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The effect of quercetin and imperatorin on programmed cell death induction in T98G cells *in vitro*

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ABSTRACT

Background: High expression of HSP27 and HSP72 in glioma cells has been closely associated with chemoresistance and decreased sensitivity to programmed cell death induction. Therefore, it is important to devise therapies that effectively target invasive cancer cells by inducing cell death. The aim of our study was to assess the effect of quercetin and imperatorin applied separately and in combinations on the apoptosis and autophagy induction in human T98G cells cultured *in vitro*.
Methods: Cell death induction was analyzed by the staining method. The Western blotting technique and fluorimetric measurements of activity were used to assess the expression of marker proteins of apoptosis and autophagy. The specific siRNA transfected method was used for blocking of the expression of HSP27 and HSP72 genes.
Results: The experiments revealed the highest percentage of apoptotic cells after using a 50 μM concentration of both compounds. Simultaneous quercetin and imperatorin administration induced apoptosis more effectively than incubation with single drugs. These results were accompanied with decreased HSP27 and HSP72 expression, and a high level of caspase-3 and capsae-9 activity. Autophagy was not observed. Additional experiments were performed on a cell line with blocked Hsp27 and Hsp72 expression and significant increase the sensitivity to apoptosis induction upon quercetin and imperatorin treatment was noticed.
Conclusions: The present study indicates that quercetin and imperatorin are potent apoptosis inducers, especially when they act synergistically, which may be a promising combination useful in glioma therapy. Our results also demonstrated that blocking the HSP27 and HSP72 gene expression might serve as a therapeutic target for the human brain cancer.

Introduction

Glioblastoma multiforme (GBM), classified by WHO as a IV grade tumor, represents the most common primary malignant brain tumor. Despite the available multimodality treatment, no cure or effective therapy for it has been developed to date. In population-based studies, most patients diagnosed with GBM survive less than a year despite intensive treatment including surgical resection, radiation, and chemotherapy. Malignant gliomas are characterized by diffusive, infiltrating invasion into normal brain tissues, which makes complete resection impossible [13,22,40,44]. Like most cancers, human brain tumors also exhibit an unusual level of HSP27 and HSP72, which is implicated in cell proliferation, differentiation, and a poor therapeutic outcome. Furthermore, numerous studies demonstrated that the high level of these proteins strongly correlates with histologic grades [10,18]. Therefore, targeting the expression of HSPs can be suggested as an effective anticancer therapy based on sensitizing cancer cells to cell death induction by reducing the level of these proteins.

In the past years, the strategy for killing cancer cells through programmed cell death has been extensively studied. Apoptosis, also called type I programmed cell death, is a physiological process leading to elimination of unwanted damaged, infected, or mutated cells. The apoptotic signal may extend by two pathways, intrinsic (mitochondrial) related to cytochrome *c* release into the cytosol

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and extrinsic associated with activation of death receptors. However, regardless of the type of apoptosis, both pathways lead to activation of caspases [20,38]. Autophagy, *i.e.* type II programmed cell death, is a phylogenetically old process used as a tool not only for death, but also for survival. The essence of autophagy is simple and it is defined as an intracellular system of degradation of cytoplasm components in particular long half-life proteins through lysosomal enzymes. The final outcome of autophagy is always the same – total and irreversible dismantling of macromolecular substrates to their basic components [27,37].

Triggering apoptosis in cancer cells is the principal mechanism by which most anticancer agents act and failure to elicit it not only leads to progression of tumors but also increases their resistance to treatment. One of the element which plays a key role in protecting the cell against spontaneous apoptosis is overexpression of heat shock proteins (HSP) occurring in a wide range of human cancers [7,19].

HSPs, well-known molecular chaperones, are the oldest cell protecting system. Their expression is induced in response to a variety of environmental and physiological stress conditions, which correlates with cell survival. HSPs are involved in assistance with native protein folding, maintenance the proper conformation of multiprotein complexes, and degradation of senescent proteins. One of the best-studied proteins are HSP27 and HSP72, the most strongly and universally synthesized chaperones, whose overexpression has been observed in many types of cancer. HSP27 and HSP72 inhibit key effectors of the apoptotic machinery; therefore, it is believed that they have a role in tumorigenesis [5,7,11,31,42].

One of the best known HSPs inhibitors is quercetin (3,3′,4′,5-7-pentahydroxyflavone). This is one of the best-described flavonoid present in daily diet. Like many compounds of this group, it has strong antioxidant, anti-inflammatory, and antiproliferative properties. In recent years, quercetin has attracted special attention as a potential anticancer agent inducing apoptosis in numerous types of cancer [9,32]. The mechanism of this reaction is correlated with inhibition of DNA topoisomerase I/II, modulation of signaling pathways, release of cytochrome *c*, and activation of caspases [15,29,41].

Imperatorin (8-isopentenyloxypsoralen), a major active furanocoumarin isolated from the root of *Angelica officinalis*, has been reported to possess a wide range of biological activities including analgesic, antiinflamatory, anticoagulant, and photosensitizing properties. Recent reports have shown pharmacological actions of imperatorin against cancer including oncogene suppression, inhibition of proliferation, arresting cell cycle in the G1/S phase, and induction of apoptosis in various cancers [1,26,34,36]. Moreover, literature data indicate that this furanocoumarin induces both the external and internal pathway of apoptosis. Microscopic observations showed typical apoptotic changes like cell shrinkage, nuclear fragmentation, or membrane blebbing [1,21].

