigma) and propidium iodide (Sigma), respectively [17]. The morphological analysis was performed under a fluorescence microscope (Nikon E-800). Cells exhibiting blue fluorescent nuclei (fragmented and/or with condensed chromatin) were interpreted as apoptotic. Cells exhibiting pink fluorescent nuclei were interpreted as necrotic. At least 1,000 cells in randomly selected microscopic fields were counted under a microscope. Each experiment was performed in triplicate.

Detection of acidic vesicular organelles with acridine orange

Autophagy is a process of sequestering cytoplasmic proteins into the lytic compartment and is characterised by formation and promotion of acidic vesicular organelles (AVOs). Vital staining with 1 µg/ml of acridine orange (AO) for 15 min was performed for detection of AVOs in the cells treated with quercetin and/or temozolomide [18]. Typical AO-positive cells exhibiting granular discretions of AVOs in the cytoplasm were interpreted as autophagic. A morphological analysis was performed under a fluorescence microscope (Nikon E-800). At least 1,000 cells in randomly selected microscopic fields were counted. Each experiment was performed in triplicate. The percentage of autophagic cells was calculated as the number of cells with AVOs versus the total number of stained cells.

Detection of apoptosis and necrosis by flow cytometry

The Annexin V-FITC apoptosis detection kit (Sigma) was used for detection of apoptosis and necrosis by flow cytometry. The samples were prepared according to the manufacturer's protocol. Briefly, the control and drug-treated cells were incubated with 5 µl of Annexin V-FITC and 10 µl of propidium iodide for 10 min. Immediately after staining, the cells were analysed on the FacsCanto instrument (Becton Dickinson, San Jose, CA, USA). A total of 30,000 events were acquired and analysed using FacsDiva software. Cells which were in the early stage of the apoptotic process were stained only with Annexin V-FITC, whereas late apoptotic

cells were stained by both fluorochromes. Cells stained only with propidium iodide were interpreted as necrotic. Live cells showed no staining by either propidium iodide or Annexin V-FITC. Each experiment was performed in triplicate.

Mitochondrial membrane potential

Fluorochrome 3,3'-dihexyloxycarbocyanine iodide DiOC₆(3) was used for studying the changes in the mitochondrial membrane potential ($\Delta\psi_m$) in cells incubated with quercetin and temozolomide. At low concentrations, it accumulates in the mitochondria. Loss in DiOC₆(3) staining indicates disruption of the mitochondrial inner transmembrane potential [19]. The control and drug-treated cells were incubated with the fluorochrome at the final concentration of 50 nM for 20 min at 37 °C in the dark, washed three times with PBS and subjected to the FacsCanto instrument (Becton Dickinson). A total of 30,000 events were acquired and analysed using FacsDiva software. Each experiment was performed in triplicate.

Isolation of the mitochondrial fraction

A commercial isolation kit was used (Sigma) for the isolation of the mitochondria. The samples were prepared according to the manufacturer's protocol. Briefly, after quercetin or/and temozolomide treatment, the cells at the density about 2×10^7 were trypsinised, centrifuged for 5 min ($600 \times g$) and washed twice with PBS. The cell pellet was resuspended in lysis buffer (Sigma), incubated for 5 min on ice and centrifuged for 10 min ($600 \times g$). The supernatant was transferred onto a fresh tube and centrifuged at $11,000 \times g$ for 10 min. The pellet was resuspended in cell lysis buffer and used for electrophoresis.

Isolation of the cytosolic fraction

After quercetin and/or temozolomide treatment, the cells were lysed in hot SDS-loading buffer (125 mM Tris-HCl pH 6.8; 4 % SDS; 10 % glycerol; 100 mM DTT), boiled in a water bath for 10 min and centrifuged at $10,000 \times g$ for 10 min, and the supernatants were collected. The protein concentration was determined by the Bradford method [20] and samples of the supernatants containing 80 μ g of proteins were used for electrophoresis.

Immunoblotting

Cytoplasmic and mitochondrial samples were separated by 10 % SDS-polyacrylamide gel electrophoresis [21] and subsequently transferred onto Immobilon P membrane (Sigma). Following the transfer, the membrane was blocked with 3 % low fat milk in PBS for 1 h and incubated

overnight with mouse anti-Hsp72 monoclonal antibody (SPA 810, StressGen) diluted 1:1,000, anti-Hsp27 (SPA 800, StressGen) diluted 1:1,000, rabbit beclin 1 antibody (Sigma) diluted 1:500, anti-caspase 12 (Cell Signaling) diluted 1:1,000 and sheep anti-cytochrome c antibody (Sigma) diluted 1:1,000. The membranes were washed three times for 10 min with PBS containing 0.05 % Triton X-100 (Sigma) and incubated for 2 h with a 1:30,000 dilution of alkaline phosphatase-conjugated goat anti-mouse IgG, anti-sheep IgG or anti-rabbit IgG (Sigma). The membranes were visualised with alkaline phosphatase substrate (5-bromo-4-chloro-3-indolylphosphate and nitro-blue tetrazolium, Sigma) in colour development buffer (DMF, Sigma). The data were normalised relative to β -actin (Sigma, working dilution 1:2,000, data not shown).

The levels of protein expression were determined using the Bio-Profil Bio-1D Windows Application V.99.03 programme. Three independent experiments were performed.

