Pharmacological Reports
2011, 63, 403–416
ISSN 1734-1140

Kinetic studies of the effects of Temodal and quercetin on astrocytoma cells

Joanna Jakubowicz-Gil[1], Ewa Langner[3], Wojciech Rzeski[2,3]

[1]Department of Comparative Anatomy and Anthropology, [2]Department of Immunology and Virology, Maria Curie-Sklodowska University, Akademicka 19, PL 20-033 Lublin, Poland

[3]Department of Medical Biology, Institute of Agricultural Medicine, Jaczewskiego 2, PL 20-950 Lublin, Poland

Correspondence: Joanna Jakubowicz-Gil, e-mail: jjgil@poczta.umcs.lublin.pl

Abstract:
The aim of the present study was to investigate the kinetics of the effects exerted by Temodal and quercetin on the survival of the human astrocytoma MOGGCCM cell line. Our results indicate that quercetin was toxic and induced necrosis, whereas Temodal induced autophagy-mediated cell death most effectively. The amount of cell death directly correlated with drug concentration and length of exposure. During combined administration of both drugs, Temodal attenuated the cytotoxic effects of quercetin. Combinations of both drugs were effective in inducing programmed cell death, but the type of cell death was concentration-dependent. Co-administration of Temodal (100 μM) with a low quercetin concentration (5 μM) resulted in a very significant induction of autophagy; however, after treatment with quercetin at a higher concentration (30 μM), apoptosis became the primary mechanism of cell death. The sequence of drug administration was also important. The highest number of dead cells was observed after simultaneous administration of both drugs or after pre-incubation with Temodal followed by treatment with quercetin. Apoptosis was identified through activation of the mitochondrial pathway including cleavage of caspase-3 and release of cytochrome c. Autophagy was identified through increased levels of LC3II. Our results indicate that Temodal and quercetin are synergistic inducers of programmed cell death, better together than applied separately. This drug combination appears to be a potent and promising therapeutic relevant to the treatment of gliomas.

Key words:
Temodal, quercetin, astrocytoma, cell death, caspases, Hsps, LC3

Introduction

Anaplastic astrocytoma (WHO grade III) is a common malignant brain tumor representing ~25% of all primary brain tumors. Despite years of extensive investigations, astrocytomas remain resistant to conventional therapy, including resection followed by radiotherapy. The prognosis for patients with this type of cancer is very poor, and survival time from diagnosis is approximately 3–8 years. Astrocytomas are characterized by diffusive infiltration that makes complete surgical resection nearly impossible. Additionally, resistance to chemotherapy is correlated with activation of protective mechanisms facilitating the escape of cells from death signals [17, 23, 25]. A potential solution towards the goal of eradicating these chemotherapeutic-resistant tumor cells is the use of agents that inhibit repair processes and induce programmed cell death.

Quercetin (3,3',4',5,7-pentahydroxyflavone) is a natural flavonoid that is found in a broad range of fruit and

vegetables with an average daily intake of about 30 mg. It possesses multiple biological, pharmacological and medical applications [26, 27, 31]. It is one of the most potent antioxidants. As an anticancer agent, it facilitates apoptosis through caspase-3 and caspase-9 activation and cytochrome c release [5, 6]. Additionally, it suppresses the level of heat shock proteins (Hsps), well known molecular chaperones responsible for chemotherapy resistance and overexpressed in nearly all classes of tumors, including gliomas [7, 14, 43].

Temodal is a leading compound in a new class of alkylating chemotherapeutic agents that enter the cerebrospinal fluid and do not require hepatic metabolism for activation [9]. With a 35% response rate in WHO grade III anaplastic astrocytomas, Temodal is trending towards increasing efficacy against lower grade tumors [3, 40]. The cytotoxicity of Temodal is due to formation of O^6-methylguanine in DNA, which mispairs with thymine during the next cell cycle of DNA replication. In consequence, astrocytomas respond by undergoing G_2/M arrest and ultimately undergo autophagy-induced cell death [16, 29, 38].

Previous studies have revealed that natural bioactive compounds may act in synergy with chemotherapeutic drugs used in clinical treatment [27, 31]. Therefore, this investigation was undertaken to examine the effects of varying Temodal and quercetin concentrations administered alone and in combination for different amounts of time on astrocytoma cell sensitivity to induction of apoptosis, necrosis and autophagy. We also examined the molecular mechanism underlying these processes based on Hsp27, Hsp72, cytochrome c, caspase-3, caspase-8, LC3 and Bcl2L12 protein expression.

Materials and Methods

Cell lines and culture conditions

Human brain astrocytoma cells (MOGGCCM, European Collection of Cell Cultures, Centre for Applied Microbiology and Research, Salisbury, UK) were grown in a 3:1 mixture of DMEM and Ham's F-12 (Sigma) supplemented with 10% FBS (Sigma), penicillin (100 U/ml) (Sigma) and streptomycin (100 μg/ml) (Sigma). Cultures were kept at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Drug treatment

Quercetin (Sigma) at final concentrations of 5 μM, 15 μM, 30 μM, and Temodal (TMZ, Temozolomide, Schering-Plough) at final concentrations of 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 or expression levels of the studied proteins. Three variants of drug treatment were employed. In the first one, MOGGCCM cells were incubated with quercetin or Temodal for 6, 12, 24, 48 and 72 h. In the second, quercetin and Temodal were added to the culture medium at the same time and incubated for 24 h. In the third variant , cells were preincubated with one of the two drugs for 6 h, followed by treatment with the other drug for the next 18 h. As a control, the cells were incubated with 0.01% of DMSO.

Immunoblotting

After quercetin and/or Temodal treatment, the MOGGCCM cells were lysed in hot SDS-loading buffer (125 mM Tris-HCl, pH 6.8; 4% SDS; 10% glycerol; 100 mM DTT), incubated in a boiling water bath for 10 min, centrifuged at 10,000 × g for 10 min, and the supernatants were collected. The protein concentration was determined using the Bradford method [4], and samples of the supernatants containing 80 μg of protein from whole cells lysates were separated by 10% SDS-polyacrylamide gel electrophoresis [18] 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 the mouse monoclonal antibodies anti-Hsp72 (SPA 810, StressGen) diluted 1:1,000, anti-Hsp27 (SPA 800, StressGen) diluted 1:1,000, anti- caspase-8 active form (Sigma) diluted 1:500, anti-Bcl2L12 (Sigma) diluted 1:500, rabbit antibodies anti-caspase-3 active form (Sigma) diluted 1:500, anti-LC3 (Sigma) diluted 1:1,000 or sheep anti-cytochrome c antibody (Sigma) diluted 1:1,000. The membranes were washed 3 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 mem-

branes were visualized with alkaline phosphatase substrate (5-bromo-4-chloro-3-indolylphosphate and nitro-blue tetrazolium, Sigma) in a color development buffer (DMF, Sigma). The data were normalized 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 program. Three independent experiments were performed.

Apoptosis detection with fluorochromes

Apoptosis and necrosis were identified using the fluorescent dyes Hoechst 33342 (Sigma) and propidium iodide (Sigma), respectively [13]. Morphological analysis was performed under a fluorescence microscope (Nikon E800). 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 the microscope. Each experiment was performed in triplicate.

Detection of acidic vesicular organelles with acridine orange

Autophagy is a process by which cytoplasmic proteins are sequestered in the lytic compartment. It is characterized by the formation and promotion of acidic vesicular organelles (AVOs). Detection of AVOs in MOGGCCM cells treated with quercetin and/or Temodal was obtained through staining with acridine orange (AO) [37]. The cells were incubated with AO at the final concentration of 1 μg/ml for 15 min. A typical orange acridine-positive cell exhibited granular discretion of AVOs in the cytoplasm, which was indicative of autophagosome formation. Morphological analysis was performed under a fluorescence microscope (Nikon E800). At least 1,000 cells in randomly selected microscopic fields were counted under the microscope, with each experiment performed in triplicate. The percentage of autophagic cells was calculated as the number of cells with AVOs *vs.* the total number of stained cells.

Statistical analysis

The data are presented as the mean ± SD of the results from three independent experiments. Statistical evaluation was performed using one-way ANOVA tests followed by Dunnett's multiple comparison test; $p < 0.05$ in comparison to the control was taken as a criterion of significance.