Therefore, the aim of the present study was to assess the effect of quercetin and imperatorin administered separately or in combinations on programmed cell death induction (apoptosis and autophagy) in the human glioma T98G cell line cultured *in vitro*. In order to determine the pathways by which apoptosis and autophagy take place in T98G cells, we investigated the expression of marker proteins involved in these processes at the molecular level. We also examined the potential of HSP27 and HSP72 expression in protecting T98G cells from cell death induction.

Materials and methods

Cells and culture conditions

The human brain glioblastoma multiforme cells T98G (European Collection of Cell Cultures, No 92090213) were grown in a 1:1 mixture of DMEM and Nutrient mixture F-12 Ham (Ham's F-12, Sigma) supplemented with 10% FBS (Life Technologies, Karlsruhe, Germany), penicillin (100 μg/ml, Sigma), and streptomycin (100 μg/ml, Sigma). The cultures were kept at 37 °C in humidified atmosphere of 95% air and 5% CO_2.

Imperatorin isolation

Imperatorin was isolated from the fruits of *Angelica officinalis* in the Department of Pharmacognosy, Medical University of Lublin, Poland. The air-dried and powdered fruits of *A. officinalis* were extracted with petroleum ether exhaustively in the Soxhlet apparatus, which yielded a fraction of furanocoumarins obtained as semi-crystalline sediment from the concentrated extract. Then the imperatorin-rich sediment obtained from the fruits of *A. officinalis* was first dissolved in hot dichloromethane and then subjected to crystallization with cold *n*-hexane. The sediment formed was recrystallized three times in methanol, which led to isolation of imperatorin. The identity and purity of imperatorin were confirmed by HPLC and H NMR analyses [35].

Drug treatment

Quercetin (Sigma) at the final concentrations 25, 50, and 100 μM and imperatorin (obtained from *A. officinalis*) at the final concentrations 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, T98G cells were incubated only with quercetin or only with imperatorin for 24 and 48 h. In the second, quercetin and imperatorin were added to the culture medium at the same time and incubated for 24 and 48 h. In the third variant, the cells were preincubated with quercetin or imperatorin for 6 or 24 h followed by administration of the other compound and incubated for the next 18 or 24 h. Control 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 [16]. The staining mixture was added in a volume of 2.5 μl/ml to the medium in which the cells were grown and incubated for 5 min at 37 °C in the dark. Apoptotic and necrotic cells were visualized and scored (at least 1000 cells from randomly selected fields) under a fluorescent microscope (Nikon E800). Cells undergoing apoptosis demonstrated blue fluorescent nuclei (intact or fragmented). Cells exhibiting necrosis pink fluorescent nuclei were interpreted as necrotic. Three independent experiments were performed.

Detection of autophagy with acridine orange

Autophagy is a process that involves sequestration and delivery of cytostolic components to the lysosome for degradation; it is characterized by formation and promotion of acidic vesicular organelles (AVOs). Vital staining with acridine orange was performed for identification of AVOs in T98G cells treated with quercetin and/or imperatorin [39]. The cells were incubated with the fluorochrome at a final concentration 1 μg/ml for 15 min in the dark. Autophagic positive cells demonstrated typical granular discretion of AVOs in the cytoplasm. Morphological analysis was performed under a fluorescent microscope (Nikon E800). Autophagic cells were visualized and counted (at least 1000 cells from

randomly selected fields). Three independent experiments were performed. The percentage of autophagic cells was calculated as the number of cells with AVOs *versus* the total number of stained cells. Each experiment was performed in triplicate.

TUNEL staining

T98G cells were cultured in 8-well chamber slides (Lab-Tek) at density of 2×10^5 cells/ml. After fixation with 4% Paraformaldehyde in PBS, the cancer cells were permeabilized for 2 min in 0.1% Triton X-100 in 0.1% sodium citrate, washed in PBS and treated to identify TUNEL-positive cells according to the manufacturer's protocol of the *In Situ* Cell death Detection Kit, Fluorescein (Roche Diagnostics, Switzerland). The percentage of TUNEL-positive cells relative to total cells was calculated for each sample (at least 1000 cells from randomly selected fields) under a fluorescent microscope (Nikon E800). Three independent experiments were performed.

Immunoblotting

After quercetin and/or imperatorin treatment, the T98G 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, centrifuged at 10,000 × *g* at 4 °C for 10 min, and the supernatants were collected. The Bradford method was used to determine the concentration of protein in the cell-free extracts obtained [3]. Samples of supernatants containing 80 μg of proteins were separated by 10% SDS-polyacrylamide gel electrophoresis [24], and subsequently transferred onto the Immobilon P membrane (Millipore). Following the transfer, non-specific binding sites on the membrane were blocked with 3% low fat milk in PBS for 1 h and incubated overnight with rabbit polyclonal anti-beclin-1 antibody (Sigma) diluted 1:1000, goat anti-HSP27 monoclonal antibody (Santa Cruz Biotechnology) diluted 1:1000, and anti-HSP72 (Santa Cruz Biotechnology) diluted 1:1000. After the incubation, 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 anti-rabbit IgG or anti-goat IgG (Sigma). The membranes were visualized by the colorimetric reaction with alkaline phosphatase substrate (5-bromo-4-chloro-3-indolylphosphate and nitro-blue tetrazolium, Sigma) in a color development buffer (DMF, Sigma). Quantitative evaluation of the expression of heat shock proteins with molecular weights of 27 kD and 72 kD and beclin-1 was determined using the Bio-Profil Bio-1D Windows Application V.99.03 program. Three independent experiments were performed.