Caspase activity assay

Caspases are cysteine proteases, which in normal conditions exist as inactive pro-forms or zymogens. They are cleaved to the active form following induction of apoptosis. The activity of caspases 3, 8 and 9 was estimated using the SensoLyte[®]AMC Caspase Substrate Sampler Kit (AnaSpec) in the control and drug-treated cells. Sample preparation and enzymatic reaction were performed according to the manufacturer's protocol. The fluorescence of AMC was monitored at Ex/Em=354 nm/442 nm in 96-well black microplates using 2030 Multilabel Reader Victor[™]x4 (Perkin Elmer).

Nuclear morphology

For the analysis of the nuclear shape in the control and drug-treated cells, the roundness factor was used [22, 23] with value 1 for a circle and smaller values for increasing irregularities of nuclear shape. For visualisation of the nucleus, the cells were stained with Hoechst 33342 at a final concentration of 80 μ g/ml for 5 min. The measurement of the shape of the nuclei was performed with the Image J programme on digital microscopic images, projected on the computer screen. The tumour cell nuclei were manually traced. A minimum of 300 tumour nuclei per experimental variant were analysed. Three experimental variants were performed.

ER staining

For identification of ER, a staining method with fluorochrome 3,3'-dihexyloxycarbocyanine iodide was used [24]. The cells were incubated with 10 μ M of DiOC₆(3) for 10 min in the dark at 37 °C. Morphological analysis was performed under a fluorescent microscope Nikon E-800.

Statistical analysis

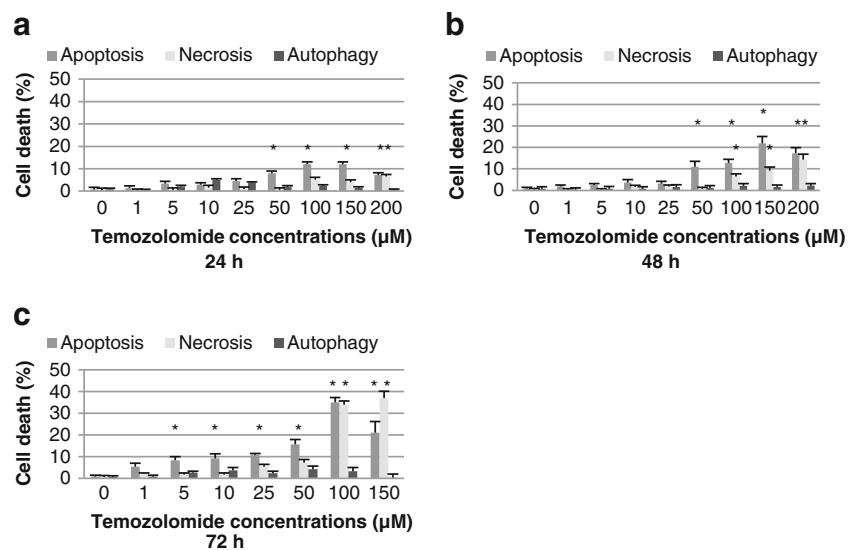
The data are presented as mean \pm standard deviation (SD). The statistical evaluation was performed with a one-way ANOVA test followed by Dunnett's multiple comparison test. $P < 0.05$ was taken as the criterion of significance.

Results

Sensitivity of T98G cells to temozolomide or quercetin treatment

To estimate the sensitivity of T98G cells to temozolomide or quercetin treatment, staining was performed with dyes specific for apoptosis, necrosis and autophagy: Hoechst 33342, propidium iodide and acridine orange, respectively (Figs. 1 and 2). Microscopic observations of stained cells revealed that temozolomide was very effective in apoptosis induction (Fig. 1a–c). A significant increase in the number of programmed dead cells was observed after 24 h of incubation, reaching a maximum (12 %) at the concentration of 100 and 150 μM (Fig. 1a). The longer incubation time was even more effective, and signs of apoptotic cell death were noticed in about 22 % of cells after 48 h of incubation with temozolomide 150 μM (Fig. 1b) and 35 % after 72 h of treatment with the drug concentration of 100 μM (Fig. 1c). Besides apoptosis, temozolomide applied to the culture medium for 48 and 72 h at concentrations higher than 100 μM induced necrosis in T98G cells. What was also interesting, the increased number of necrotic cells was accompanied by a decreased number of apoptotic ones, when the cells were incubated with temozolomide starting with 200 μM applied for 48 h and 150 μM after 72 h of treatment. Temozolomide had no effect on autophagy induction.

Fig. 1 The level of apoptosis, necrosis and autophagy induction in the glioblastoma multiforme T98G cell line treated with temozolomide (0–200 μM) for 24 (a), 48 (b) and 72 h (c), stained with Hoechst 33342, propidium iodide and acridine orange. $*P < 0.05$