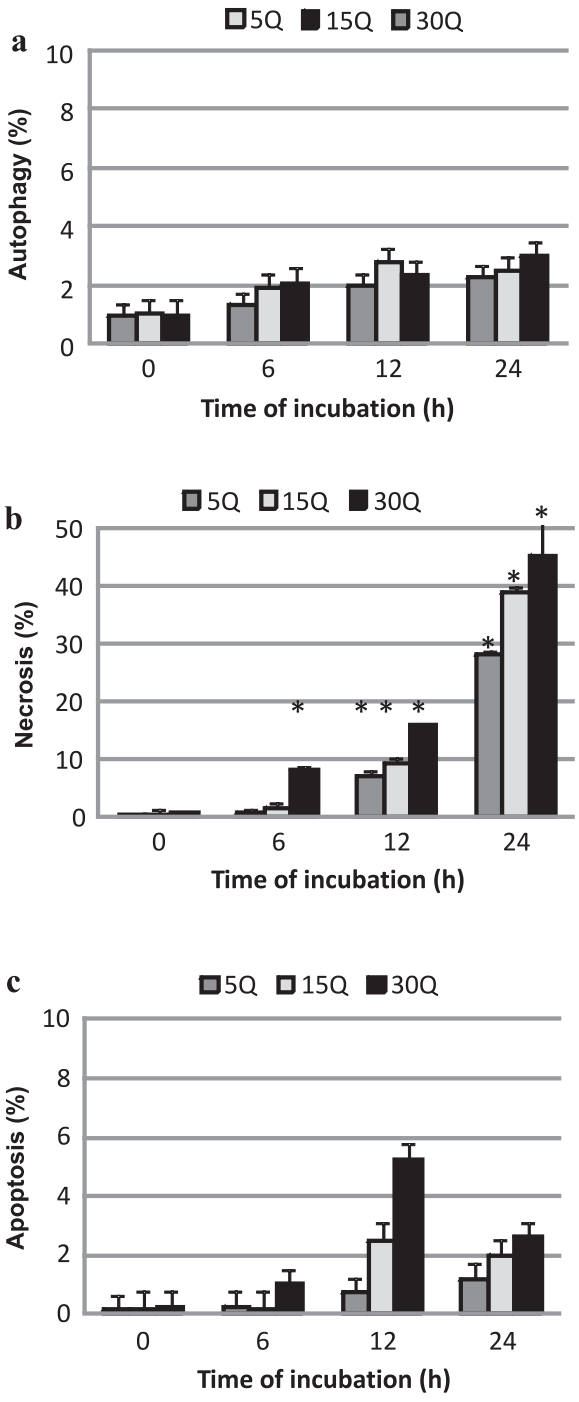

Fig. 1. The time-dependent effect of quercetin (Q) on autophagy (**a**), necrosis (**b**) and apoptosis (**c**) induction in the MOGGCCM cell line. The cells were incubated with the drug (5, 15 and 30 μM) for 6, 12 and 24 h; * $p < 0.05$

Results

The effects of Temodal and quercetin on cell death induction

To distinguish the type of cell death in MOGGCCM cells after treatment with quercetin or Temodal, a staining method with specific fluorochromes (Hoechst 33342, propidium iodide and acridine orange) commonly used for identification of apoptosis, necrosis or autophagy was performed.

Quercetin

Our experiments indicated that quercetin was not very effective in the induction of programmed cell death in MOGGCCM cells (Fig. 1). The highest number of apoptotic cells (5.25%) was observed after 12 h incubation with 30 μM of quercetin. Extending the incubation time to 24 h did not result in an increase in the number of apoptotic cells. At the three concentrations studied (5, 15 and 30 μM), the amount of apoptotic cells did not exceed 4.5% (Fig. 1c). In the case of autophagy, a 24 h incubation with the flavonoid at 15 μM was the most effective (4.35%) (Fig. 1a). On the other hand, astrocytoma cells underwent necrotic cell death after drug treatment. During the first 6 h of incubation with quercetin at 5 μM and 15 μM, the number of necrotic cells did not change significantly, whereas flavonoid treatment at 30 μM induced this type of death in ~9% of cells. Longer treatment correlated with gradual, concentration-dependent increases in the cytotoxic effect of the flavonoid. For example, a 24 h incubation with 5 μM of quercetin produced a population containing 29% necrotic cells, whereas 30 μM induced necrosis in 45% of the cells (Fig. 1b). After a longer incubation time with higher drug concentrations, necrosis was observed in more than 50% of the cells (data not shown).

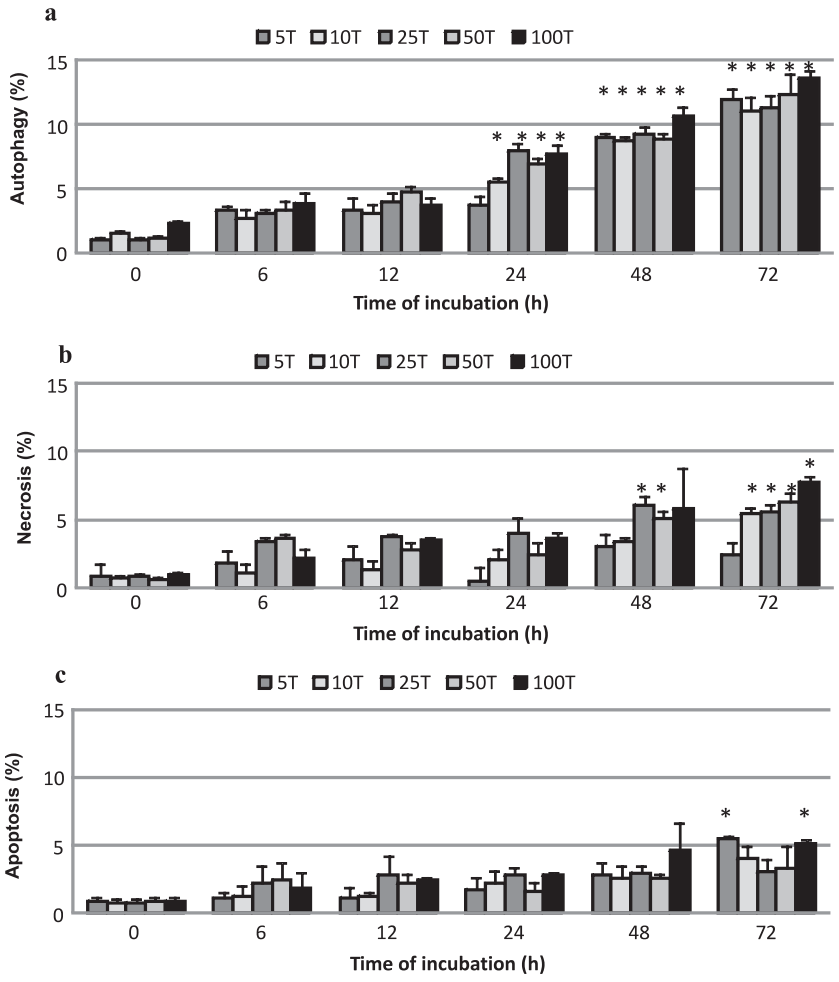

Fig. 2. The time-dependent effect of Temodal (T) on autophagy (**a**), necrosis (**b**) and apoptosis (**c**) induction in the MOGGCCM cell line. The cells were incubated with the drug (5, 10, 25, 50 and 100 μM) for 6, 12, 24, 48 and 72 h; * $p < 0.05$

Temodal

In contrast to quercetin, Temodal was less effective at the induction of necrosis in MOGGCCM cells. The highest number of necrotic cells (7.73%) was observed after 72 h of treatment at 100 μM (Fig. 2b). The sensitivity of MOGGCCM cells to the induction of apoptosis was similar to quercetin treatment, and the ratio of apoptotic cells did not exceed 5.5% (Fig. 2c). We observed that astrocytoma cells responded to Temodal treatment by autophagy most effectively (Fig. 2a). This was directly correlated with a longer incubation time and a higher drug concentration. The highest number of autophagic cells (13.66%) was observed after 72 h of incubation with Temodal at 100 μM.

