

Different sensitivity of neurons and neuroblastoma cells to quercetin treatment

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Quercetin is a natural flavonoid with pro-apoptotic and antiproliferative properties. In this study, we determined the sensitivity of neurons and neuroblastoma cells on apoptosis and necrosis induction upon quercetin treatment. No expression of Hsp72 was observed in neurons, which were more sensitive to cell death upon quercetin treatment than neuroblastoma cells, where Hsp72 expression was observed. Reduction of *Hsp72* gene expression in neuroblastoma cells by antisense oligonucleotides made them more sensitive to pro-apoptotic action of quercetin. Moreover, the flavonoid decreased Hsp27, procaspase-3, MRP and PKB expression in neuroblastoma cells and in neurons. Nuclear localization of mainly cytoplasmic Hsp27 was observed in neuroblastoma cells after treatment with high quercetin concentrations, while in neurons, the protein was present in nuclei both in control and quercetin treated cells. Our results suggest that quercetin induce apoptosis more effectively in cells with low level of Hsp72 expression. Higher sensitivity of neurons for cell death after treatment with high quercetin concentrations in comparison to neuroblastoma cell line should also be taken into consideration in further studies on using studied flavonoid as therapeutic agent.

Key words: quercetin, neuroblastoma, neurons, Hsps, MRP, procaspase-3, PKB, cell death

INTRODUCTION

Quercetin (3,3',4',5,7-pentahydroxyflavone) is a natural flavonoid commonly present in many edible fruits and vegetables like: apples, grapes, lemons, onion, kale, tomatoes. It has been reported that quercetin has a broad range of biological, pharmacological and medical applications (Duthie et al. 2000, Birt et al. 2001, Marchand 2002, Liesveld et al. 2003, Schültke et al. 2005, Braganhol et al. 2006, Ma et al. 2006). It exerts antiproliferative effects on different malignant cells by several mechanisms: arresting the cell cycle at control points G0/G1, G1/S and G2/M; interaction with estrogen II binding sites; inhibiting glycolysis and activity of several enzymes like PKC, MAPK, CDC2,

tyrosine kinases, phosphoinositide -3, -4, and -5 kinases. It diminishes the expression of genes necessary for cell proliferation, such as *H-ras*, *K-ras*, *N-ras* and *c-myc* (Weber et al. 1997, Ferrandina et al. 1998, Balabhadrapathruni et al. 2000, Ranelletti et al. 2000, Walker et al. 2000, Lee TJ et al. 2006, Lee WJ et al. 2006, Murtaza et al. 2006, Wung et al. 2006, Mu et al. 2007). Quercetin also facilitates apoptosis of tumour cells by caspase-3 and caspase-9 activation and cytochrome C release (Wang et al. 1999, Jakubowicz-Gil et al. 2002, Chowdhury et al. 2005).

Tumour cell resistance for chemotherapy is correlated with over-expression of heat shock proteins (Hsps), especially Hsp72 a well known molecular chaperone (Beissinger and Buchner 1998, Clark 2001, Voellmy and Boellmann 2007). Enhanced expression of Hsps has been reported for nearly all classes of tumours. It is known that tumour cells are very resistant to cell death, and the reason for that can be the

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ability of Hsps to protect cells from apoptosis. This may explain the fact that Hsps overexpression in tumour cells indicates poor prognosis and resistance to chemotherapy (Creagh et al. 2000, Jolly and Morimoto 2000, Sarto et al. 2000, Sreedhar and Csermley 2004). Thus, decreasing Hsps level in cancer cells would be beneficial.

Protein kinase B (PKB) belongs to the family of serine/threonine kinases, which is downstream of phosphoinositide 3-kinase (PI3K) in the growth factor signal transduction pathway. This is a pivotal kinase in neuronal survival (Spencer et al. 2003). Amplification of genes encoding PKB isoforms has been found in several types of human cancers. It has anti-apoptotic properties, activates proliferation, and may promote cancer invasiveness. Reduction of PKB activity would be useful in tumour treatment (Hill and Hemmings 2002).

Other proteins responsible for tumour cells resistance for therapy are a group of multi-drug resistance proteins (MRP). MRPs are integral membrane P-glycoproteins, which belong to the family of the ATP-binding cassette (ABC) of transmembrane transporter proteins. They expel cytotoxic drugs and diminish their concentration to the levels which are not lethal for cells (Ding et al. 2000).

Quercetin is known to exert beneficial effects on different tumour cells, acting as a sensitizer to apoptosis induction upon chemotherapeutics (Jakubowicz-Gil et al. 2005a,b, Ramos 2007). However the potential side effects of the flavonoid are still uncertain, especially in the light of observations concerning its occasional pro-oxidative (Metodiewa et al. 1999) and cytotoxic properties (Spencer et al. 2003). Unfortunately, little is known about the effect of quercetin on apoptosis induction in neuroblastoma cells and normal neurons. Thus, the aim of the study was to investigate the sensitivity of mentioned cells for quercetin treatment in correlation with Hsp72, Hsp27, procaspase-3, MRP and PKB expression. The effect of quercetin on the localization of the studied proteins in cells was also investigated.

METHODS

Cells and culture conditions

The human neuroblastoma cell line (SK-N-AS) was obtained from the European Collection of Cell Cultures

(Center for Applied Microbiology and Research, Salisbury, UK). Cells were grown in 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 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. The cells were seeded on Lab-Tech Chamber Slides (Nunc) (for apoptosis detection and indirect immunofluorescence), or in Falcon vessels (for heat shock proteins, procaspase-3, MRP and PKB identification).