T98G cell transfection

In order to block the expression of HSP27 and HSP72 genes, the specific siRNA (Santa Cruz Biotechnology) transfection method was used. The blocking procedure was performed in 6-well tissue culture plates at cell density of 2×10^5. Prior to transfection, a mixture containing specific siRNA and Transfection Reagent was prepared and left at room temperature for 45 min. During this time, the medium was removed from the above-mentioned the cells and the cells were washed once with 2 ml of the Transfection Medium containing no serum or antibiotics. Next, the previously prepared transfection mixture was added to the cell culture and incubated for 5 h at 37 °C. After this time, 1 ml of growth medium containing serum and antibiotics at a 2-fold higher concentration than normal was added without removing the transfection mixture and incubated for the next 24 h at 37 °C. After the incubation, the whole mixture was replaced with fresh normal growth medium and quercetin and imperatorin were added to the cells at appropriate concentrations for 24 h. The effectiveness of the blocking of HSP27 and HSP72 genes expression was assessed by immunoblotting. Three independent experiments were performed.

Detection of caspase activity

T98G cells were cultured in 6-well plates at the density 2×10^5 cells/well and treated with quercetin (50 μM) and imperatorin (50 μM) administered separately or simultaneously. For detection of activity of caspase-3, -8, and -9, a commercially available assay kit SensoLyte® AMC Caspase Substrate Sampler Kit Fluorimetric (AnaSpec) was used according to the manufacturer's protocol. The intensity of fluorescence was measured by a Perkin Elmer 2030 Multilabel Reader VICTOR™ x4 at Ex/Em = 354 nm/442 nm. Each experiment was performed in triplicate.

Statistical analysis

The quantitative data were expressed as the mean ± standard deviation (SD). All statistical analyses of the data were performed using one-way ANOVA tests followed by a Dunett's multiple comparison test. Differences with p-values of <0.05 were considered statistically significant.

Results

The effect of quercetin and imperatorin on cell death induction

Quercetin

The T98G cells were incubated with quercetin at the concentrations 25, 50 and 100 μM for 24 and 48 h. As shown in Fig. 1A, the 48-h incubation of glioblastoma cells with the flavonoid resulted in effective apoptosis induction, and the highest percentage of dead cells (25.3%) was observed after the treatment with 100 μM of the flavonoid. Unfortunately, this was accompanied by significant necrosis induction (8.9%). The lower concentrations of quercetin, 25 and 50 μM, were less effective but the level of apoptotic cells was still higher than that observed in the control. Necrosis after the incubation with 25 and 50 μM was similar to that in the control cells, thus 50 μM of quercetin, resulting in 23.2% of dead cells, was chosen for further investigation. The shorter treatment with quercetin was much less efficient. Quercetin at all the tested concentrations both after 24- and 48-h incubations did not result in autophagy induction (Fig. 1A).

The sensitivity of T98G cells to apoptosis induction upon quercetin treatment were further verified by TUNEL method based on the use of fluorescently or enzimaticaly labeled dUTP fragments which, in the presence of DNA fragments with a free 3′-OH group generated during apoptosis, bind to them and are detected using a fluorescence microscope, flow cytometer or immunohistochemical methods. Conducted experiments confirmed that 48-h incubations resulted in stronger apoptosis induction in comparison to the 24-h incubations (Fig. 2A). Furthermore the 50 μM concentration of flavonoid appeared to be the most effective apoptosis initiator, generating 17.96% of dead cells. Treatment with the other concentrations tested revealed a slightly weaker but statistically significant percentage of apoptotic cells.

Imperatorin

In contrast to quercetin, imperatorin was more effective to induce apoptosis in T98G cells after the 24-h incubations (Fig. 1B). Starting from 25 μM, an increasing number of apoptotic cells was observed, reaching a maximum (26.76%) after the treatment with 100 μM. However, this concentration also generated the strongest cytotoxic effect (7.57%). The lower concentration also effectively