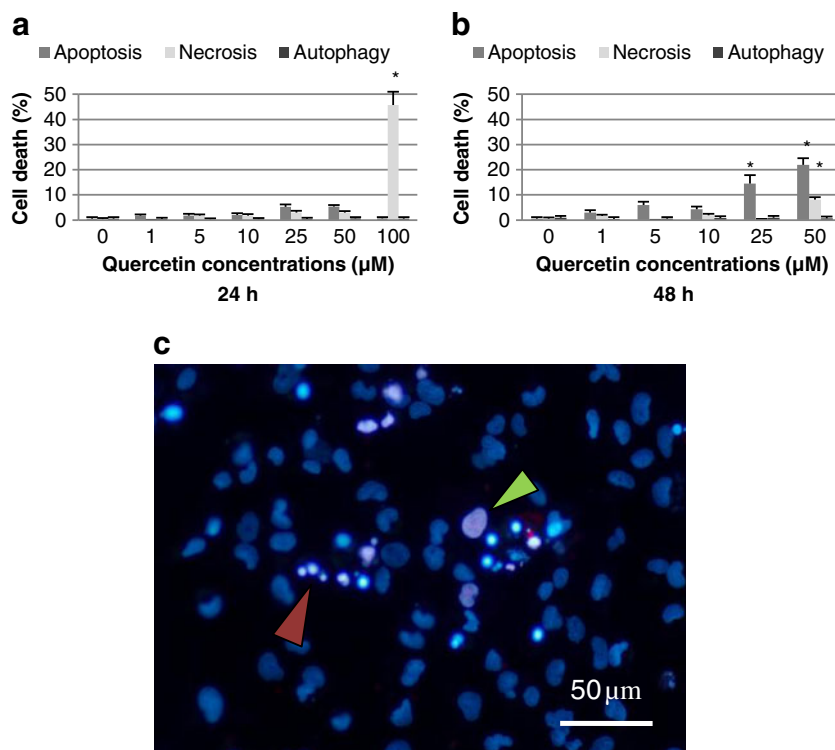


Similar to temozolomide, quercetin was also effective in apoptosis induction, but in comparison to the alkylating drug, its pro-apoptotic properties were weaker (Fig. 2a–c). Quercetin induced apoptosis very effectively after the 48-h-long treatment with the concentrations of 25 (14 %) and 50 μM (22 %) (Fig. 2b, c). The longer period of incubation with the higher flavonoid concentration induced mainly necrosis (data not shown). The necrotic type of cell death also dominated after the 24-h-long incubation with the concentration above 100 μM (Fig. 2a). Similar to TMZ, quercetin had no significant effect on autophagy induction.

Sensitivity of T98G cells to combined temozolomide and quercetin treatment

To estimate the combined effect of quercetin and temozolomide on cell death, two possible variants of the drug treatment were performed. In the first variant, the cells were incubated simultaneously with both drugs, and in the second one, the cells were pre-incubated with one of the drugs followed by incubation with the other one. The type of death was estimated through observation of cells after staining with Hoechst 33342, acridine orange and propidium iodide (Fig. 3). Incubation of T98G cells with temozolomide at the concentration of 50 μM with increasing quercetin concentrations (0–50 μM) for 24 (Fig. 3a–c) and 48 h (Fig. 3d–f) revealed that such a combination induced mainly apoptosis and the effectiveness of such a treatment was time and concentration dependent. The number of apoptotic cells rose gradually with the increased concentration of quercetin, being the most effective in a combination containing 50 μM of the flavonoid. Increased effectiveness was observed after the longer incubation time. When T98G cells were treated simultaneously with the combination of 50 μM of quercetin and 50 μM of temozolomide for 24 h, apoptosis was observed in 6 % of the cells, while after the 48-h-long incubation,

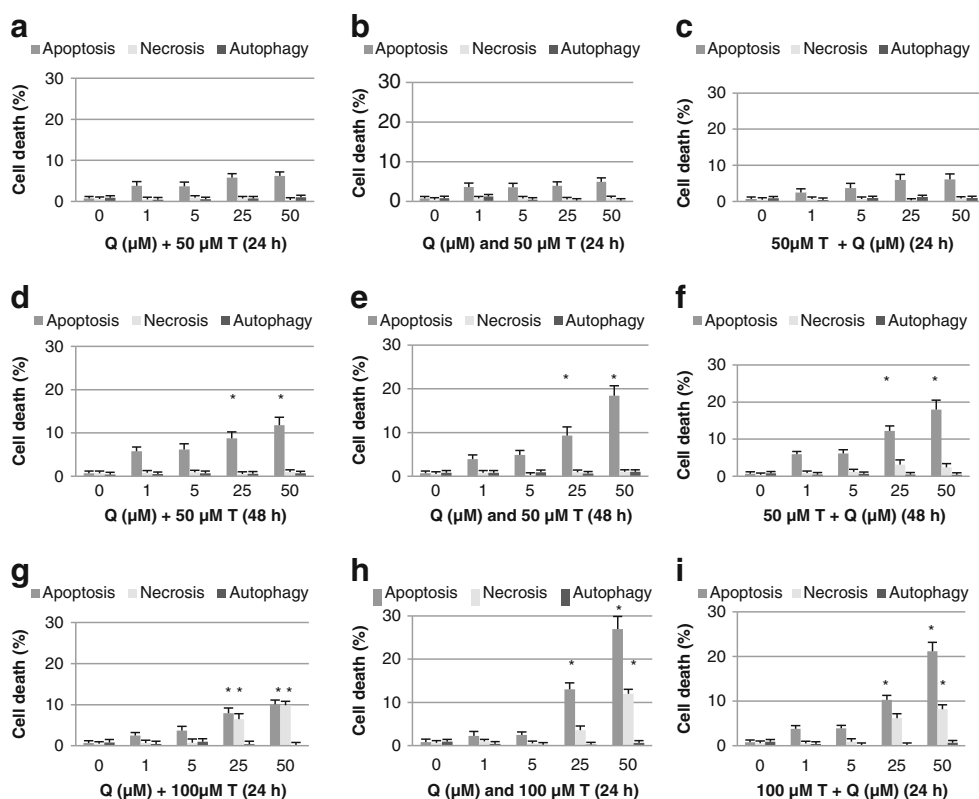
Fig. 2 The time-dependent effect of quercetin (0–100 μ M) on apoptosis, necrosis and autophagy induction in the T98G cell line incubated with the drug for 24 (a) and 48 h (b). **c** A picture of T98G cell stained with Hoechst 33342 and propidium iodide after 48 h of treatment with 50 μ M of quercetin. *Green arrow* indicates necrotic cell, while *red arrow* points apoptotic cell. * $P < 0.05$



apoptosis was observed in 18 % of the cells. Although the percentage of apoptotic cells after simultaneous drug application was similar to the level observed after separate quercetin treatment, the number of necrotic cells was significantly lower

when flavonoid was accompanied by temozolomide. In the case of separate quercetin treatment, the level of necrosis was high and reached 10 %. When flavonoid was combined with temozolomide, the percentage of necrotic cells was about 2.