A neuronal cell culture was prepared from cortices of 18-day-old Wistar rat fetuses as previously described (Rzeski et al. 2004). The tissue was pooled into ice-cold glucose (33 mM) Hank's Balanced Salt Solution (HBSS, Sigma, St. Louis, MO), cut into small pieces and incubated for 30 min at 37°C with 0.25% trypsin-EDTA solution (Sigma, St. Louis). A single cell suspension was obtained by gentle pipetting the cortex fragments in the presence of 10% FBS (Life Technologies, Karlsruhe, Germany) and 0.01% DNase I (Sigma, St. Louis). Cells were then sieved through a 70 µm cell strainer (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ), centrifuged at 800 rpm for 10 min and plated at a density of 5×10^5 cells/ml on poly-L-lysine (MW 70 000–150 000) coated 96-multiwell plates or Lab-Tek Chamber Slides (NUNC, Roskilde, Denmark). The culture medium consisted of B-27 supplemented Neurobasal Medium (Life Technologies, Karlsruhe, Germany), 0.5 mM L-glutamine and 1% of antibiotic-antimycotic solution (Life Technologies, Karlsruhe, Germany). For the initial plating the culture medium was supplemented with 25 µM of glutamate. Incubation was carried out for 8 days at 37°C in humidified 95% air and 5% CO₂ atmosphere. The culture medium was changed every three days. Neuronal identity was confirmed by positive staining with mouse anti-neuron specific enolase γγ monoclonal antibody (Chemicon International, Temecula, CA). The presence of functional NMDA receptors was verified by NMDA receptor subunit NR1 immunoreactivity test, using a mouse anti-NR1 monoclonal antibody (Chemicon International, Temecula, CA).

Neuroblastoma cells transfection

For neuroblastoma cells transient transfection, antisense oligonucleotides (Sigma, Genosys) anti-Hsp72

(5' CGCGGCTTTGGCCAT 3', according to Wei et al. 1994), conjugated with fluorescein at 5' ends were inserted into the cells using cationic lipid Lipofectin Reagent (Gibco) (Evans et al. 1999). The cells were seeded on the day preceding transfection. Just before transfection, the medium from above the cells was replaced by 2 ml of fresh serum free medium. Subsequently, samples containing 1.5 µg of oligonucleotide in 100 µl of serum free medium and 10 µl of Lipofectin Reagent in 100 µl of serum free medium were mixed together and incubated for 15 min at room temperature. After incubation, the formed oligonucleotide-Lipofectin Reagent complexes were overlaid on the cell cultures and incubated at 37°C in 5% CO₂ for 8 h. The medium containing oligonucleotide was replaced with normal growth medium containing serum. The cells were incubated for the next 48 h at 37°C. Three independent experiments were performed.

Quercetin treatment

Quercetin(3,3',4',5,7-pentahydroksyflavone)(Sigma) at the final concentrations 3, 15, 30, 45 µM were used in the experiments. The flavonoid was dissolved in dimethyl sulfoxide (DMSO, Sigma). The final concentration of DMSO in the culture medium did not exceed 0.1%, which as indicated in preliminary experiments, did not influence cell viability and the expression of studied proteins. Neuroblastoma cells and neurons were incubated with quercetin for 6, 12, 24, or 48 h. As control, cells were incubated with 0.1% of DMSO.

Hsp72, Hsp27, MRP and PKB detection by indirect immunofluorescence

After quercetin treatment the cells were washed three times with PBS and fixed in 3.7% paraformaldehyde in PBS for 10 min. After extensive washing with PBS the cells were treated with 0.2% Triton X-100 (Sigma) for 7 min and then washed three times with PBS, all at room temperature. Subsequently, a blocking step of 30 min in 5% low fat milk at room temperature was included. Then the cells were incubated with: mouse monoclonal antibody anti-Hsp72 (SPA 810, StressGen) diluted 1:200, anti-Hsp27 (SPA 800, StressGen) diluted 1:100, anti MRP (QCRL-1, Calbiochem) diluted 1:40 and rabbit antibodies anti-PKB (Cell Signaling) diluted 1:100. The primary antibodies were detected with FITC (Fluorescein

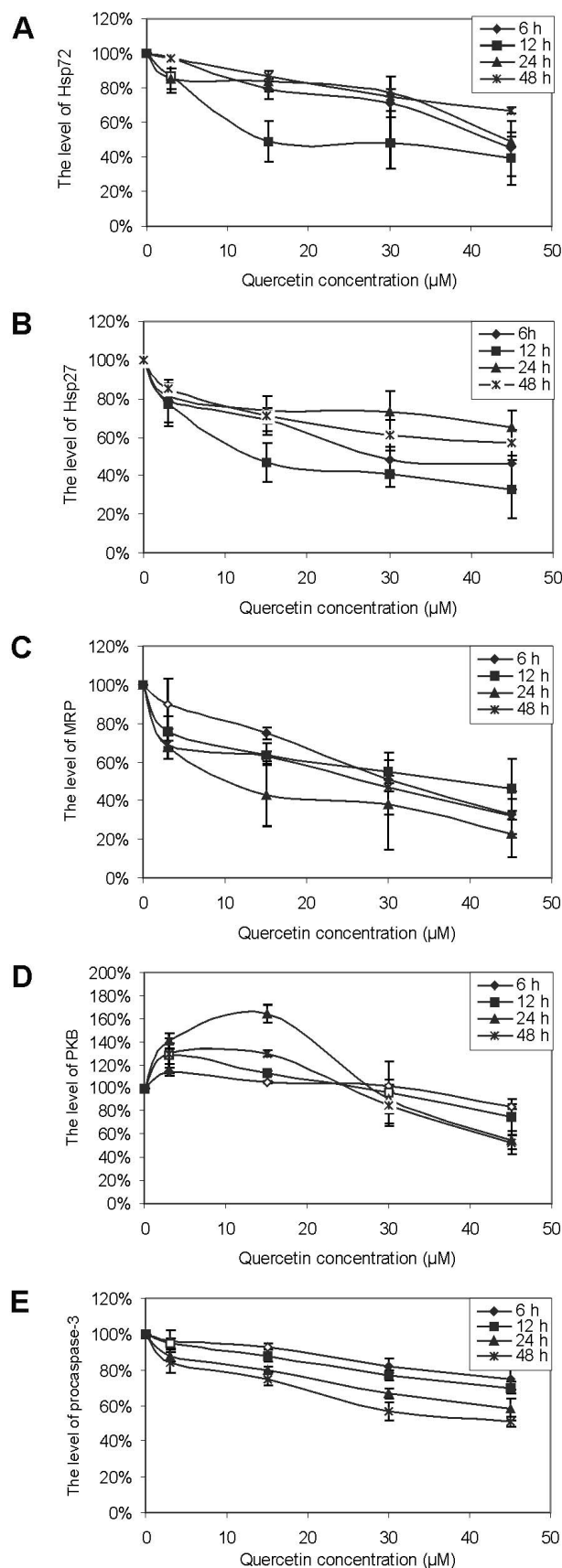
Isothiocyanate)-conjugated goat anti-mouse antibody (Sigma) or with anti-rabbit alkaline phosphatase conjugated antibodies (Sigma) and visualized with alkaline phosphatase substrate (5-bromo-4-chloro-3-indolylphosphate and nitro-blue tetrazolium, Sigma) in a colour development buffer (DMF, Sigma).