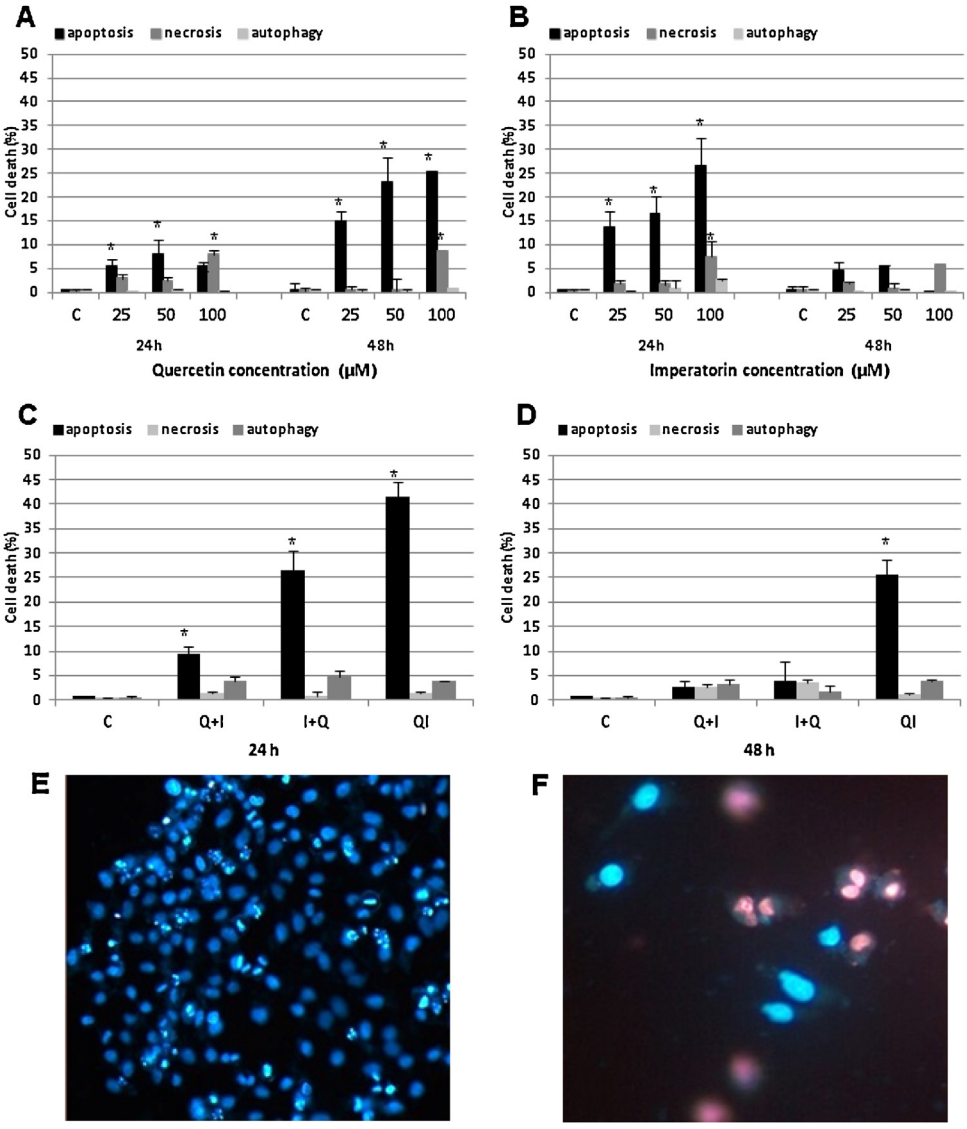

Fig. 1. The effect of quercetin (A), imperatorin (B) and combined treatment with both drugs (C–D) on apoptosis, necrosis and autophagy induction in the T98G cell line. The cells were incubated with the drugs for 24 and 48 h; *C* – control; *Q* – quercetin; *I* – imperatorin; *Q* + *I* – cells pre-incubated with quercetin followed by imperatorin administartion; *I* + *Q* – cells pre-incubated with imperatorin followed by quercetin administration; *QI* – cells treated with both drugs administered simultaneously; $^{*}p < 0.05$. Apoptotic (E; magnification 200×) and necrotic (F; magnification 400×) cells after 24 h incubation with 50 μM of quercetin.

induced apoptosis reaching 13.61% after incubation with 25 μM and 16.61% after incubation with 50 μM, respectively. The percentage of necrotic cells did not exceed 2%. Therefore, the 50 μM concentration of the compound was used for further experiments. Extending the incubation to 48 h resulted in a strong cytotoxic effect caused by imperatorin. The cell culture was extremely sparse and a high percentage of detached cells were observed. The treatment with imperatorin as well as quercetin had no a significant effect on autophagy induction in T98G cells; the percentage of dead cells were comparable to that in the control.

Similar results were achieved by using TUNEL assay. As can be seen in Fig. 2B the 24-h incubations indeed displayed an effective apoptosis initiation and the highest percentage of dead cells was observed after treatment with 50 μM of imperatorin (19.67%). TUNEL measurements, likewise morphological analysis allowed us to observe a remarkably lower number of cells undergoing apoptosis after 48-h incubations, and the level of dead cells did not exceed 8%.

Quercetin plus imperatorin

As mentioned before, quercetin at the concentration 50 μM and imperatorin at the concentration 50 μM were the most effective in apoptosis induction, and therefore they were used for further investigations. To evaluate the combined effect of the tested compounds on apoptosis and autophagy induction in T98G cells, two variants of the experiments were performed: (1) the cells were pre-incubated with quercetin or imperatorin for 6 or 24 h followed by administration of the other drug for the next 18 or 24 h, and (2) the cells were treated with both drugs simultaneously for 24 and 48 h. As shown in Fig. 1C–D, apoptosis induction was dependent on time and the drug application procedure. We noticed that the 24-h incubation proved to be more effective in apoptosis induction reaching maximum 41.45% of dead cells after incubation with quercetin and imperatorin administered simultaneously, and the level of necrotic cells did not exceed 1.3% (Fig. 1C). Induction of apoptosis in cells pretreated with quercetin or imperatorin was less effective. As can be seen in Fig. 2B, the 48-h incubation resulted in weaker induction of apoptosis and reached 25% of dead cells