Temodal plus quercetin

Experiments with Temodal and quercetin as described above revealed that the drugs induced different types of death in MOGGCCM cells. Thus, we were interested to evaluate the effects of combined administration of both drugs on cell sensitivity to cell death (Figs. 3–5). Astrocytoma cells were incubated with quercetin and Temodal at different concentrations, administrated simultaneously, at the same time or the cells were pre-incubated with quercetin or Temodal for 6 h followed by administration of the other drug for the next 18 h. The combined drug treatment was more effective in the induction of programmed cell death than separate drugs, and induced necrosis to the smaller extent, suggesting that quercetin in combination with Temodal loses its pro-necrotic properties. The order of drug application was also important. We observed that pre-incubation with Temodal followed by quercetin treatment (Figs. 3a, 4a, 5a), as well as simultaneous drug administration (Figs. 3c, 4c, 5b), were the most effective at inducing apoptosis and autophagy. Pre-incubation with quercetin was less effective (Figs. 3b, 4b), however, it still resulted in ~25% autophagic cells. The intensity of the programmed cell death was also concentration-dependent. After treatment with a low quercetin concentration (5 μM) and Temodal, the autophagic type of cell death dominated at all the concentrations studied and varied from 18.85% (Fig. 3b) to 38% (Fig. 3a). Few apoptotic and necrotic cells were observed, not exceeding 4% (Figs. 3a,b,c). In the case of experiments with higher flavonoid concentrations (15 μM and 30 μM), and Te-

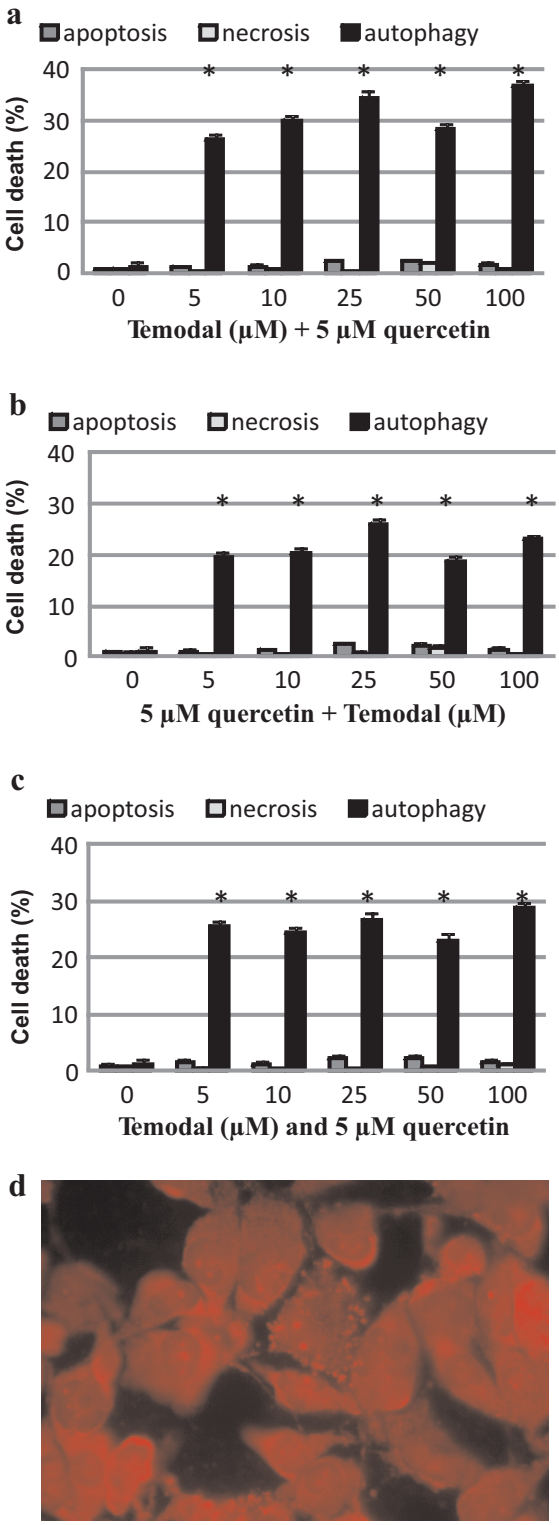

Fig. 3. Estimation of the level of apoptosis, necrosis and autophagy in MOGGCCM cells incubated with 5 μM of quercetin and increasing Temodal concentration (0–100 μM); (**a**) cells pre-incubated with Temodal followed by quercetin administration; (**b**) cells pre-incubated with quercetin followed by Temodal administration; (**c**) cells treated with both drugs administered at the same time; (**d**) an autophagic cell after simultaneous treatment with 5 μM of quercetin and 100 μM of Temodal stained with acridine orange, magnification 400×; * $p < 0.05$

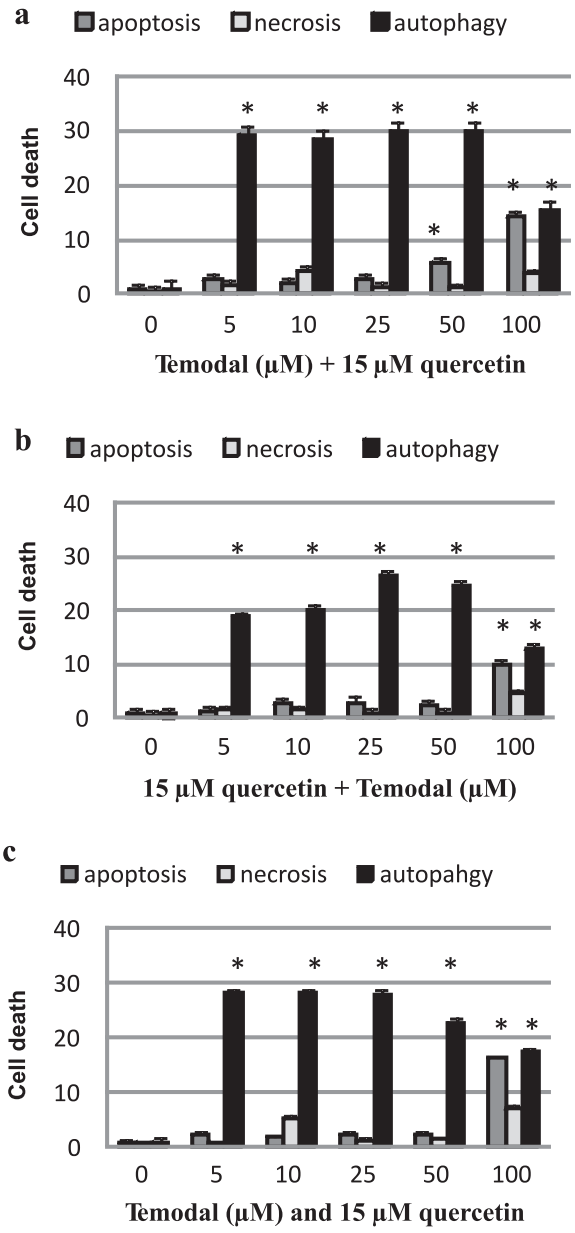

Fig. 4. Estimation of the level of apoptosis, necrosis and autophagy in MOGGCCM cells incubated with 15 μM of quercetin and increasing Temodal concentration (0–100 μM); (**a**) cells pre-incubated with Temodal followed by quercetin administration; (**b**) cells pre-incubated with quercetin followed by Temodal administration; (**c**) cells treated with both drugs administrated at the same time; * $p < 0.05$