Hsp27, Hsp72, MRP and PKB localization in cells was analyzed using a Nikon E-800 microscope. The fluorescent channel was $\lambda = 488$ nm. Three independent experiments were performed. Over 100 cells were analyzed in each experimental variant.

Hsp72, Hsp27, procaspase-3, MRP and PKB detection by immunoblotting

After quercetin treatment, neuroblastoma cells and neurons were lysed in hot SDS-loading buffer (125 mM Tris-HCl pH 6.8; 4% SDS; 10% glycerol; 100 mM DTT), boiled in water bath for 10 min, centrifuged at 10000 g for 10 min, and the supernatants were collected. The protein concentration was determined by the Bradford method (1976), and samples of the supernatants containing 80 µg of proteins were separated by 10% SDS-polyacrylamide gel electrophoresis (Laemmli 1970), 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 monoclonal antibodies against Hsp72, Hsp27 (StressGen, Canada) diluted 1:1 000, anti-MRP antibodies (QCRL-1, Calbiochem) diluted 1:100, or rabbit antibodies against PKB (Cell Signaling) diluted 1:2 000 and procaspase-3 (Sigma) diluted 1:2 000. The membrane was 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 or anti-rabbit IgG (Sigma). The membrane was visualized with alkaline phosphatase substrate (5-bromo-4-chloro-3-indolylphosphate and nitro-blue tetrazolium, Sigma) in a color development buffer (DMF, Sigma). Data were normalized relative to β -actin (Sigma, working dilution 1:2 000).

Levels of heat shock proteins, MRP, procaspase-3 and PKB were determined using Bio-Profil Bio-1D Windows Application V.99.03 programme. Three independent experiments were performed and the level of proteins was expressed as percentage of the initial (control) loading.



Apoptosis detection

For apoptosis and necrosis identification the cells were stained with fluorescent dye Hoechst 33342 (Sigma) and propidium iodide (Sigma), respectively (Jankowska et al. 1997). 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 the microscope. Each experiment was performed in triplicate.

Statistical analysis

Data are presented as mean \pm standard deviation (SD). Statistical evaluation was performed with one-way Anova test followed by Dunnet's multiple comparison test. $P < 0.05$ was taken as criterion of significance.

RESULTS

The effect of quercetin on apoptosis and necrosis induction in neurons and in neuroblastoma cells

Neurons and neuroblastoma cell line were incubated with quercetin (3, 15, 30, 45 μM) for 6, 12, 24, and 48 h. Staining with specific dyes for apoptosis and necrosis, Hoechst 33342 and propidium iodide, respectively, revealed that cell sensitivity for apoptosis and necrosis induction depended on the flavonoid concentration and incubation time (Table I and II).

In neuroblastoma cells (Table I), the highest number of apoptotic cells was observed after 24 h and 48 h incubation with quercetin at a concentration of 45 μM (11.9% and 9.85%, respectively). Shorter treatments with lower quercetin concentrations were less effective. The sensitivity of cancer cells to

Fig. 1. The effect of different quercetin concentrations (3, 15, 30, 45 μM) on Hsp72 (a), Hsp27 (b), MRP (c), PKB (d) and procaspase-3 (e) expression in the neuroblastoma cell line. Cells were incubated with the drug for 6, 12, 24, and 48 h. Each point represents the mean \pm SD. of three results coming from three independent experiments. Filled symbols indicate a significant difference from control ($P < 0.05$).

Table I

The effect of quercetin on apoptosis and necrosis induction in neuroblastoma cells								
Quercetin μM	Time of incubation							
	6 h		12 h		24 h		48 h	
	Apoptosis (%)	Necrosis (%)	Apoptosis (%)	Necrosis (%)	Apoptosis (%)	Necrosis (%)	Apoptosis (%)	Necrosis (%)
Control	0.7 ± 0.2	0.6 ± 0.2	0.7 ± 0.1	0.6 ± 0.2	0.6 ± 0.1	0.4 ± 0.2	0.9 ± 0.1	0.6 ± 0.3
3	0.7 ± 0.1	0.4 ± 0.1	0.75 ± 0.1	$2.2^{**} \pm 0$	1.1 ± 0.1	$2.1^{*} \pm 0.2$	$2.2^{*} \pm 0.4$	$3^{*} \pm 0.4$
15	$2.8^{*} \pm 0.6$	$3.5^{**} \pm 0.3$	$2.4^{*} \pm 0.4$	$2.3^{**} \pm 0.1$	$3.5^{**} \pm 0.1$	$2.9^{**} \pm 0.1$	$4.5^{**} \pm 0.1$	$5^{*} \pm 1.2$
30	$3.6^{**} \pm 0.1$	$2.9^{**} \pm 0.1$	$3.15^{*} \pm 0.5$	$3^{**} \pm 0.1$	$6.3^{**} \pm 0.1$	$5^{**} \pm 0.4$	$6.1^{***} \pm 0.1$	$6^{*} \pm 1.6$
45	$5^{**} \pm 0.3$	$6.8^{**} \pm 0.2$	$4.4^{*} \pm 0.8$	$5.5^{**} \pm 0.3$	$11.9^{***} \pm 0$	$4.6^{**} \pm 0.2$	$9.9^{**} \pm 0.5$	$9.8^{*} \pm 1.8$

Each value represent mean \pm SD ($n=3$). Values significantly different from control are indicated $^{*}P<0.05$, $^{**}P<0.005$, $^{***}P<0.001$.