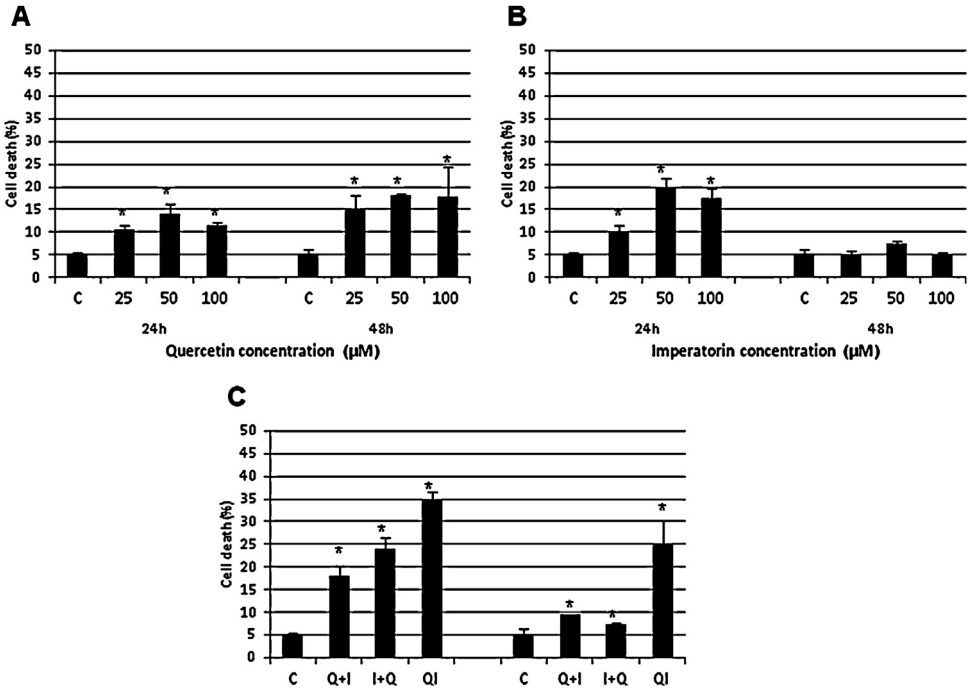

Fig. 2. The effect of quercetin (A), imperatorin (B) and combined treatment with both drugs (C) on apoptosis induction in the T98G cell line, indentified by TUNEL method. *C* – control; *Q* – quercetin; *I* – imperatorin; *Q* + *I* – cells pre-incubated with quercetin followed by imperatorin administartion; *I* + *Q* – cells pre-incubated with imperatorin followed by quercetin administration; *QI* – cells treated with both drugs administered simultaneously; $^{*}p < 0.05$.

after the treatment with quercetin and imperatorin applied at the same time. The other combinations had no significant effect on apoptosis induction. The combined drug treatment in all the variants had no impact on autophagy induction.

TUNEL assay confirmed previously obtained results. The 24-h incubations provoked stronger apoptosis initiation, resulting in maximum 34.57% of dead cells after simultaneous administration of quercetin and imperatorin (Fig. 2C). In turn, the pre-treatment experimental variants generated lower percentage of apoptotic cells, but still higher than that observed in the control. As shown in Fig. 2 C in the case of 48-h incubations statistically significant induction of apoptosis was noticed only after simultaneous quercetin and imperatorin treatment (24.93%). The other variants of the 48-h incubations initiated apoptosis at levels comparable to the control cells.

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

Apoptosis and autophagy are controlled at the molecular level by changes in the expression of marker proteins that either mediate or suppress the process of cell death. In present study, we examined the effect of quercetin and imperatorin on the activation of pro-apoptotic caspase-3, caspase-8, caspase-9, expression of anti-apoptotic heat shock proteins HSP27, HSP72, and pro-autophagic beclin-1.

The activation of caspase-3, caspase-8, and caspase-9

The best-studied mediators of apoptosis include caspases, *i.e.* aspartate-specific cysteine proteases, which function as ultimate effectors during apoptosis.

Cell staining revealed that quercetin and imperatorin induced apoptosis in the T98G cells most effectively at the 50 μM concentration. These results were closely associated with the increasing level of activity of caspases. As can be seen in Fig. 3A, our experiments showed that T98G cells treated with quercetin and imperatorin administered simultaneously for 24 h exhibited the highest increase in caspase-3 and caspase-9 activity. Lower levels of the activity of caspase-3 and caspase-9 after incubation with drugs administered separately were noticed. No increase of caspase-8 activity was observed.

The expression of beclin-1

Beclin-1, belonging to the Bcl-2 family, plays a central role in autophagy. This protein recruits lot of key autophagic proteins required for proper initiation of autophagosome formation. Morphological analysis revealed that neither quercetin nor imperatorin have the ability to induce autophagy in T98G cells (Fig. 3B). Verification of these results at the molecular level resulted in no significant changes of beclin-1 expression after quercetin and/or imperatorin treatment.

The expression of HSP27 and HSP72 proteins

Western blot analysis showed that imperatorin and quercetin administered simultaneously appeared to be effective inhibitors of HSP27 expression (Fig. 3 C) in T98G cells. The inhibitory effect after the treatment with the drugs applied separately was slightly weaker. Similar results were found in HSP72 expression (Fig. 3D). Treatment of the T98G cells with quercetin and imperatorin administered at the same time resulted in the strongest inhibition of HSP72 expression. In turn, after incubation with single drugs, the inhibitory effects were less effective.

The effect of quercetin and imperatorin on cell death induction in transfected T98G cells

To verify the relationship between HSP27 and HSP72 over-expression in cancer cells and their protective role against