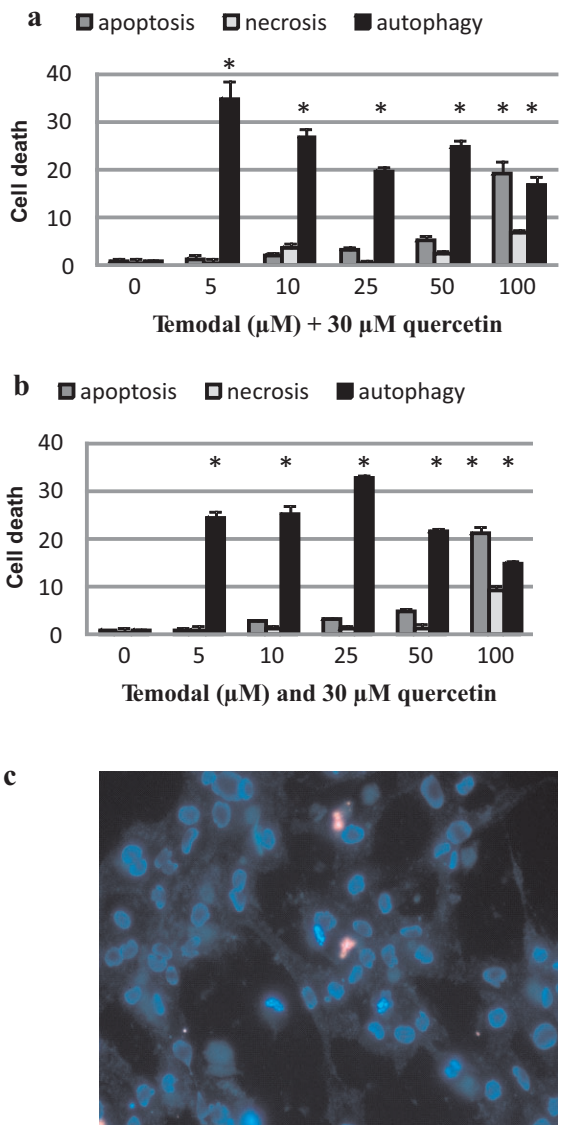

Fig. 5. Estimation of the level of apoptosis, necrosis and autophagy in MOGGCCM cells incubated with 30 μM of quercetin and increasing Temodal concentration (0–100 μM); (**a**) cells pre-incubated with Temodal followed by quercetin administration; (**b**) cells treated with both drugs administered at the same time; (**c**) apoptotic cells after simultaneous treatment with 30 μM of quercetin and 100 μM of Temodal, magnification 200×; * $p < 0.05$

modal at concentrations above 50 μM, we observed more apoptosis than autophagic cell death (Fig. 4a,b,c, Fig. 5a,b). The results obtained after pre-incubation with quercetin at 30 μM were not presented, because a high percentage of necrotic and unstuck cells were observed during pre-incubation.

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

Cell death is characterized by the expression and activation of typical marker proteins. We decided to evaluate the effects of Temodal and quercetin on the activation of cleavable pro-apoptotic caspase-3, expression of anti-apoptotic and pro-necrotic protein Bcl2L12, pro-apoptotic caspase-8 and cytochrome c, anti-apoptotic molecular chaperones Hsp27 and Hsp72 and pro-autophagic LC3.

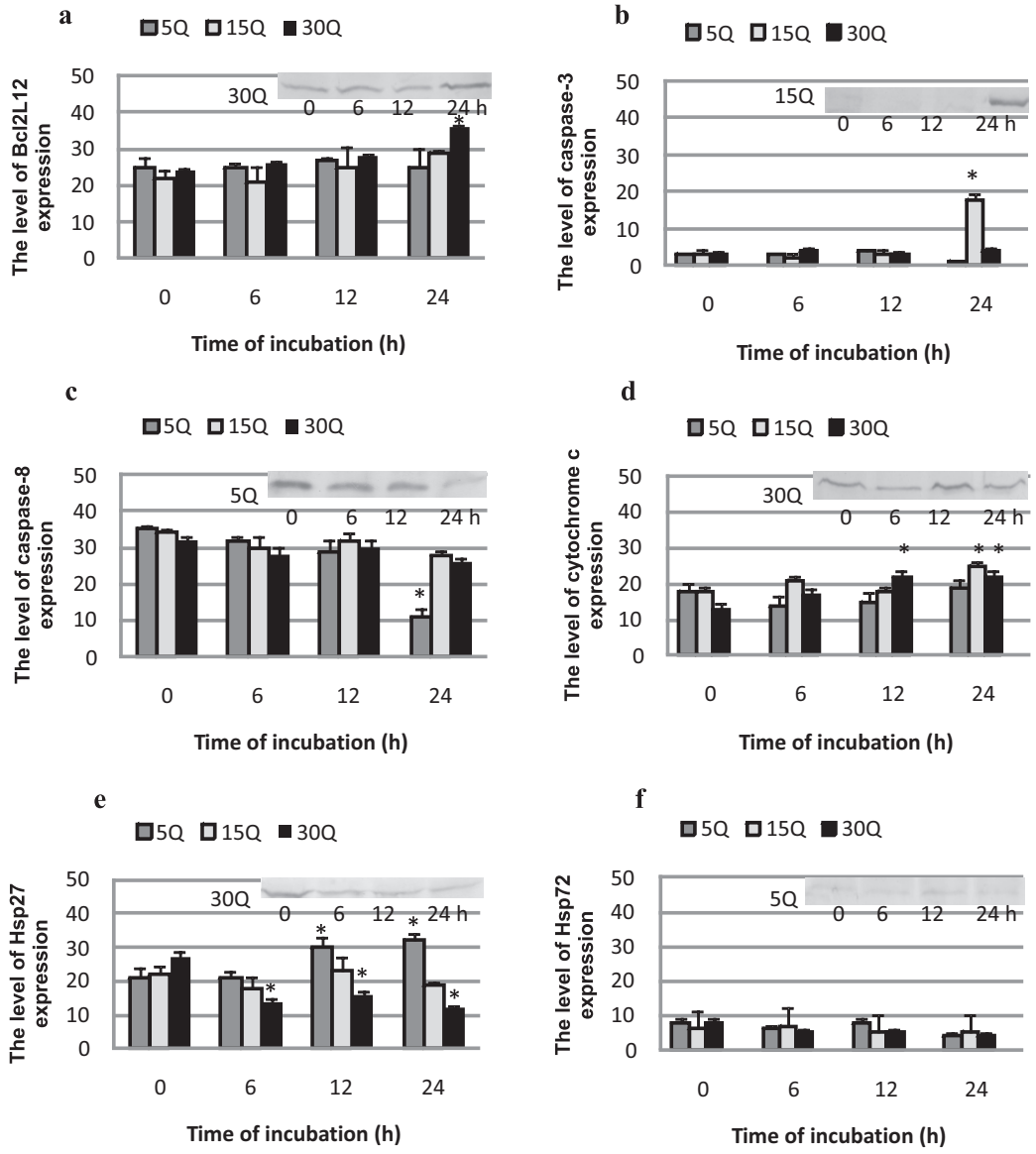

Fig. 6. The effect of different quercetin (Q) concentrations (0–30 μM) on the expression of Bcl2L12 (**a**), caspase-3 (**b**), caspase-8 (**c**), cytochrome c (**d**), Hsp27 (**e**) and Hsp72 (**f**) after 6, 12 and 24 h of treatment. Representative western blots are included; * $p < 0.05$

Quercetin

Cell staining revealed that quercetin induces necrosis rather than programmed cell death in MOGGCCM cells. This was associated with overexpression of Bcl2L12 especially after 24 h of incubation with the flavonoid at 30 μM (Fig. 6a). Levels of the apoptotic marker caspase-3 did not significantly vary after 6 and 12 h of treatment with 5 μM, 15 μM and 30 μM of quercetin compared to the DMSO-only control (Fig. 6b). After a 24 h incubation with 15 μM of quercetin, strong overexpression of this marker protein was observed. In contrast, the levels of caspase-8 decreased in time- and concentration-dependent manners, most noticeable after 24 h with 5 μM of the flavonoid (Fig. 6c). In the case of cytochrome c, quercetin increased the level of the protein in a stepwise manner, being the most effective after 12 h of incubation with 30 μM and 24 h of incubation with 15 μM and 30 μM (Fig. 6d). Treatment with 5 μM of the flavonoid for 6 and 12 h reduced the level of cytochrome c, which reached the level of the control sample after 24 h incubation. Quercetin suppressed the levels of Hsp72 at all concentrations used, but the results were