Table II

The effect of quercetin on apoptosis and necrosis induction in neurons								
Quercetin μM	Time of incubation							
	6 h		12 h		24 h		48 h	
	Apoptosis (%)	Necrosis (%)	Apoptosis (%)	Necrosis (%)	Apoptosis (%)	Necrosis (%)	Apoptosis (%)	Necrosis (%)
Control	0.7 ± 0.2	2.5 ± 0.1	0.7 ± 0.2	2.9 ± 0.1	1 ± 0.2	3 ± 0.1	0.9 ± 0.2	3.2 ± 0.1
3	0.6 ± 0.1	3 ± 0.2	$4^{**} \pm 0.1$	3.3 ± 0.2	$5.3^{**} \pm 0.1$	$6.4^{**} \pm 0.3$	$12.1^{*} \pm 2.3$	$22.3^{***} \pm 0.5$
15	0.6 ± 0.1	2.9 ± 0.4	$4.6^{**} \pm 0.0$	$3.7^{*} \pm 0.2$	$12.5^{*} \pm 2.2$	12.4 ± 3.5	$21.9^{**} \pm 1.9$	$19.4^{**} \pm 2.1$
30	1 ± 0.2	2.4 ± 0.4	$5.1^{*} \pm 1.3$	3.7 ± 0.6	$19.1^{**} \pm 1.2$	$17^{**} \pm 0.6$	$42.3^{***} \pm 1.1$	$31.1^{**} \pm 1.8$
45	0.9 ± 0.2	2.7 ± 0.4	$6^{*} \pm 0.9$	$3.9^{**} \pm 0.1$	$33.2^{***} \pm 0.6$	$35^{**} \pm 4.2$	$29.7^{*} \pm 5.4$	$53.2^{**} \pm 3.4$

Each value represent mean \pm SD ($n=3$). Values significantly different from control are indicated $^{*}P<0.05$, $^{**}P<0.005$, $^{***}P<0.001$.

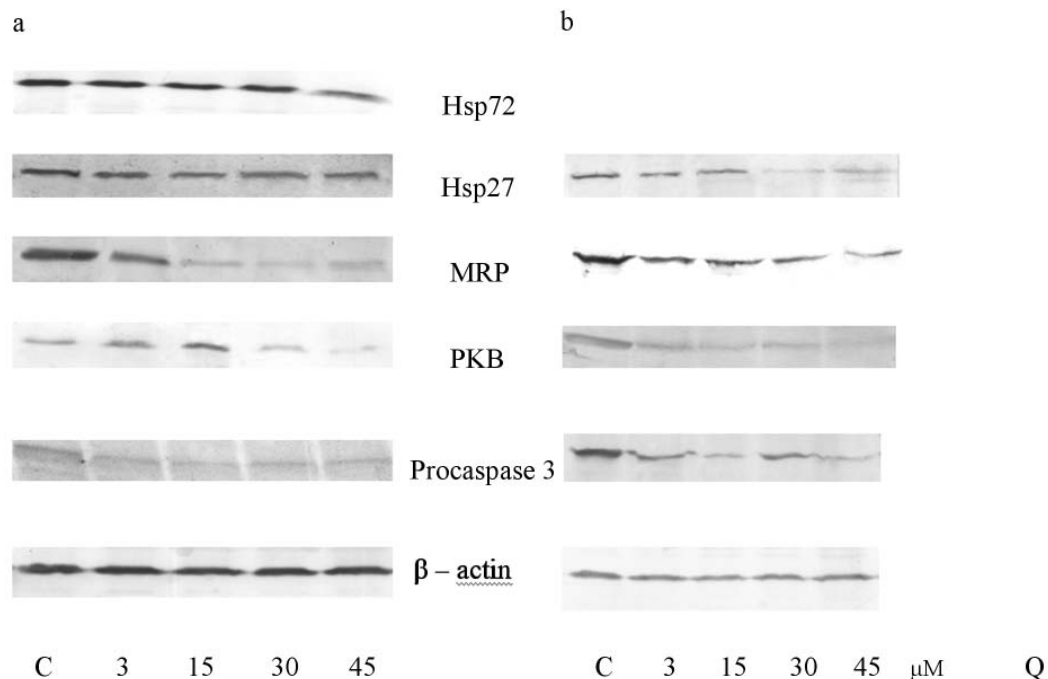


Fig. 2. Immunoblots presenting the level of Hsp72, Hsp27, procaspase-3, MRP, PKB and β -actin expression in neuroblastoma cells (a) and neurons (b) after incubation with quercetin (Q) for 24 h

necrosis induction upon quercetin treatment was also concentration-dependent. The highest number of necrotic cells (9.8%) was observed after 48 h incubation with 45 μ M of quercetin. It is noteworthy that after quercetin treatment the number of necrotic cells was generally similar or exceeded the number of apoptotic ones. Only 24 h long incubation with 45 μ M of quercetin was the exception – the number of necrotic cells was about 2.5 times lower than apoptotic ones.

Similarly to the neuroblastoma cells, 24 h and 48 h incubation of neurons at different quercetin concentrations appeared to be most effective in apoptosis and necrosis induction (Table II). The highest number of apoptotic cells (42%) was observed after treatment with 30 μ M of the tested compound for 48 h. The treatment with 45 μ M of the flavonoid resulted in a smaller number of apoptotic cells (29.7%), but in a stronger necrosis induction (53.2%). After 24 h incubation with quercetin at a concentration of 45 μ M, 33.2% of the cells were noticed to be apoptotic. The number of apoptotic and necrotic cells was similar.