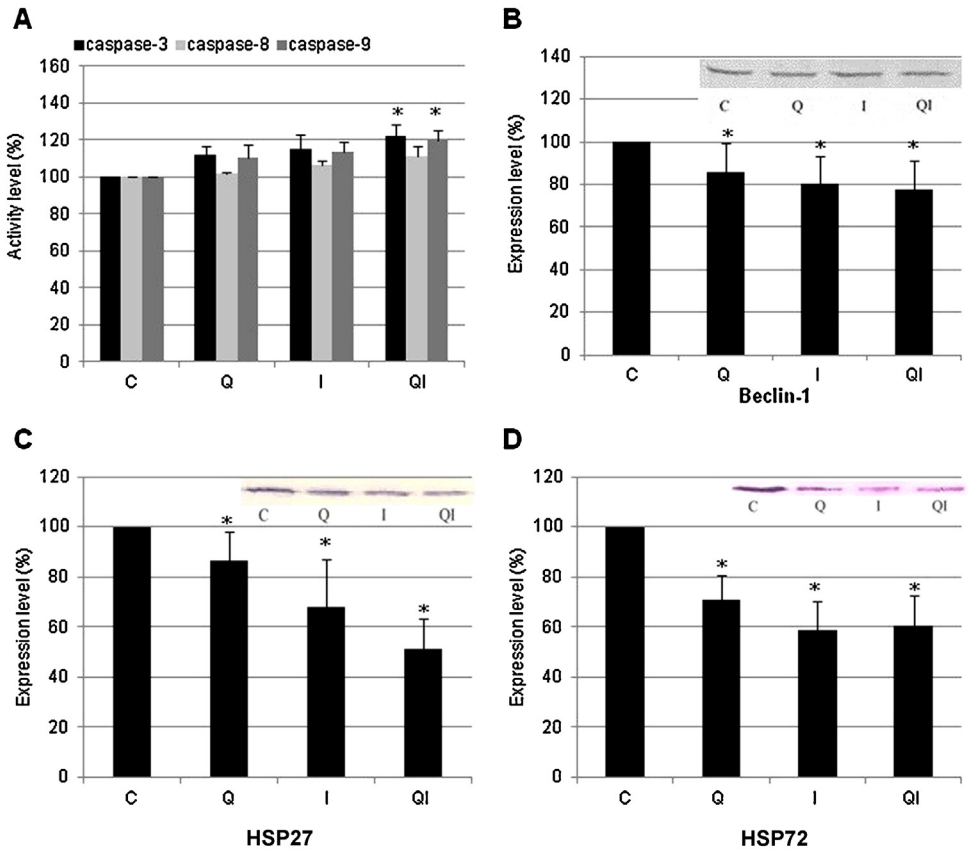

Fig. 3. The effect of quercetin and imperatorin administered separately or simultaneously for 24 h on the activation of caspases (A), expression of beclin-1 (B), HSP27 (C), HSP72 (D). *C* – control, *Q* – quercetin, *I* – imperatorin, *QI* – drugs applicated at the same time. Representative western blots are included; $^{*}p < 0.05$.

apoptosis, the cells were first stably transfected with specific siRNA constructs according to the manufacturer's instruction.

Apoptosis and necrosis induction upon quercetin and imperatorin treatment in transfected T98G cells

To determine whether the blocking of HSP27 and HSP72 gene expression is associated with cell death induction, the transfected cells were exposed to quercetin and/or imperatorin treatment. Our investigations demonstrated that the silencing of HSP27 and HSP72 gene expression was strictly correlated with increased sensitivity of T98G cells to apoptosis induction upon quercetin and imperatorin. Morphological analysis demonstrated that the simultaneous application of quercetin and imperatorin resulted in the highest percentage of dead cells (25.25%) after down-regulation of HSP27 expression (Fig. 4A), and 22.93% after downregulation of HSP72 expression (Fig. 4B). The incubation with single drugs revealed less effective apoptosis induction, however much higher than the one observed in the control. No significant effects were observed in necrosis induction. Furthermore, blocking of the HSP27 and HSP72 gene expression had no impact on autophagy induction in the T98G cells.

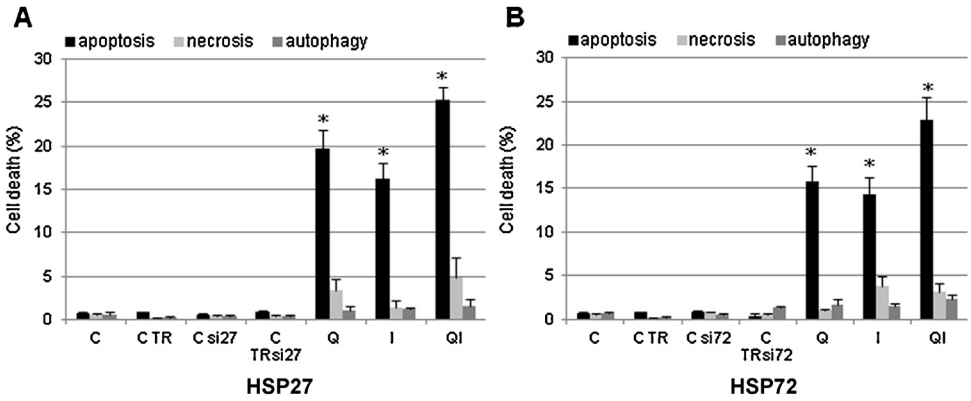

Fig. 4. The level of apoptosis, necrosis and autophagy induction in T98G cells after blocking the HSP27 (A) and HSP72 (B) expression subsequently treated with quercetin and imperatorin for 24 h. *C* – control, TR – transfection reagent introductoring siRNA into cells, TRsi – complex of transfection reagent and siRNA, *Q* – quercetin, *I* – imperatorin, *QI* – drugs administered simultaneously; $^{*}p < 0.05$.