Fig. 3 Estimation of the level of apoptosis, necrosis and autophagy in T98G cells incubated with 50 (a–f) or 100 μ M (g–i) of temozolomide and an increasing quercetin concentration (0–50 μ M) for 24 h (a–c, g–i) and 48 h (d–f), stained with Hoechst 33342 and propidium iodide; **a, d, g** cells pre-incubated with quercetin followed by temozolomide administration; **b, e, h** cells treated with both drugs administered at the same time; **c, f, i** cells pre-incubated with temozolomide followed by quercetin administration



When the T98G cells were treated with 100 μM of TMZ combined with different quercetin concentrations for 24 h, the number of apoptotic cells was higher than that observed after incubation with 50 μM of the alkylating drug (Fig. 3g–i). Application of 100 μM TMZ and 50 μM of quercetin induced apoptosis in 27 % of cells; unfortunately, it was accompanied by a significant necrosis, and after 48 h of incubation, necrosis was dominant (data not shown). Therefore, temozolomide and quercetin at the concentration of 50 μM applied to the culture medium for 48 h were chosen for further experiments. Considering the precedence of drug addition, the simultaneous treatment and pre-incubation variant with temozolomide were the most effective. Pre-incubation of cells with quercetin followed by the TMZ treatment was less effective. Incubation with both drugs had no effect on autophagy induction.

Evaluation of cell death and the mitochondrial transmembrane potential by flow cytometry

To confirm the reliability of the results obtained from the microscopic observations, the T98G cells treated with quercetin (50 μM) and temozolomide (50 μM) separately as well as in combination of both drugs for 48 h were analysed by flow cytometry according to the type of cell death (apoptosis or necrosis) and mitochondrial membrane potential.

Incubation of T98G cells with temozolomide resulted in significant induction of apoptosis (Table 1). Early apoptosis was observed in 3.82 % of the cells, while late apoptosis was observed in 9.16 %. Quercetin induced programmed cell death in 16.74 % of the cells, where 8.18 % were at the early stage of the apoptotic process and 8.56 % at the late one. The combination of both drugs was more effective and the percentage of apoptotic cells reached 22.75 (11.75 % for early apoptosis and 11 % for late apoptosis) in the case of

Table 1 The effect of 48-h-long incubation of T98G cells with quercetin (Q, 50 μM) and/or temozolomide (T, 50 μM) on apoptosis and necrosis induction

	Necrosis (%)	Early apoptosis (%)	Late apoptosis (%)
Control	0.25±0.07	0.75±0.2	0.14±0.1
Q	2.7±0.4	8.18±1.2*	8.56±0.07*
T	0.5±0.14	3.82±0.14	9.15±0.2*
QT	0.8±0.07	9.95±0.49*	8.2±0.42*
Q+T	0.8±0.0	11.75±0.07*	11±1*
TQ	0.8±0.14	11.26±0.77*	10.3±0.6*

Samples were analysed by flow cytometry with Annexin V-FITC detection kit. Each experiment was performed in triplicate

QT cells pre-incubated with quercetin, *Q+T* simultaneous drug treatment, *TQ* pre-incubation with temozolomide

* $P < 0.05$

simultaneous application. A similar effect was observed when the T98G cells were pre-incubated with temozolomide followed by quercetin treatment (21.8 %). In the case of quercetin pre-incubation, the number of dead cells was slightly lower but still reached 17.8 %.

It is known that a decreased mitochondrial membrane potential is a good indicator of apoptotic cell death [4]. In our experiments, both quercetin and temozolomide applied alone or in combination decreased this potential (Fig. 4). The lowest value was observed after treatment with the simultaneous application of quercetin and temozolomide.

The effect of temozolomide and quercetin on the expression of marker proteins

Apoptosis and autophagy at the molecular level are characterised by changes in the expression of typical marker proteins. Using the immunoblot technique, we studied the effect of temozolomide and quercetin applied separately or in combinations (Fig. 5) on the level of expression of pro-apoptotic cytochrome c [in the cytoplasmic (Fig. 5c) and mitochondrial (Fig. 5d) fractions], anti-apoptotic molecular chaperones Hsp27 (Fig. 5b) and Hsp72 (Fig. 5a) and pro-autophagic beclin 1 (Fig. 5g). Additionally, we analysed fluorimetrically the activity of caspases 3, 8 and 9, i.e. aspartate-specific cysteine proteases being part of the caspase cascade system, which plays a vital role in apoptosis (Fig. 5f). Using the immunoblot technique, we also studied the level of expression of caspase 12, a well-known enzyme mediating apoptosis under ER stress (Fig. 5e).