The effect of quercetin on heat shock proteins, procaspase-3, PKB, MRP expression and cellular localization

Heat Shock Proteins

Western blot analysis revealed that 6, 12, 24, and 48 h incubation of neuroblastoma cells with quercetin at 3–45 μ M concentrations, significantly decreased Hsp72 (Fig. 1a, 2a) and Hsp27 (Fig. 1b, 2a) expression in a concentration-dependent way. The strongest effect (inhibition by about 60%) was observed when the cells were treated with 45 μ M of quercetin for 6 and 12 h. Time-dependent blunting of the flavonoid efficiency in lowering the level of Hsps was noticed. Incubation of cancer cells at the highest quercetin concentration for 24 and 48 h resulted in 50% and 30% inhibition of Hsp72, and 35%, and 40% of Hsp27, respectively. As was revealed by indirect immunofluorescence in non-treated neuroblastoma cells, Hsp72 was detected in cytoplasm, and quercetin did not change its localization (Fig. 5e). However, quercetin affected Hsp27 distribution in neuroblas-

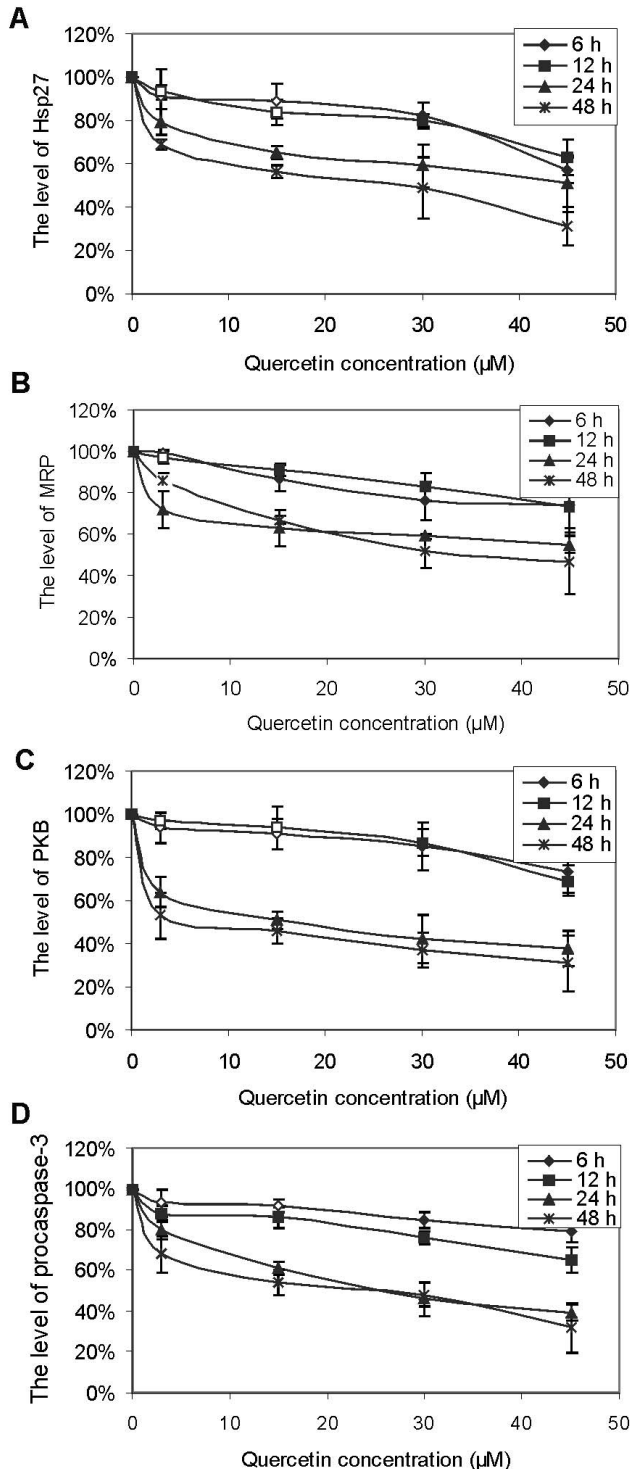


Fig. 3. The effect of different quercetin concentrations (3, 15, 30, 45 μ M) on Hsp27 (a), MRP (b), PKB (c) and procaspase-3 (d) expression in neurons incubated with the drug for 6, 12, 24, and 48 h. Each point represents the mean \pm SD of three results coming from three independent experiments. Filled symbols indicate a significant difference from control ($P < 0.05$).

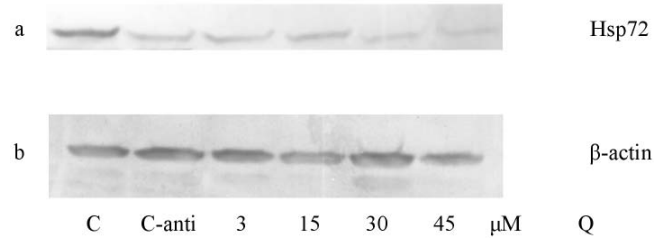


Fig. 4. The level of Hsp72 expression in neuroblastoma cells transfected with antisense oligonucleotides blocking Hsp72 expression, subsequently treated with quercetin (Q) (3, 15, 30, 45 μ M); (C) control of not transfected cells, (C-anti) control of transfected cells. For cancer cells transfection oligonucleotides were inserted into the cells using Lipofectin Reagent.

ma cells (Fig. 5 a–d). In non-treated control cells and after incubation with 3 μ M of quercetin, the protein was present only in cytoplasm. After incubation with quercetin at a concentration of 15 μ M, Hsp27 was distributed uniformly both in the cytoplasm and nucleus. Cell treatment with 30 μ M of the flavonoid resulted in increased nuclear level of Hsp27, which exceeded the cytoplasmic one. In cells incubated with 45 μ M of quercetin, Hsp27 was present mainly in the nucleus.

What deserved to be noted was that no Hsp72 expression was observed in neurons. A gradual, time- and concentration-dependent decrease of Hsp27 level was observed (Fig. 2b, 3a). The strongest effect (the level of the protein lowered by about 70%) was observed after 48 h incubation with 45 μ M of quercetin.