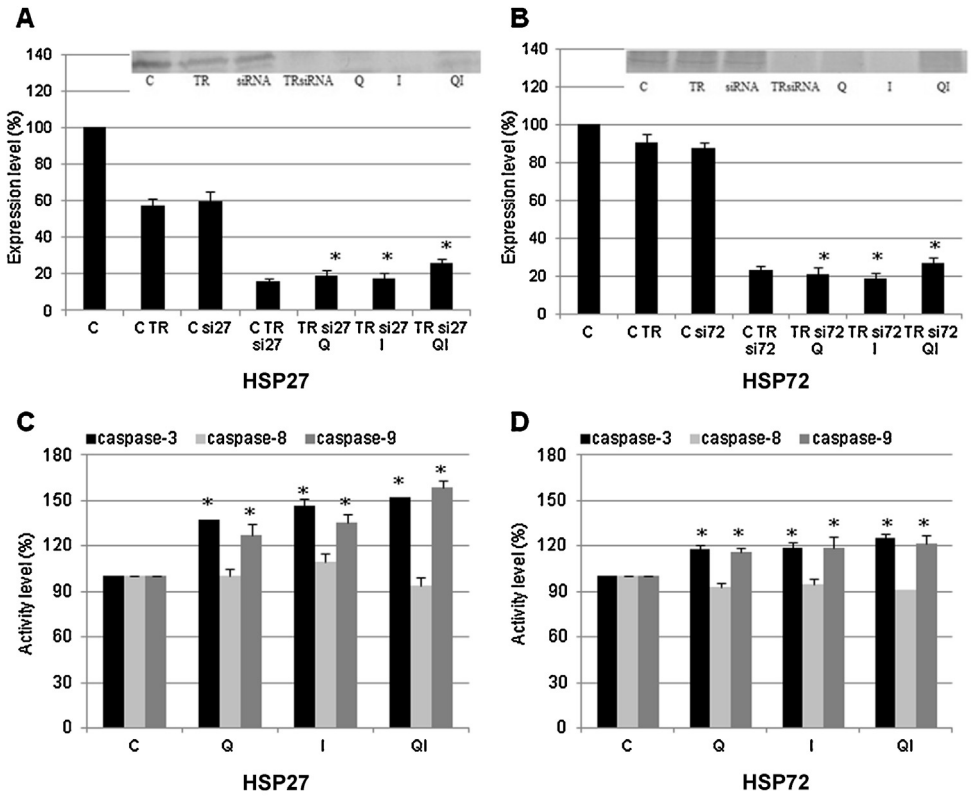

Fig. 5. The expression of Hsp27 (A), Hsp72 (B) in transfected T98G and the level of activity of caspase-3, caspase-8 and capase-9 after blocking HSP27 (C) and HSP72 (D) expression in cells subsequently treated with quercetin and imperatorin for 24 h. *C* – control, TR – transfection reagent introductoring siRNA into cells, TRsi – complex of transfection reagent and siRNA, *Q* – quercetin, *I* – imperatorin, *QI* – drugs administered simultaneously. Representative western blots are included; $^{*}p < 0.05$.

The expression of HSP27 and HSP72 proteins in transfected T98G cells

The efficiency of blocking the expression of HSP27 and HSP72 genes was further investigated by Western blotting analysis. The strongest inhibitory effect of HSP27 expression was observed after incubation with quercetin and imperatorin administered separately. The combined treatment of the tested drugs resulted in less effective inhibition of HSP27 expression (Fig. 5A). Similar effects were observed in the changes in the expression of HSP72 (Fig. 5B). The strongest inhibition of the HSP72 level was achieved in the treatment with quercetin and imperatorin applied separately. The incubation with the drugs administered simultaneously inhibited the expression of HSP72 slightly more weakly.

The activation of caspase-3, caspase-8, and caspase-9 in transfected T98G cells

In order to understand whether silencing of the HSP27 and HSP72 expression interferes with the activation of caspases during apoptosis, the processing of procaspase-3, -8 and -9 was determined. Our experiments revealed that incubation with quercetin and/or imperatorin caused a stronger increase in the activity of caspases in cells with silenced HSP27 expression than in cells with downregulated HSP72 expression. The treatment with quercetin and imperatorin administered simultaneously resulted in maximum activity of caspase-3 and caspase-9 in cells with blocked HSP27 expression (Fig. 5C), as well as in cells with a reduced level of HSP72 (Fig. 5D). The incubations with single drugs were less effective in activation of caspases. Activation of caspase-8 was unaffected.

Discussion

Among various types of human cancer, GBM is accepted as the leading cause of cancer mortality worldwide and most deaths are associated with cancer metastasis. Like most cancers, it is characterized by hypoxia, pH imbalances, altered metabolism, and relatively undamaged DNA synthesis checkpoints leading to heavy mutation load. Additionally, the human glioma cancer often overexpresses HSPs, probably because of the stressful atmosphere in which tumors reside, but also because of the benefits of HSPs cytoprotection [10,46]. Numerous types of heat shock proteins are involved in the development of human glioma cancer, among which HSP27 and HSP72 have attracted a great deal of attention as apoptotic chaperone proteins [12,23,46]. It has been reported that HSP27 and HSP72 regulate apoptosis through their ability to interact with the key components of the apoptotic-signaling pathways, particularly those involved in activation of caspases. However, the mechanism by which this is achieved still remains unclear. Nevertheless, it is known that overexpression HSP27 and HSP72 in tumor cells negatively regulates apoptosis by preventing recruitment of procaspase-9 and procaspase-3 and sequestration of cytostolic cytochrome *c* from Apaf-1 after its release from the mitochondrion, thereby preventing the assembly of the functional apoptosome [2,30,31,38].

Quercetin has emerged as a potential therapeutic agent for treatment of various types of cancers owing to its ability to induce apoptosis. Several articles have suggested mechanisms implicated in the proapoptotic properties of this compound involving activation of kinases, downregulation of survival pathways, ROS production, mitochondrial potential perturbation, release of cytochrome *c*, and, in consequence, activation of caspase-3 and caspase-9 [4,8,9,20,28,41]. A body of literature also describes

quercetin as an effective inhibitor of HSPs synthesis. It has been reported that it blocks HSP27 and HSP72 expression at the transcriptional level by preventing the binding of heat shock factors 1 and 2 (HSF1, HSF2) to the conserved DNA sequence known as the heat shock element (HSE). Another theory suggests that quercetin blocks additional modifications needed to activate HSF, such as post-translational phosphorylation, or causes changes in the conformation of the factor and inhibiting its interaction with other proteins binding to DNA [15]. Additionally Yang et al. demonstrated that also liposomal form of quercetin combined with radiofrequency ablation occurred to be an effective suppressor of HSPs expression, thereby contributing to increase apoptosis [45].