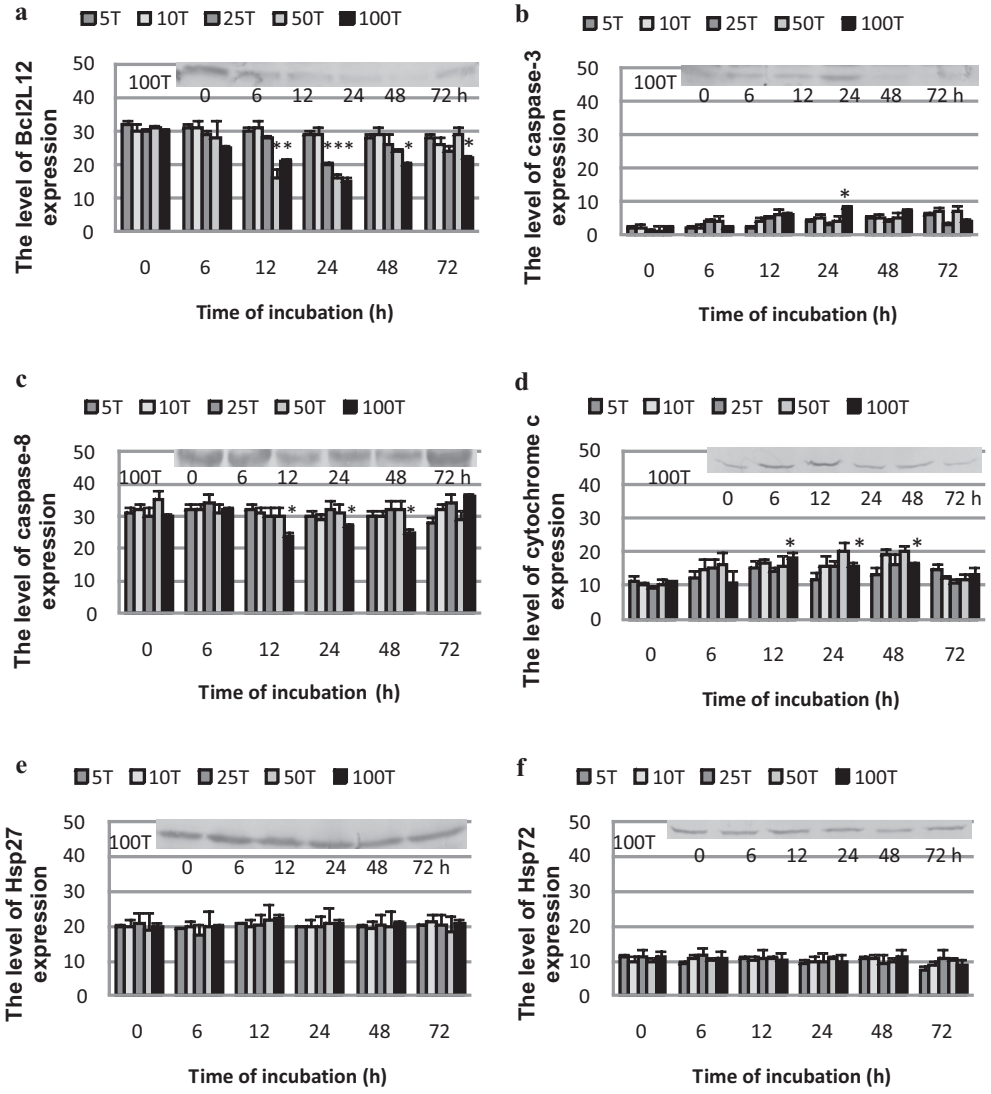

Fig. 7. The effect of different Temodal (T) concentrations (0–100 μM) on the expression of Bcl2L12 (**a**), caspase-3 (**b**), caspase-8 (**c**), cytochrome c (**d**), Hsp27 (**e**) and Hsp72 (**f**) after 6, 12, 24, 48 and 72 h of treatment. Representative western blots are included; * $p < 0.05$

not significant (Fig. 6f). The effect of the flavonoid on Hsp27 levels was concentration-dependent (Fig. 6e). Five μM of the flavonoid increased expression, and 15 μM and 30 μM of the drug decreased expression. The hallmark of autophagy is the conversion of LC3I into a smaller form – LC3II that participates in the formation of autophagosomes and is associated with its structure. Accordingly, autophagy is associated with an inverse level of LC3, and an increased LC3II to LC3I ratio [15]. A greater than 3-fold increase in the level of LC3II was observed after quercetin treatment at all incubation times examined, but the level of the lipidated form did not exceed the level of LC3I (Fig. 9a).

Temodal

In cell staining experiments, Temodal appeared to be a potent inducer of autophagy at all concentrations that were investigated. The more than 2-fold increase in the lipid-bound form of LC3, LC3II, confirms Temodal as an inducer of autophagy (Fig. 9b). Additionally, 6, 12 and 24 h of incubation with 100 μM of Temodal caused the level of LC3II to exceed that of LC3I. Such a ratio between the forms of LC3 was also observed after 48 h of incubation with 25 μM of Temodal and after 72 h with 10 μM of the drug. In regard to expression of caspases, Temodal only had a significant effect on protein expression at 100 μM.

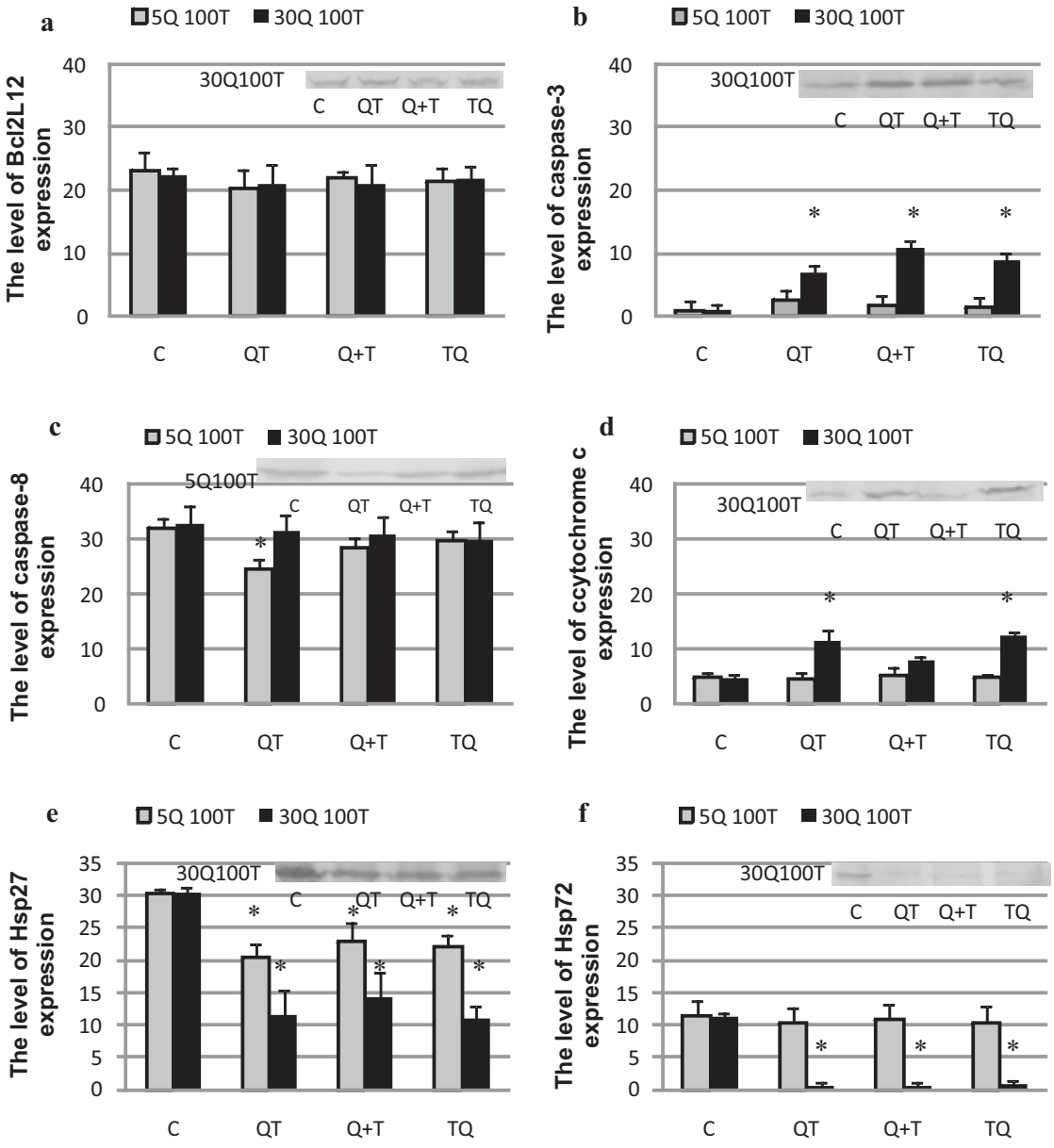

Fig. 8. The effect of simultaneous treatment of MOGGCCM cells with Temodal (T) and quercetin (Q) on the levels of Bcl2L12 (**a**), caspase-3 (**b**), caspase-8 (**c**), cytochrome c (**d**), Hsp 72 (**e**) and Hsp27 (**f**) expression. Representative western blots are included. C – control, QT – cells pre-incubated with quercetin and subsequently treated with Temodal, Q+T – both drug treatments at the same time, TQ – Temodal pre-treatment followed by quercetin incubation. * $p < 0.05$