The studied flavonoid had no effect on Hsp27 distribution in neurons (Fig. 6a). The protein was located both in the cytoplasm and nucleus, but the nuclear level exceeded the cytoplasmic.

MRP

Quercetin decreased MRP expression in neuroblastoma cells in a concentration-dependent way (Fig. 1c, 2a). Incubation with the flavonoid at a concentration of 45 μ M was most effective, and the level of the protein decreased by about 70%, 55%, 75%, and 70% after 6, 12, 24, and 48 h treatment, respectively. Time- and concentration-dependent inhibition of MRP level after incubation with quercetin, was observed in neurons, but to a smaller extent than it was observed in neuroblastoma cells (Fig. 2b, 3b). Quercetin had no effect on MRP

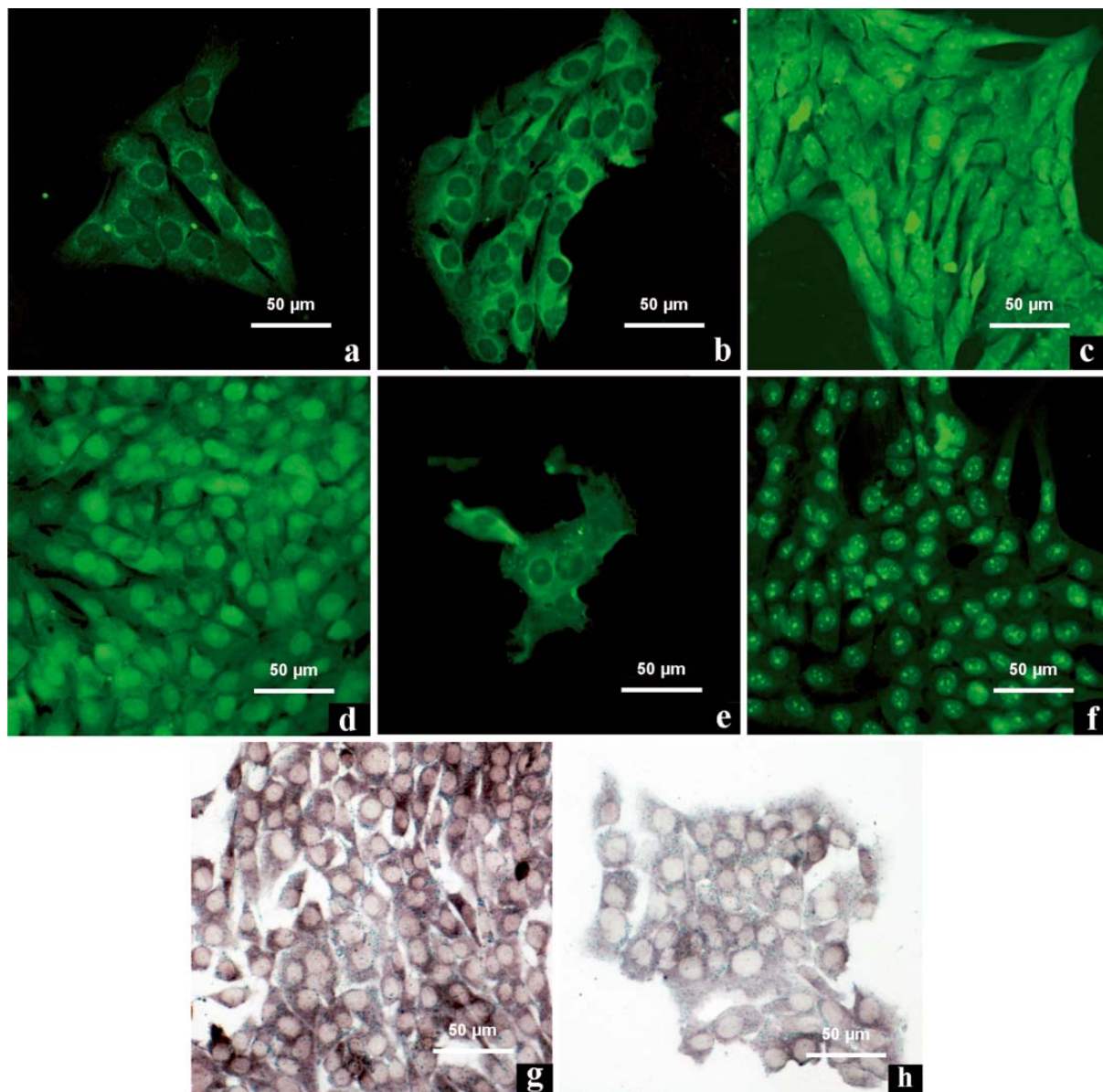


Fig. 5. Localization of Hsp27, Hsp72, MRP and PKB in neuroblastoma cells incubated with quercetin for 24 h, revealed by indirect immunofluorescence. Distribution of Hsp27 in non treated neuroblastoma cells (a), cells treated with quercetin at the concentrations: 3 μ M (b), 30 μ M (c), and 45 μ M (d); Hsp72 in cells treated with 30 μ M of quercetin (e); MRP in cells treated with 30 μ M of quercetin (f); PKB in cells treated with 15 μ M (g) and 45 μ M (h) of quercetin; magnification 400 \times . The drug did not change the localization of Hsp72, MRP and PKB, thus pictures of protein distribution only in quercetin treated cells are presented.

localization in neuroblastoma (Fig. 5f) and neurons (Fig. 6b). In both cell lines the protein was located in nuclei.

PKB

The level of PKB expression in neuroblastoma cells depended on quercetin concentration (Fig. 1d, 2a). Incubation with 3 and 15 μ M of the tested compound,

increased PKB expression, especially after 24 h treatment, when 40% and 62% induction was noticed, respectively. The treatment with 45 μ M generally inhibited PKB expression. Quercetin reduced PKB expression in neurons in time- and concentration-dependent way (Fig. 2b, 3c). Incubation of cells at quercetin concentration of 45 μ M for 48 h appeared to be most effective and reduced the level of studied protein by about 70%.