The morphological analysis performed indicated that quercetin was an effective apoptosis inducer in T98G cells at the 50 μM concentration after 24 h of treatment yielding the highest percentage of dead cells (8.25%). Conducted TUNEL analysis additionally confirmed that 50 μM of quercetin is a potent apoptosis initiator. At the molecular level, the apoptosis induction in the T98G cells was closely correlated with the expression of HSP27 and HSP72 proteins, well-known apoptosis inhibitors. Our experiments demonstrated a strong inhibitory effect of the flavonoid on both HSP27 and HSP72 expression in the cells studied, resulting in sensitizing T98G cells to apoptosis induction, which corresponded with an increase in the activation of caspase-3 as well as caspase-9. Based on these results, it can be hypothesized that quercetin induces mitochondrial-dependent apoptosis in T98G cells.

Several studies have reported that quercetin has the ability to trigger autophagy in cancer cells [37,43]. Paradoxically, our morphological analysis revealed that the flavonoid was a weak autophagy inducer in the T98G cells. These results were further verified at the molecular level by measurement of the beclin-1 level, which showed no significant changes in its expression. Thus, quercetin has no ability to induce autophagy in T98G cells.

An increasing number of reports have described imperatorin as a compound with anticancer properties manifested in induction of apoptosis in several cancer cell lines, based on both death receptor and mitochondria mediated pathways [1,21,25,29]. However, the underlying mechanism of this proapoptotic activity is scarcely studied. Our experiments demonstrated that imperatorin proved to be an effective apoptosis inducer for the first time in T98G cells, resulting in maximum 16.61% of dead cells after the treatment with 50 μM for 24 h. The validity of results mentioned above were corroborated by TUNEL analysis, which clearly indicate on pro-apoptotic activity of imperatorin on T98G cell line. At the molecular level, this was accompanied with efficient inhibition of the expression of both HSP27 and HSP72, which made the cells more sensitive to apoptosis induction. Indeed, we observed distinct increasing activity of caspase-3 and caspase-9. Based on these results it can be hypothesized that imperatorin induces apoptosis in T98G cells *via* the mitochondrial pathway. Furthermore, although currently there is very little understanding about a possible mechanism of HSPs inhibition by imperatorin, our experiments demonstrated imperatorin as a possible suppressor of HSPs expression. On the other hand, imperatorin turned out not to be a potent autophagy inducer in T98G cells. Microscopic analysis as well as measurement of the beclin-1 expression gave no support to challenge this hypothesis.

Choochuay et al. [6] observed that imperatorin stimulates the molecular mechanisms leading to atypical form of apoptosis that is anoikis. This type of cell death, characteristic for adherent cells, is a mechanism essential for maintaining the proper position of cells within tissues. Anoikis induction occurs when cell lose attachment to extracellular matrix (ECM), or adhere to an inappropriate ECM [6]. In our experiments two independent methods used for apoptosis identification demonstrated much less amount of apoptotic cells, which may be correlated with anoikis induction and detaching cells from substrate.

Many studies have demonstrated that natural plant extracts and natural compounds exhibit a synergistic anti-tumor effect [14]. Herein we decided to evaluate, for the first time, the effect of the combined treatment of quercetin and imperatorin on cell death induction in T98G cells. We measured apoptosis and autophagy in cells pretreated with quercetin or imperatorin followed by imperatorin or quercetin administration and then compared to the effect on cell death induced by quercetin and imperatorin applied simultaneously. Two independent methods of apoptosis detection revealed the combination of both drugs given at the same time was the most effective in apoptosis induction, reaching 41.45% of dead cells as a result of morphological analysis, and 34.57% as a result of TUNEL assays, respectively. These results were consistent with the strongest inhibition of HSP27 and HSP72 expression and the highest activation of caspase-3 and caspase-9. Neither drug at all the combination variants had a significant effect on autophagy induction.

Various studies have reported that the unusual expression of HSP27 and HSP72 is crucial for progression of different types of human cancer and is closely associated with resistance to apoptosis by enhancement of cell growth. Indirect experimental evidence suggests that downregulation of HSPs expression in cancer cells may either sensitize them to chemotherapy or commit to apoptosis [5,17,33,46,47]. Our earlier experiments demonstrated that both quercetin and imperatorin inhibited the expression of HSP27 and HSP72. Thus, to obtain direct evidence of the participation of HSP27 and HSP72 in resistance of T98G cells to apoptosis, silencing of the HSP27 and HSP72 gene expression by specific siRNA was performed. Our experiments showed that downregulation of the HSP27 and HSP72 expression greatly increased the sensitivity of T98G cells on apoptosis induction by quercetin and imperatorin. The combined drug treatment proved to be more effective in apoptosis induction in transfected cells than the single agents were. At the molecular level, these results corresponded with strongly reduced HSP27 and HSP72 expression, and a much higher level of caspase-3 and caspase-9 activity. These results suggest that quercetin and imperatorin induce apoptosis in transfected cells *via* the mitochondrial pathway. The intriguing blocking of HSP27 and HSP72 gene expression still had no effect to autophagy induction upon quercetin and imperatorin treatment.

In conclusion, in the present study we provided for the first time the evidence supporting the potential of quercetin and imperatorin as effective apoptosis inducers in T98G cells. For the first time, the combined treatment with quercetin and imperatorin administered simultaneously induced apoptosis more effectively than the incubation with single drugs. Furthermore, we reported herein that silencing of HSP27 and HSP72 gene expression remarkably sensitizes human glioblastoma multiforme cells to apoptosis induced by the tested drugs. These findings illustrate the potential use of HSP27 and HSP72-silencing therapy as a highly effective tool to eliminate various types of cancer. However, further studies are needed to elucidate the precise nature of this phenomenon.

Conflict of interest

No conflict of interest to disclose.

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