The quantitative and qualitative analyses of the immunoblots revealed that quercetin and temozolomide inhibited Hsp72 and Hsp27 expression in all the experimental variants. The studied drugs applied alone or in combinations decreased the level of cytochrome c in the mitochondrial fraction, which was accompanied by increased accumulation of the protein in the cytoplasm. Quercetin and temozolomide had no significant effect on the expression of the autophagy marker beclin 1 as its level was similar to

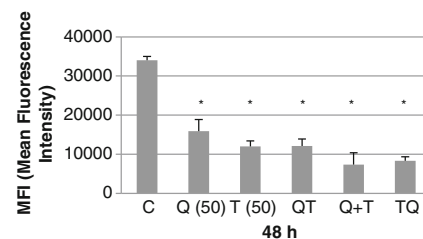


Fig. 4 The mitochondrial membrane potential in T98G cells stained with DiOC₆(3), incubated separately with 50 μM of quercetin (Q) and 50 μM of temozolomide (T) or in combination of both drugs for 48 h and analysed by flow cytometry. C control cells, QT cells pre-incubated with quercetin, Q+T simultaneous drug treatment, TQ pre-incubation with temozolomide. * $P < 0.05$

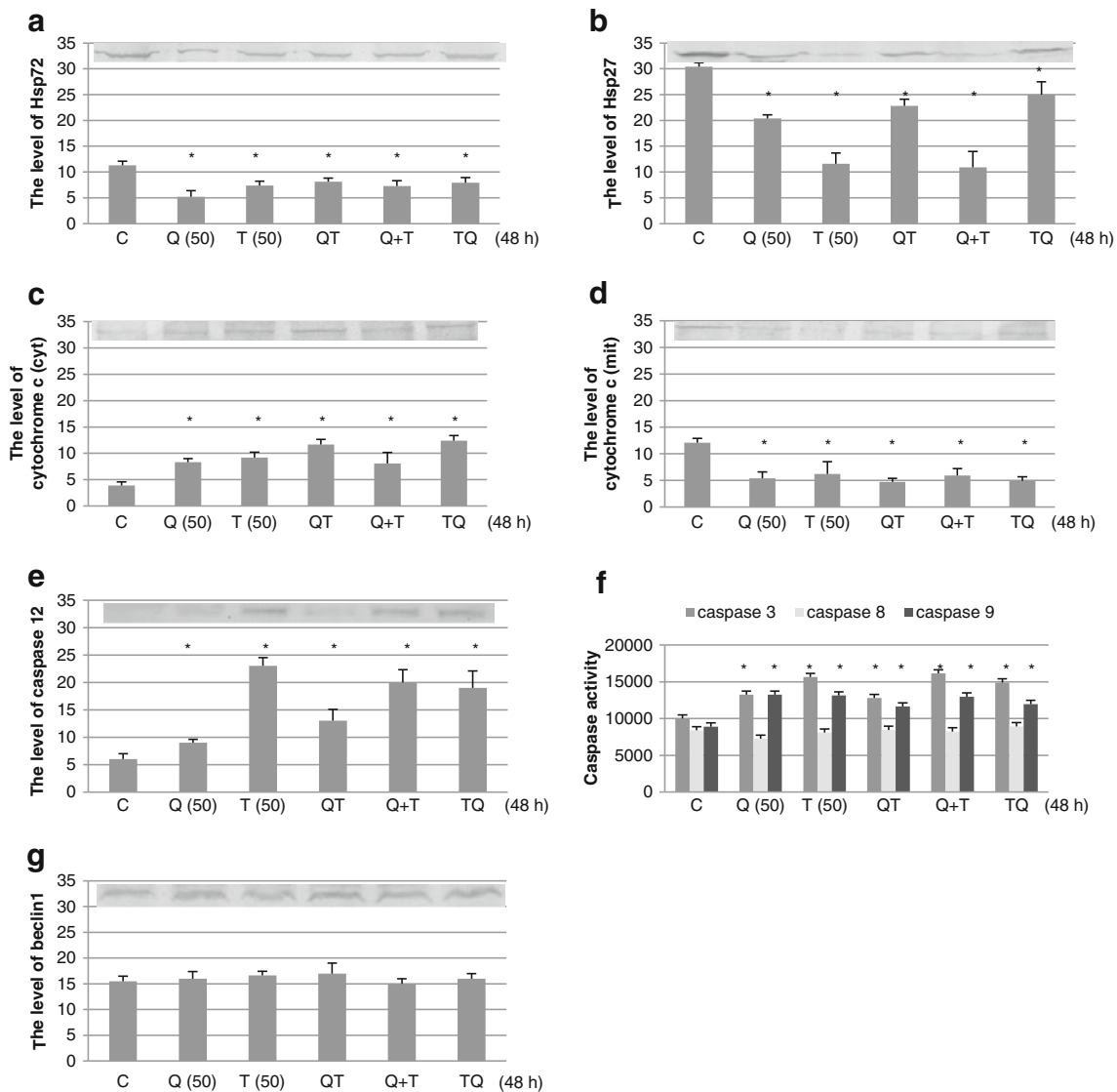


Fig. 5 Level of Hsp72 (a), Hsp27 (b), cytochrome c (c cytoplasmic, d mitochondrial fraction), caspase 12 (e) and beclin 1 (g) expression with representative blots and the activity of caspases 3, 8 and 9 (f) after

temozolomide (T) and quercetin (Q) treatment for 48 h. C control cells, QT cells pre-incubated with quercetin, Q+T simultaneous drug treatment, TQ pre-incubation with temozolomide. * $P < 0.05$