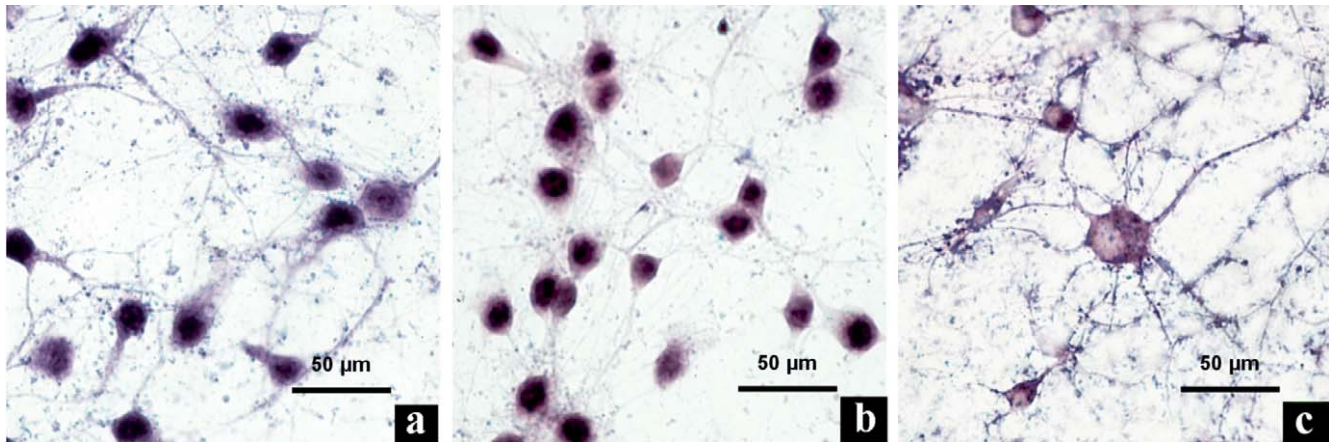


Fig. 6. Localization of Hsp27 (a), MRP (b), and PKB (c) in neurons incubated for 24 h with 30 μ M of quercetin; magnification 400 \times . The drug did not change the localization of Hsp 27, MRP and PKB, thus pictures of protein distribution only in quercetin treated cells are presented.

Indirect immunofluorescence revealed that PKB was present in the cytoplasm of neuroblastoma cells (Fig. 5g, h) and neurons (Fig. 6c), both in control and quercetin treated cells. Changes in the intensity of immunocytochemical reaction with regard to the studied protein were observed: increased reaction after treatment with 3 and 15 μ M of quercetin, and diminished after 6–48 h treatment with 45 μ M of quercetin.

Procaspase-3

Quercetin diminished the level of the inactive form of caspase-3 (procaspase-3) both in neuroblastoma cells and neurons, being more effective in neurons (Fig. 1e, 2, 3d). Correlation between a higher flavonoid concentration, longer incubation time and lower level of procaspase 3 was noted. In neuroblastoma cells and neurons treated with 45 μ M of quercetin for 6 h the level of protein was diminished by 15% and 32%, respectively, while 48 h incubation reduced procaspase-3 expression by 49% and 68%.

Apoptosis and necrosis induction by quercetin in transfected neuroblastoma cells

To obtain neuroblastoma cells with decreased Hsp72 level we transfected cancer cells with antisense oligonucleotides anti-Hsp72 (5' CGCGGCTTTGGCCAT 3'). Transfected cancer cells showed a diminished expression of Hsp72 in comparison to non-transfected ones. Additional treatment of the studied cells with querce-

tin (3, 15, 30, 45 μ M) inhibited studied protein expression even more effectively (Fig. 4).

Reduction of Hsp72 expression in transfected cells was accompanied by increased sensitivity of cancer cells to apoptosis induction after quercetin treatment in comparison to non-transfected neuroblastoma cells (Table III). The flavonoid at a concentration of 3 μ M

Table III

Apoptosis and necrosis induction in neuroblastoma cells transfected with antisense oligonucleotides anti Hsp72 and incubated with quercetin for 24 h, stained with Hoechst 33342 and propidium iodide

	Apoptosis (%)	Necrosis (%)
Control	0.7 \pm 0	0.8 \pm 0.1
DNA control	1 \pm 0	0.9 \pm 0.7
Lipofectin control	1.3 \pm 0.6	1.2 \pm 0.4
Quercetin 3 μ M	10.2 \pm 1.6*	1.2 \pm 0.7*
Quercetin 15 μ M	12.5 \pm 0.5*	2.1 \pm 2.1*
Quercetin 30 μ M	16.8 \pm 1.1*	1 \pm 0.6*
Quercetin 45 μ M	23.7 \pm 3.3*	0.8 \pm 0.7

Each value represent mean \pm SD ($n=3$). Values significantly different from control are indicated * $P<0.005$.

induced apoptosis in 10% of cells. The number of programmed dead cells rose gradually with quercetin concentration increase. The treatment with 45 μ M appeared to be most effective, causing programmed cell death in 24% of transfected cells. It is worth noting that this value is similar to the data obtained in neurons, where Hsp72 was not detected, and the number of apoptotic cells reached 33%.

Reduction of Hsp72 expression had no effect on necrosis induction. No significant differences between the number of necrotic cells in control (non transfected and non treated) cells and transfected neuroblastoma cells treated with quercetin were observed.

DISCUSSION

We observed that quercetin induced apoptosis and necrosis in both the neuroblastoma cell line and neurons. Neurons were much more sensitive to cell death (both, apoptotic and necrotic) upon quercetin treatment than the cancer cell line. After 48 h incubation with 45 μ M of quercetin the number of necrotic cells exceeded the number of apoptotic ones, which may suggest even a toxic effect of quercetin on neurons what may limit the usefulness of using the flavonoid as therapeutic agent (Jakubowicz-Gil et al. 2008). However, it is difficult to relate these observations to what occurs *in vivo*. Cells in culture accumulate high amounts of quercetin (Spencer et al. 2003). *In vivo*, its daily intake is estimated at about 25 mg. Only 30–50% of oral quercetin is absorbed, the rest passes through gastro-intestinal tract where is metabolized to O-methylated and glucuronide metabolites (Boulton et al. 1999, Spencer et al. 1999, Ross and Kasum 2002). Baseline quercetin concentrations generally vary between 50 and 80 nmol/l (Conquer et al. 1998). Quercetin toxicity toward neurons may be limited by the blood-brain barrier (BBB) – an obstacle to reaching the brain (Youdim et al. 2004).