After 24 h of treatment, an increased level of activated caspase-3 (Fig. 7b) and after 12, 24 and 48 h of incubation, a decreased level of activated caspase-8 were observed (Fig. 7c). After 72 h of incubation, the level of caspase-3 reached the control level. In the case of caspase-8, overexpression was observed but the result was not significant. The expression of cytochrome c increased after 6, 12, 24 and 48 h at all concentrations, but similarly to results observed with caspases, the protein level reached that of the control after 72 h (Fig. 7d). The level of pro-necrotic Bcl2L12 was generally decreased in time- and concentration-dependent manners (Fig. 7a). Five μM and 10 μM decreased the level of the protein gradually without significant correlation with increased incubation time. Concentrations higher than 10 μM decreased the protein level most effectively after 24 h of incubation. Temodal had no effect on Hsp72 and Hsp27 expression (Fig. 7e,f).

Temodal and quercetin

It is known from previously described experiments that the sensitivity of MOGGCCM cells to autophagy, apoptosis and necrosis after Temodal and quercetin treatment was concentration-dependent. Temodal at 100 μM with quercetin at 5 μM induced autophagy most effectively, whereas in combination with quercetin at 15 μM or 30 μM, the fraction of autophagic cells decreased, which was accompanied by an increased fraction of apoptotic cells. Therefore, we decided to use quercetin at 5 μM or 30 μM in combination with Temodal at 100 μM in future experiments. At a low quercetin concentration, the expression of Hsp27 was significantly inhibited, whereas no effect on Hsp72 expression was detected (Fig. 8e,f). No

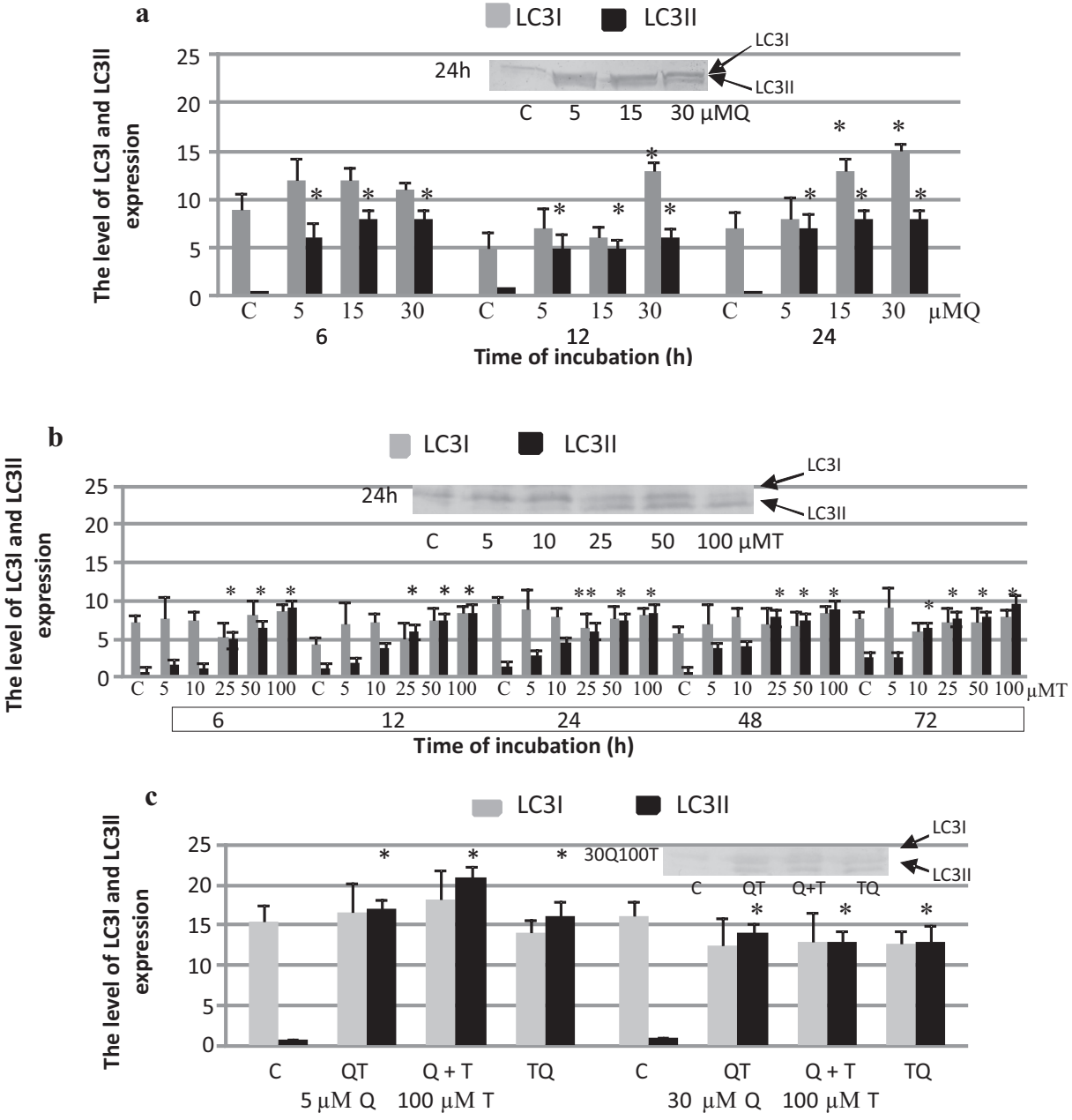

Fig. 9. The levels of LC3I and LC3II expression after quercetin (Q) (**a**), Temodal (T) (**b**) and both drugs (**c**) treatment. Representative western blots are included. C – control, QT – cells pre-incubated with quercetin and subsequently treated with Temodal, Q + T – both drug treatments at the same time, TQ – Temodal pre-treatment followed by quercetin incubation. * $p < 0.05$

changes in caspase-3, -8, cytochrome c, or Bcl2L12 were observed (Fig. 8a–d). The level of LC3II protein exceeded the level of LC3I (Fig. 9c). In the experiments with quercetin at 30 μM, the inhibition of Hsp72 and Hsp27 expression was accompanied by an induction of cytochrome c release (especially in the cells pre-incubated with Temodal and quercetin) and caspase-3 activation (after simultaneous drug administration) (Fig. 8a–f). The effectiveness of LC3I lipidation to LC3II was not as effective as that observed in the case of quercetin at 5 μM (Fig. 9c).