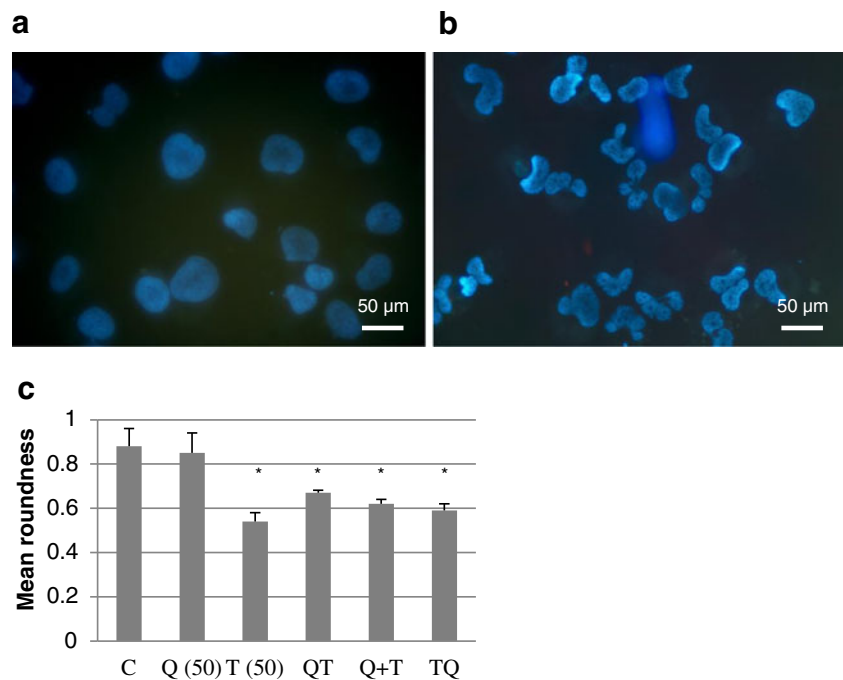
the control one in all the experimental variants. In the case of caspases, increased expression of caspase 12 was observed after treatment with temozolomide separately as well as in combination with quercetin. Both drugs applied alone or in combination significantly increased the activity of caspase 3 and caspase 9. The simultaneous drug treatment was the most potent. TMZ applied separately as well as in combination with quercetin did not change the caspase 8 activity. Its inhibition was observed after the quercetin treatment.

The effect of temozolomide and quercetin on the shape of nuclei and cytoplasm organisation

Staining of the cells with Hoechst 33342 revealed that temozolomide alone and together with quercetin changed

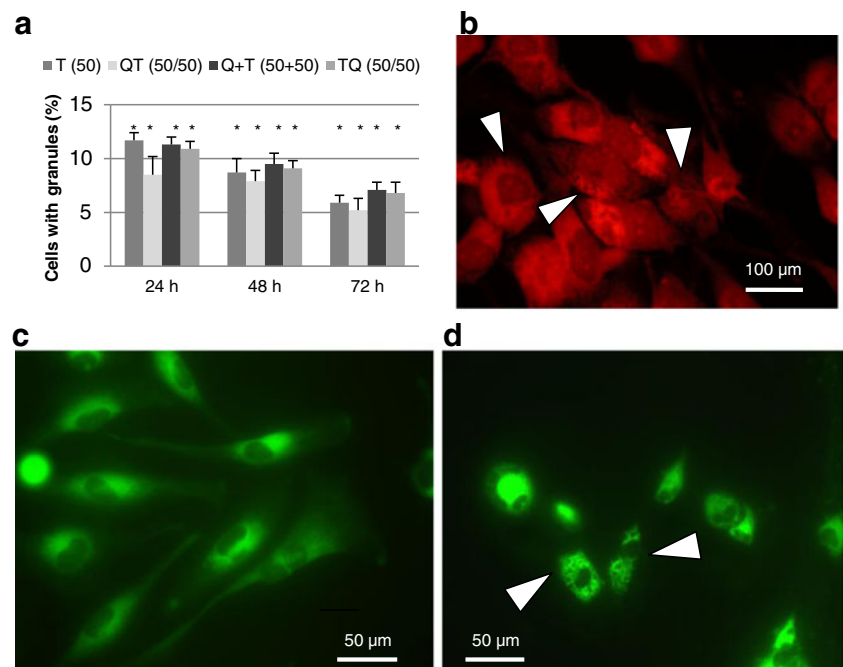
the morphology of glioblastoma nuclei from circular, as observed in the control (Fig. 6a), to a more curved ‘croissant-like’ shape (Fig. 6b). The mean roundness factor used for the histomorphometrical analysis of the shape of the nuclei after TMZ with or without the flavonoid decreased from 0.86 (control) to about 0.6 (Fig. 6c). Quercetin did not change the shape of the nuclei and the mean roundness factor was similar to that in the control (0.85) (Fig. 6c). Staining of the T98G cells treated with temozolomide with orange acridine facilitated distinguishing round-shaped granules, which were not stained with the fluorochrome used (Fig. 7). The percentage of cells where such structures were noticed was the highest after 24 h of incubation (11 %) and decreased gradually with a longer incubation time. Granules were observed in about 9 % of cells after a 48-h-

Fig. 6 The effect of temozolomide (T) and quercetin (Q) on the shape of the nuclei expressed by the mean roundness factor (MRF) after staining with Hoechst 33342. **a** Control cells, **b** nuclei after treatment with quercetin and temozolomide, **c** quantitative analysis. *QT* cells pre-incubated with quercetin, *Q+T* simultaneous drug treatment, *TQ* pre-incubation with temozolomide. * $P < 0.05$



long incubation and 6 % after 72 h after incubation with only TMZ and simultaneously with quercetin (Fig. 7a, b, d). No granules in the cytoplasm were noticed in the control nontreated cells and cells incubated with quercetin (data not shown). Additional staining of the T98G cells treated with the alkylating drug with or without flavonoid with DiOC₆(3), a fluorochrome that binds to ER, revealed the presence of round bulky structures within the reticulum that were similar in shape and size to the granules observed in the cytoplasm (Fig. 7d).