It is known that quercetin can induce apoptosis by several mechanisms, among others, *via* inhibition of the expression of Hsp70 and Hsp27 proteins, which stimulate cytochrome C release, apoptosome formation and procaspase-9 and procaspase-3 activation (Wang et al. 1999). Reduction of Hsps expression also makes cells more vulnerable to necrotic death after quercetin treatment by a dramatic decrease of the total cellular ATP content (Agullo et al. 1994, Kabakov and Gabai 1994, 1995). In our experiments, quercetin

diminished Hsp27 expression in neurons and neuroblastoma cells and Hsp72 expression in cancer cells. The strongest reductive effect was observed in cells treated with 45 μ M of quercetin. Longer than 24 h incubation of cancer cells with the tested compound diminished the reductive properties of quercetin. This might be explained by a low stability of the flavonoid in cell culture media (van der Woude et al. 2003). Quercetin decreases Hsp27 and Hsp72 expression by preventing the Heat shock factor 1 and 2 (Hsf1 and Hsf2), binding to the Heat Shock Element (HSE) in the promoter region of *hsp* genes (Hosokawa et al. 1992, Hansen et al. 1997, Nagasaka and Nakamura 1998).

Our results have clearly shown that Hsp72 is not expressed in neurons. This confirms the observations of other authors, who were not able to detect mRNA encoding Hsp70 in hippocampal neurons even after heat shock. It is also known that in hippocampal neurons, there is the lack of Hsf1 expression, which may be the reason for incomplete Hsp70 expression and increased cellular vulnerability under stress (Kaarniranta et al. 2002). It is possible, that the lack of Hsp72 expression in neurons is responsible for increased cellular sensitivity to apoptosis and necrosis induction after treatment with high concentrations of quercetin, in comparison to neuroblastoma cells, where Hsp72 expression was observed. Thus, to find Hsp72 engagement in the programmed cell death process upon quercetin treatment, we inhibited this protein expression in the neuroblastoma cell line using antisense oligonucleotides, complementary to the initiation codon and four downstream codons of human gene *Hsp72*. The results of our studies clearly indicate that transfected cells showed a diminished heat shock protein level, in comparison to control non-transfected cells. This was correlated with significantly increased number of apoptotic cells in the transfected neuroblastoma cell line after quercetin treatment, in comparison to non-transfected cancer cells. There was no significant increase in the number of necrotic cells after incubation of transfected neuroblastoma cells with quercetin, in comparison to control, which suggests that a low level of Hsp72 is not responsible for increased sensitivity of cancer cells to the pro-necrotic action of quercetin.

It is known that nuclear localization of Hsp72 is an indicator of cellular stress and is correlated with cell protection against cell death (Ellis et al. 2000, Nonaka et al. 2003, Steel et al. 2004). In control and quercetin treated neuroblastoma cells, Hsp72 was detected main-

ly in cytoplasm but not in the nucleus. Our earlier experiments indicated that quercetin revealed an ability to block Hsp72 translocation through nuclear membrane contributing to decrease in cell protection against cell death (Jakubowicz-Gil et al. 2005 a,b). In contrast to Hsp72, Hsp27 gradually translocated from cytoplasm to nucleus upon quercetin treatment. As it was observed by other authors in non stress conditions, Hsp27 is located mainly in cytoplasm. Under stress conditions, small Hsps translocate to the nucleus, where functional chaperone complexes are formed to protect nuclear proteins (Geum et al. 2002, de Graauw et al. 2005). This is the first observation that quercetin induced translocation of Hsp27 to the nucleus in the neuroblastoma cells line and caused increased resistance of cancer cells to cell death. In neurons quercetin had no effect on Hsp27 localization.

As it was mentioned earlier, inhibition of Hsp70 and Hsp27 expression stimulates procaspase-3 activation (Wang et al. 1999). Our experiments seem to confirm this observation. Processing procaspase-3 into its active form was enhanced after quercetin treatment and accompanied by increased number of apoptotic cells.

A strong activation of caspase-3 after quercetin treatment might also result from inhibition of PKB phosphorylation and activation, that subsequently lead to PKB cleavage and reduction of the total protein content (Matter et al. 1992, Vlahos et al. 1994, Spencer et al. 2003). As revealed by Spencer and coauthors (2003), 30 μ M of quercetin diminished the PKB level, while at lower concentrations (about 10 μ M) the PKB reduction was transient. Our results confirmed that the level of PKB expression was concentration dependent. In neuroblastoma cells, 3 and 15 μ M of quercetin increased the level of PKB. These concentrations had weak effects on apoptosis induction, which may suggest protective involvement of PKB against programmed cell death. After treatment with 45 μ M of quercetin, reduction of PKB expression as well as the highest percentage of apoptotic cells was observed. In neurons quercetin decreased PKB expression at all concentrations used.

Increased sensitivity of neurons and neuroblastoma cells for apoptosis induction observed after treatment with quercetin may also correlate with the reduction of MRP expression. It was reported by other authors that a strong correlation exists between poor prognosis in childhood neuroblastoma and the high level of the expression of MRP1 protein (Peaston et al. 2001).

CONCLUSIONS

Our results indicate that the sensitivity of neuroblastoma cells and neurons to apoptosis and necrosis induction after quercetin treatment is different and that flavonoid induces cell death more strongly in neurons than in cancer cells. Increased vulnerability of neuroblastoma cells with inhibited Hsp72 expression to proapoptotic action of quercetin may suggest the involvement of heat shock protein in cell resistance but the precise mechanism of this phenomenon requires more investigations. It also suggests that further studies on using quercetin in treatment of brain tumors should be carried out with regard to its possible cytotoxic effect on neurons.

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