Discussion

Chemoprevention includes the use of natural or pharmacological agents that suppress, arrest or reverse carcinogenesis. Many phytochemicals present in a diet rich in fruit and vegetables have been proposed as potential chemopreventive agents. Quercetin is one of the most potent antioxidant, anti-inflammatory, anti-proliferative and apoptosis-inducing agents, as demonstrated in different *in vivo* and *in vitro* studies [31]. Quercetin is highly enriched in cultured cells [33]. *In vivo*, the flavonoid is extensively metabolized in the small intestine and the liver, therefore low amounts of quercetin and higher levels of its metabolites are found in the circulation [1, 2, 24]. On the other hand, the elimination of quercetin and its metabolites from the organism is quite slow, which could favor its accumulation in plasma with repeated intake. Baseline quercetin concentrations generally vary between 50 and 80 nmol/l. After 28 days of supplementation with over 1 g/day of quercetin, the baseline concentration increased to 1.5 μM [8]. An important benefit of quercetin in anticancer treatment is its selectivity for transformed cells. The flavonoid had no cytotoxic effects on normal fibroblasts (BG-9), human gingival fibroblasts (HGF), human periodontal ligament fibroblasts (HPLF), human skin fibroblasts (HSF), human pulp cells (HPC), and green monkey kidney cells (GMK) [6, 12, 22]. It protects hippocampal neurons against ischemia and blocks apoptosis in mesencephalic dopamine neurons induced by oxidative stress [5, 21]. However, quercetin has been observed to impart neurotoxicity; *in vitro*, the flavonoid induced severe apoptosis and necrosis in the cultured neurons, as well as diminished neuronal arborization [12, 34]. *In vivo*, the concentration of quercetin in brain tissues might not reach the toxic level. Quercetin and some of its metabolites (especially O-methylated metabolites) are able to enter the central nervous system (CNS) but its transport may be limited by the blood-brain barrier (BBB). Additionally, the flavonoid is a substrate for BBB efflux transport, especially the P-glycoprotein transporter [44]. The neurotoxicity of quercetin may also be diminished by rapid conjugation to glutathione once it is within glial cells [41].

In our experiments, we observed that quercetin had a toxic effect on the astrocytoma MOGGCCM cell line, inducing necrosis rather than programmed cell death. Necrosis is considered to be the hallmark of the most common malignant primary brain tumor, GBM, and is used to differentiate GBM from lower grade astrocytomas. Clinical observations suggest that the presence of biological necrosis has a negative overall impact on survival. On the cellular level, cells undergo lysis, surrounding cells are alarmed to the possibility of injury, and local inflammation occurs [28]. On the protein level, we observed overexpression of the pro-necrotic marker protein Bcl2L12, a Bcl2 family member that is significantly expressed in primary human gliomas. It confers marked apoptosis resistance and provokes a pro-necrotic state *in vitro* by inhibiting post mitochondrial apoptosis signaling at the level of effector caspase activation. Bcl2L12 does not affect cytochrome c release and caspase-9 activation, but inhibits caspase-3 maturation [36]. We observed low levels of caspase-3 expression, with the exception of a 24 h incubation with 15 μM of quercetin where we observed overexpression. Overexpression of caspase-3 did not correlated with a significant increase in the level of apoptotic cells. It is known that caspase-3 activity is modulated by various proteins including Hsp27, a well known molecular chaperone that protects cells from apoptosis and necrosis [32, 42]. Heat shock proteins are also responsible for resistance to cell death after chemotherapy. This may explain why overexpression of Hsp family proteins in tumor cells indicates poor prognosis [11, 14, 35]. One mechanism may be that the interaction between Hsp27 and caspase-3 blocks enzyme activity and therefore apoptosis [42]. Quercetin, as an inhibitor of Hsp family proteins, may release caspase-3 from complex and initiate cell death. In our experiments, Hsp27 inhibition was observed after treatment with flavonoid at 30 μM, but not observed at lower drug concentrations. This may explain the lack of apopto-

sis induction observed with quercetin at 15 μM when caspase-3 levels were high. We also observed that MOGGCCM cells responded to Hsp inhibition with necrosis rather than apoptosis.

Similar to its effects on induction apoptosis, quercetin was weak at inducing autophagy. Unlike apoptosis, autophagy is a caspase-independent form of programmed cell death, characterized by the accumulation of autophagic vacuoles in the cytoplasm, the degradation of the Golgi apparatus and ER, which precedes destruction of the nucleus. Autophagy is suppressed during the early stages of tumor progression. Therapeutically increasing autophagy could represent an alternative cancer therapy [10, 19]. In light of recent investigations that gliomas naturally resist apoptosis, therapies that target alternative pathways would be beneficial [37]. The flavonoid induced the lipidation of LC3I to LC3II, but the level of the cleaved form did not exceed the level of the initial form. Thus, the induction of autophagy was weak.

Pharmacokinetic studies revealed that *in vivo,* Temodal accumulates mainly in transformed cells. After a single oral dose of 75–200 mg/m^2, higher drug concentrations in human glioma tumors (2.9 to 6.7 μg/ml) relative to normal brain (1.8 to 3.7 μg/ml) were observed [30]. In MOGGCCM cells, Temodal induced autophagy, as well as necrosis and apoptosis but to smaller extents. Autophagy correlated with the increased amount of the lipidated form of LC3I. Generally, the drug had no significant effect on Hsp72 and Hsp27 expression levels. Caspase-8 and Bcl2L12 protein expression levels were inhibited, and Temodal had no effect on cytochrome c release. Temodal induced the activation of caspase-3, probably being responsible for induction of a small fraction of apoptotic cells in the culture.

Many studies demonstrated that flavonoids exhibited a synergistic anti-tumor effect with chemotherapeutics. Quercetin may effects several smaller insults within the cells without inducing cytotoxicity, which increases their sensitivity to chemotherapeutic drugs. Such combinatorial therapy may allow the use of lower concentrations of drugs and may minimize toxicity, generating a synergistic effect [27, 31]. Therefore, we decided to examine the effects of a combination of quercetin and Temodal on cell death induction. Our results indicate that the use of both drugs simultaneously was much more effective in the induction of programmed cell death than the use of either drug by itself. The sensitivity of MOGGCCM cells to cell death and the type of cell death was drug concentration-dependent. When the cells were incubated with low quercetin concentrations and Temodal, strong autophagy was observed, accompanied by a small fraction of necrosis and apoptosis. This was correlated with increased lipidation of LC3I to LC3II. Treatment with higher quercetin and Temodal concentrations resulted in an increased fraction of apoptotic cells but a smaller fraction of autophagic cells. This strongly correlated with increased caspase-3 activation, release of cytochrome c, and inhibition of Hsp27 and Hsp72 expression. The level of LC3II was also increased. It is known that apoptosis and autophagy may occur simultaneously in certain tissues, even in the same cell. Apoptosis may begin or end with autophagy, and the blockage of caspase activity can induce autophagy [20]. The mechanism responsible for decreasing chemoresistance of cancer cells to Temodal treatment by quercetin may be correlated with the p53 antagonist, p73. In melanoma cells, Temodal induces the truncated form of p73 (ΔNp73), which in turn prevents p53-mediated apoptotic cell death. Chemoresistance was reversed by quercetin, which abrogated the inhibitory effect of ΔNp73 by modulating the protein level and altering its localization [39].

Our results indicate that Temodal and quercetin acting together are synergistic programmed cell death inducers in MOGGCCM astrocytoma cells. The type of cell death was concentration-dependent; low drug concentrations mainly induced autophagy whereas treatment with higher drug concentrations activated intracellular mechanisms responsible for apoptotic cell death. Temodal and quercetin combinatorial therapy seems to be a promising candidate for treatment of glioma.

References:

1. Azuma K, Ippoushi K, Ito H, Higashio H, Terao J: Combination of lipids and emulsifiers enhances the absorption of orally administered quercetin in rats. J Agric Food Chem, 2002, 50, 1706–1712.
2. Boulton DW, Walle UK, Walle T: Fate of the flavonoid quercetin in human cell lines: chemical instability and metabolism. J Pharm Pharmacol, 1999, 51, 353–359.
3. Brada M, Hoang-Xuuan K, Rampling R: Multicenter phase II trial of Temozolomide in patients with glioblastoma multiforme at first relapse. Ann Oncol, 2001, 12, 259–266.