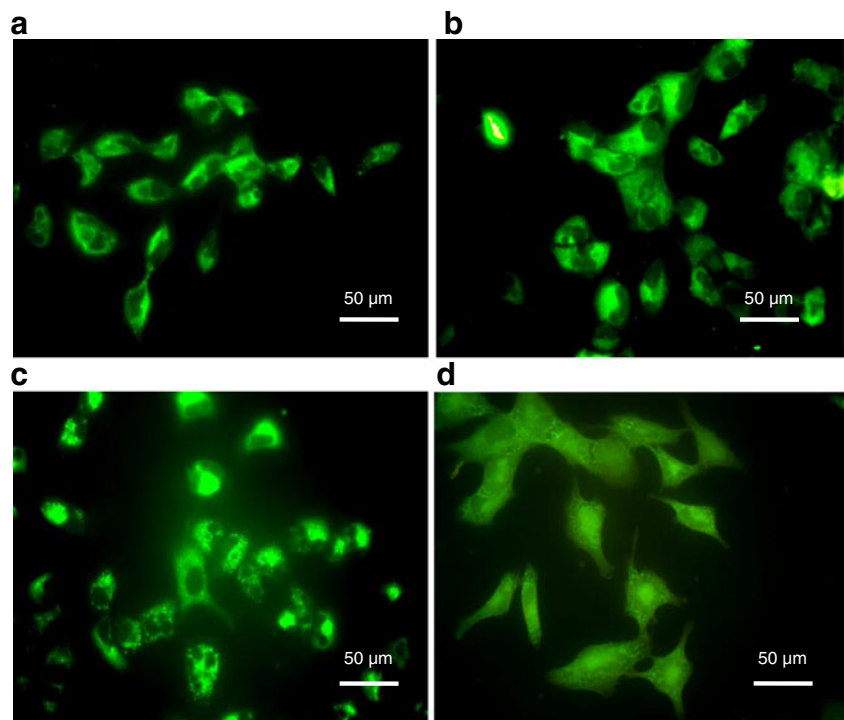
Fig. 7 The effect of temozolomide (T) and quercetin (Q) on the cytoplasmic granule formation (white arrows) in the T98G cells. **a** Quantitative analysis, **b** cells stained with OA after temozolomide treatment, **c** ER structure in control cells stained with DiOC₆(3), **d** ER structure in cells treated with temozolomide and stained with DiOC₆(3). *QT* cells pre-incubated with quercetin, *Q+T* simultaneous drug treatment, *T+Q* pre-incubation with temozolomide. * $P < 0.05$



Localisation of Hsp27 and Hsp72 in T98G cells

The indirect immunofluorescence technique used to examine the localisation of Hsp27 (Fig. 8b, d) and Hsp72 (Fig. 8a, c) revealed cytoplasmic distribution of both proteins in the untreated cells as well as after temozolomide and quercetin application. On the basis of the colour and light intensity of the fluorochrome conjugated with the secondary antibody, it was noticed that the localisation of both proteins in the cytoplasm was different. The proteins in the control

Fig. 8 The effect of temozolomide and quercetin on Hsp72 (a, c) and Hsp27 (b, d) in untreated cells (a, b) or treated with both drugs (c, d) for 48 h



cells were distributed quite uniformly throughout the cytoplasm. After the temozolomide treatment, alone or with quercetin, Hsp27 was localised in association with cytoplasmic round-shaped similar sized granules near the nuclei as well as with the lamellar structures. In the cells with croissant-like nuclei, the protein was located in the curved part of the organelle. In the case of Hsp72, two ways of protein distribution were distinguished. In some cells, the molecular chaperone was arranged in small groups localised around the nuclei and throughout the whole cytoplasm. In the rest of the cells, the localisation of Hsp72 was similar to the control one. Quercetin had no effect on Hsp72 localisation (data not shown).

Discussion

Most strategies for high grade gliomas are aimed to maximise PCD type I apoptosis or type II autophagy. These are predominantly distinctive processes, but many studies suggest a cross-talk between the two. Apoptosis may occur via death receptor-dependent (extrinsic, cytoplasmic) and receptor-independent (intrinsic, mitochondrial) pathways. The mitochondrial pathway is mediated by Bcl2 family proteins, which regulate disruption of the mitochondrial membrane and release of cytochrome c to the cytosol and lead to the activation of caspases and cell death in consequence. The extrinsic pathway is triggered through the activation of death receptors and involves caspase 8 maturation. Both pathways converge to a final common pathway correlated with activation of

caspase 3 [4]. Unlike apoptosis, which is a caspase-dependent process, autophagy is a caspase-independent programmed cell death, characterised by accumulation of autophagic vacuoles (AVOs) in the cytoplasm accompanied by degradation of Golgi apparatus and ER, which precedes the destruction of the nucleus. Autophagy is suppressed during the early stages of tumour progression. Therapeutically, increased autophagy could represent an alternative way to destroy cancer cells. It would be beneficial in the light of recent investigations, as gliomas naturally resist apoptosis [5, 25]. On the other hand, many studies provide data suggesting that autophagy represents a protective mechanism helping cancer cells to get rid of toxic particles and increase the survival in consequence. In the light of these observations, redirecting cancer cells to enter the apoptotic pathway would be beneficial [26, 27]. Our earlier experiments conducted on the MOGGCCM anaplastic astrocytoma cell line revealed that temozolomide induced autophagy more efficiently than apoptosis [15, 16]. We demonstrated for the first time that combinations of TMZ with quercetin were much more effective in programmed cell death induction in glioma cells. At a low (5 μ M) drug concentration, quercetin potentiated the pro-autophagic effect of temozolomide, while after treatment with a higher drug concentration (30 μ M), autophagy switched to apoptosis. Therefore, we decided to evaluate the effect of temozolomide applied alone or in combination with quercetin on induction of apoptosis and autophagy in the human glioblastoma multiforme T98G cell line. Our results indicated that temozolomide and quercetin were very effective apoptosis inducers in the studied cells. The combination of both drugs