4. Bradford MM: A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem, 1976, 72, 248–254.
5. Braganhol E, Zamin LL, Canedo AD, Horn F, Tamajusuku AS, Wink MR, Salbego C, Battastini AM: Antiproliferative effect of quercetin in the human U138MG glioma cell line. Anticancer Drugs, 2006, 17, 663–671.
6. Chowdhury SA, Kishino K, Satoh R, Hashimoto K, Kikuchi H, Nishikawa H, Shirataki Y, Sakagami H: Tumor-specificity and apoptosis-inducing activity of stilbenes and flavonoids. Anticancer Res, 2005, 25, 2055–2063.
7. Clark JI: Therapeutic applications of heat shock proteins and molecular chaperones. Exp Op Ther Pat, 2001, 11, 1153–1160.
8. Conquer JA, Maiani G, Azzini E, Raguzzini A, Holub BJ: Supplementation with quercetin markedly increases plasma quercetin concentration without effect on selected risk factors for heart disease in healthy subjects. J Nutr, 1998, 128, 593–597.
9. Friedman HS, Kerby T, Calvert H: Temozolomide and treatment of malignant glioma. Clin Cancer Res, 2000, 6, 2585–2597.
10. Ghobrial IM, Witzig TE, Adjei A: Targeting apoptosis pathways in cancer therapy. CA Cancer J Clin, 2005, 55, 178–194.
11. Graner MW, Cumming RI, Binger DD: The heat shock response and chaperone/heat shock proteins in brain tumors: surface expression, release and possible immune consequences. J Neurosci, 2007, 27, 11214–11227.
12. Jakubowicz-Gil J, Rzeski W, Zdzisinska B, Dobrowolski P, Gawron A: Cell death and neuronal arborization upon quercetin treatment in rat neurons. Acta Neurobiol Exp (Wars), 2008, 68, 139–146.
13. Jankowska A, Skonieczna D, Rommerts FFC, Warchoł JB: Investigations on apoptosis in Leydig cells cultured *in vitro*. Folia Histochem Cytobiol, 1997, 33, 99–110.
14. Jolly C, Morimoto RI: Role of heat shock response and molecular chaperones in oncogenesis and cell death. J Nat Cancer Inst, 2000, 92, 1564–1572.
15. Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, Kominami E et al.: LC3 a mammalian homologue of yeast Apg8p is localized in autophagosome membranes after processing. EMBO J, 2000, 19, 5720–5728.
16. Kanzawa T, Germano IM, Komata T, Kondo Y, Kondo S: Role of autophagy in temozolomide-induced cytotoxicity for malignant glioma cells. Cell Death Differ, 2004, 11, 448–457.
17. Kleihues P, Louis DN, Scheithauer BW, Rorke LB, Reifenberger G, Burger PC, Cavenee WK: The WHO classification of tumors of the nervous system. J Neuropathol Exp Neurol, 2002, 61, 215–225.
18. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 1970, 227, 680–685.
19. Lefranc F, Facchini V, Kiss R. Proautophagic drugs: A novel means to combat apoptosis-resistant cancers, with a special emphasis on gliomas. Oncologist, 2007, 12, 1395–1403.
20. Lefranc F, Kiss R: Autophagy, the Trojan horse to combat glioblastomas. Neurosurg Focus, 2006, 20, E7.
21. Mercer LD, Kelly BL, Horne MK, Beart PM: Dietary polyphenols protect dopamine neurons from oxidative insult and apoptosis: investigations in primary rat mesencephalic cultures. Biochem Pharmacol, 2005, 69, 339–345.
22. Nair H, Rao K, Aalinkeel R, Mahajan S, Chawda R, Schwartz S: Inhibition of prostate cancer cell colony formation by the flavonoid quercetin correlates with modulation of specific regulatory genes. Clin Diagn Lab Immunol, 2004, 11, 63–69.
23. Ohgaki H, Kleihues P: Population-based studies on incidence, survival rates and genetic alterations in astrocytic and oligodendroglial gliomas. J Neuropathol Exp Neurol, 2005, 64, 479–489.
24. Oliveira EJ**,** Watson DG, Grant MH: Metabolism of quercetin and kaempferol by rat hepatocytes and the identification of flavonoid glycosides in human plasma. Xenobiotica, 2002, 32, 279–287.
25. Omuro AM, Faivre S, Raymond E: Lessons learned in the development of targeted therapy for malignant gliomas. Mol Cancer Ther, 2007, 6, 1909–1919.
26. Perez-Vizcaino F, Duarte J, Jimenez R, Santos-Buelga C, Osuna A: Antihypertensiv effects of the flavonoid quercetin. Pharmacol Rep, 2009, 61, 67–75.
27. Ramos S: Effects of dietary flavonoids on apoptotic pathways related to cancer chemoprevention. J Nutr Biochem, 2007, 18, 427–442.
28. Raza SM, Lang FF, Aggarwal BB, Fuller GN, Wildrick DM, Sawaya R: Necrosis and glioblastoma: A friend or a foe? Neurosurgery, 2002, 51, 2–13.
29. Roos WP, Batista LF, Naumann SC, Wick W, Weller M, Menck CF, Kaina B: Apoptosis in malignant gliomas cells triggered by temozolomide-induced DNA lesion O^6-methylguanine. Oncogene, 2007, 26, 186–197.
30. Rosso L, Brock CS, Gallo JM, Saleem A, Price PM, Turkheimer FE, Aboagye EO: A new model for prediction of drug distribution in tumor and normal tissues: pharmacokinetinetics of temozolomide in glioma patients. Cancer Res, 2009, 69, 120–127.
31. Russo GL: Ins and outs of dietary phytochemicals in cancer chemoprevention. Biochem Pharmacol, 2007, 22, 317–320.
32. Sőti C, Sreedhar AS, Csermely P: Apoptosis, necrosis and cellular senescence: chaperone occupancy as a potential switch. Aging Cell, 2003, 2, 39–45.
33. Spencer JP, Kuhnle GG, Williams RJ, Rice-Evans C: Intracellular metabolism and bioactivity of quercetin and its in vivo metabolites. Biochem J, 2003, 372, 173–181.
34. Spencer JPE, Rice-Evans C, Williams RJ: Modulation of pro-survival Akt/Protein Kinase B and ERK1/2 signaling cascades by quercetin and its in vivo metabolites undrline thair action on neuronal viability. J Biol Chem, 2003, 278, 34783–34793.
35. Sreedhar AS, Csermley P: Heat shock proteins in the regulation of apoptosis: new strategies in tumor therapy. A comprehensive review. Pharm Ther, 2004, 101, 227–257.
36. Stegh AH, Kesari S, Mahoney JE, Jenq HT, Forloney KL, Protopopov A, Louis DN et al.: Bcl2L12-mediated

inhibition of effector caspase-3 and caspase-7 via distinct mechanisms in glioblastoma. Proc Natl Acad Sci USA, 2008, 105, 10703–10708.
37. Takeuchi H, Kondo Y, Fujiwara K, Kanzawa T, Aoki H, Mills GB, Kondo S: Synergistic augmentation of rapamycin-induced autophagy in malignant glioma cells by phosphatidylinositol 3-kinase/protein kinase B inhibitors. Cancer Res, 2005, 65, 3336–3346.
38. Tentori L, Graziani G: Recent approaches to improve the antitumor efficacy of temozolomide. Curr Med Chem, 2009, 16, 254–257.
39. Thangasamy T, Sittadjody S, Mitchell GC, Mendoza EE, Radhakrishnan VM, Limesand KH, Burd R: Quercetin abrogates chemoresistance in melanoma cells by modulating deltaNp73. BMC Cancer, 2010, 10, 282.
40. Uzzman M, Keller G, Germano IM: Enhanced proapoptotic effects of tumor necrosis factor-related apoptosis-inducing ligand on temozolomide-resistant glioma cells. J Neurosurg, 2007, 106, 646–651.
41. Vafeiadou K, Vauzour D, Rodriguez-Mateos A, Whiteman M, Williams RJ, Spencer JPE: Glial metabolism of quercetin reduces its neurotoxic potential. Arch Biochem Biophys, 2008, 478, 195–200.
42. Vasconsuelo A, Milanesi L, Boland R: Participation of Hsp27 in the antiapoptotic action of 17 -estradiol in skeletal muscle cells. Cell Stress Chaperones, 2010, 15, 183–192.
43. Voellmy R, Boellmann F: Chaperone regulation of the heat shock protein response. Adv Exp Med Biol, 2007, 594, 89–99.
44. Youdim KA, Qaiser MZ, Begley DJ, Rice-Evans CA, Abbott NJ: Flavonoid permeability across an in situ model of the blood-brain barrier. Free Radic Biol Med, 2004, 36, 592–604.

Received: February 13, 2010; **in the revised form:** October 8, 2010; **accepted:** October 13, 2010.