was even more effective. At the molecular level, it was correlated with the decreased mitochondrial membrane potential, release of cytochrome c from the mitochondrion to the cytoplasm and activation of caspase 3 and caspase 9. No activation of caspase 8 was observed. All these observations suggest that both drugs initiate apoptosis via the internal (mitochondrial) pathway. In contrast to the MOGGCCM cell line, neither temozolomide alone nor its in combination with quercetin had an effect on autophagy induction in the T98G cells. This was confirmed by the unchanged level of autophagy marker beclin 1 in comparison to the control. This is a critical component involved in autophagosome formation at the early stage of autophagy, which at the basal level of expression is not able to initiate the process of death [28]. It has also been shown that activation of caspase 3 inhibits autophagosome formation and may block beclin 1 activity or even cleave the protein [29]. Furthermore, the expression of beclin 1 is reduced during gliomagenesis, being progressively lower through grade I to grade IV gliomas [30].

Staining the cells with Hoechst 33342 for visualisation of signs of apoptosis additionally revealed that temozolomide alone and together with quercetin changed the morphology of glioblastoma nuclei from circular, observed in the control, to more curved, or ‘croissant like’. It is known that tumour cell nuclei belong to the most important histological structures in the assessment of tumour biology including typing and grading of gliomas. Histomorphometry of tumour cell nuclei could also be a promising approach for estimation of the prognosis for individual patients. It was shown that the nuclei had a tendency toward a more circular shape in short survivors [22, 23].

The microscopic observation also revealed small round granules in the cytoplasm after temozolomide treatment, both alone and in combination with quercetin. They were not stained with acridine orange—a fluorochrome typical in the detection of autophagosomes. Staining of cells with DiOC₆(3), a dye that recognises ER within cells, revealed the presence of rounded structures in the reticulum. They were visibly surrounded by Hsp27. This was accompanied by increased caspase 12 expression. All those observations may suggest that this might be correlated with the ER stress response. As tumours increase in size, cells are exposed to several environmental stressors like hypoxia, limited nutrients and acidosis. This leads to accumulation of misfolded and aggregated proteins in the cytoplasm. Such perturbations in protein folding are also observed after exposure to chemotherapy. When molecular chaperones cannot keep up with denatured and misfolded proteins, they aggregate in larger, uniform in size, membrane free aggresomes present throughout the cytoplasm, leading

to ER stress. In response to this, the unfolded protein response is activated. It initially tries to restore normal ER homeostasis by activation of the proteasome complex or autophagy, when aggresomes fuse with lysosome and create autophagolysosomes. In this context, autophagy is a pro-survival process [31]. If damage is too severe, the death pathways are activated especially through the mitochondrial apoptotic pathway [32]. The precise molecular mechanisms of ER stress-induced apoptosis, however, have not been fully elucidated. It is known that ER stress leads to caspase 12 activation [33]. Processed caspase 12 reportedly activates caspase 9, which is followed by activation of caspase 3 [34]. On the other hand, some reports demonstrate that in fact the mitochondrial pathway of apoptosis plays a significant role in ER stress-induced death, while activation of caspase 12 plays a minor role in the process [35]. In our experiments, the presence of granules and a cleaved form of caspase 12 followed by apoptosis induction was shown in the cells treated with temozolomide alone or with quercetin. In the cells incubated with quercetin, we noticed neither granules nor cleavage of caspase 12, which may suggest that the ER stress-activated apoptosis in glioma cells after temozolomide treatment is correlated with activation of caspase 12. The lack of ER stress signs in cells incubated with quercetin may be explained by the fact that the antioxidant protects cells from ER stress-induced cell death [36].

Hsps are molecular chaperones whose enhanced expression inhibits ER stress, induces apoptosis and leads to cell survival [32]. The mechanism of Hsp27 and Hsp72 apoptosis blockade is complex. They prevent cytochrome c release, mitochondrial membrane permeabilisation, recruitment of procaspase 9 to the apoptosome and its subsequent maturation to the active form. Hsp72 migrates to the nucleus to protect nuclear proteins from caspase 3 cleavage. Hsp27 may bind to F-actin, thereby preventing cytoskeleton disruption. It has also been observed that Hsps may be sometimes fused to aggresomes, but they are not directly engaged in misfolded protein degradation. They only recognise them. Depletion of Hsp27 and Hsp72 severely decreases degradation of misfolded proteins and decreases cell survival [31, 32, 37, 38]. All those protective effects of Hsps are beneficial in normal cells. In the case of cancer cells where the level of Hsps is abnormally high, these molecular chaperones act as negative prognostic markers. It was also observed that in many cases, the expression of the proteins rose with the grade of the tumour. It has even been suggested that heat shock proteins may participate in oncogenesis. Therefore, inhibition of Hsps expression has become a novel strategy for cancer therapy [39–41]. In our experiments, both temozolomide and quercetin partially inhibited Hsps expression in T98G cells.

In summary, our results indicate that temozolomide and quercetin are very potent apoptotic but not autophagic inducers in glioma multiforme T98G cells. It seems that the apoptotic process is preceded by ER stress followed by activation of the mitochondrial death pathway. Our observations may form a basis for further studies on using this drug combination in clinical application.

Conflicts of interest None

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