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The effect of quercetin on pro-apoptotic activity of cisplatin in HeLa cells

J. Jakubowicz-Gil [a,*], R. Paduch [b], T. Piersiak [a], K. Głowniak [c], A. Gawron [a], M. Kandefer-Szerszeń [b]

[a] Department of Comparative Anatomy and Anthropology, Maria Curie-Skłodowska University, Akademicka 19, 20-033 Lublin, Poland
[b] Department of Virology and Immunology, Maria Curie-Skłodowska University, Akademicka 19, 20-033 Lublin, Poland
[c] Department of Pharmacognosy with Medicinal Plant Laboratory, Skubiszewski Medical University, Chodźki 1, 20-093 Lublin, Poland

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Abstract

It is well known that some tumour cells are very resistant to chemotherapy-induced cell death which indicate poor prognosis for patients. Thus the aim of the present study was to investigate the effect of quercetin on pro-apoptotic activity of cisplatin in human cervix carcinoma cells (HeLa).

Three variants of experiments were performed. In the first one cells were incubated with studied drugs separately for 8 and 24 h. In the second, drugs were added to the culture medium simultaneously. In third cisplatin or quercetin addition was followed by subsequent quercetin or cisplatin treatment, respectively.

We observed different apoptotic effects, dependent on the drug succession. Preincubation of cells with quercetin followed by cisplatin treatment appeared to be the most effective and was correlated with strong activation of caspase-3 and inhibition of both heat shock proteins (Hsp72) and multi-drug resistance proteins (MRP) levels.

Our results indicate that quercetin pretreatment sensitizes HeLa cells to cisplatin-induced apoptosis in HeLa cells.

Keywords: Quercetin; Cisplatin; Apoptosis; Hsp72; MRP; HeLa cells

1. Introduction

Apoptosis is a physiological process, which occurs during embryonic development as well as during maintenance of tissue homeostasis, tumour development and metastasis. It can be induced by a variety of treatments like UV irradiation and chemotherapeutic drugs [1,2]. The transduction and execution of apoptotic signals requires coordinated activation of the cascade of caspases (cysteine proteases) [3,4]. One of the critical enzyme is caspase-3. It belongs to the CED-3 subfamily and specifically cleave substrates including the poly-(ADP-ribose) polymerase (PARP) or inhibitor of caspase-activated DNase (ICAD). It also plays a major role in the chromatin condensation or DNA fragmentation [5].

It is well known that some tumour cells are very resistant to chemotherapy-induced cell death which indicate poor prognosis for patients [6]. It has been reported that cisplatin (*cis*-dichlorodiammine-platinum (II) CDDP) may induce apoptosis through caspase-3-dependent and -independent signal pathways [5,7]. Cisplatin is widely used chemotherapeutic agent in the treatment of several malignances like ovarian, testicular, bladder, lung, head and neck cancers. It interacts with cellular proteins and lipids and forms DNA adducts causing cell cycle arrest in G2/M phase [8–10]. It can also disturb cytoskeleton organization [11]. However, drug resistance compromise its clinical effectiveness. It has been shown that alteration in caspase-3 expression and activity is associated with the development of cellular resistance to cisplatin. It has also been shown that the mechanism of the relative resistance to apoptosis may be connected with reduced cytochrome c release and PARP cleavage [5,7]. There are also several other factors responsible for such resistance. One of them are heat shock proteins. These are molecular chaperones, controlling the proper folding of newly synthesized polypeptides, the refolding of missfolded proteins, and controlling trans-

* Corresponding author. Tel.: +48 81 537 59 96; fax: +48 81 537 51 01.
E-mail address: jjgil@biotop.umcs.lublin.pl (J. Jakubowicz-Gil).

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location through cell membranes [12–15]. Their expression can be modulated by factors leading to apoptosis in such pathologic states as ischemia, fever, inflammation, infections and cancers [16,17]. The enhanced expression of Hsps has been reported for nearly all classes of tumours [6,18–20]. Therefore, tumour cells resistance to apoptosis is thought to be closely associated with Hsps overexpression. This may also indicate poor prognosis. Thus decreasing Hsps level in cancer cells would be beneficial for patients.

Tumour cells resistance to cisplatin treatment may be also connected with multi-drug resistance proteins expression. MRPs are an integral membrane P-glycoprotein which belongs to the family of the ATP-binding casette (ABC) of transmembrane transporter proteins. They expel cytotoxic drugs and diminish their concentration to intercellular level which is not lethal for cells in consequence [7].

It was previously shown that antiproliferative effect of cisplatin was enhanced by quercetin in vivo and in vitro [21,22]. Quercetin (3,3′,4′,5,7-pentahydroksyflavone) belongs to flavonoids. It is present in many edible fruits and vegetables like: apples, grapes, lemons, onion, kale, tomatoes. The daily intake of it is estimated to be about 25 mg. Quercetin has a broad range of biological, pharmacological and medical applications [23–25]. It has been reported to be an anticancer agent because it exerts antiproliferative effects on different malignant cells by several mechanisms: freezing the cell cycle in control points G0/G1, G1/S and G2/M, interaction with estrogen II binding sites, inhibiting glycolysis and activity of several enzymes like protein kinase C (PKC), mitogen-activated protein (MAP) kinase, cell division cycle (CDC) kinase 2, tyrosine kinases, phosphoinositol-3, -4 and -5 kinases. It diminishes the expression of genes necessary for cell proliferation like: H-ras, K-ras, N-ras i c-myc [26–35]. Like other members of flavonoids quercetin facilitates the apoptosis of tumour cells [36].

Thus the aim of the present study was to investigate the effect of quercetin on pro-apoptotic activity of cisplatin in human cervix carcinoma cells (HeLa). The level of MRP, procaspase-3 and Hsp72 were also examined.

2. Materials and methods

2.1. Cells and culture conditions

Human cervix carcinoma cell line (HeLa B, ECACC No. 85060701) cultured in RPMI 1640 medium supplemented with 5% FBS (fetal bovine serum) (v/v) was used in this study. Cells were seeded on cover slides (for apoptosis detection and indirect immunofluorescence) or in Falcon vessels (for heat shock proteins, MRP and procaspase-3 identification), at a density of 1×10^5 cells/ml and incubated at 37 °C in humidified atmosphere with 5% CO_2.

2.2. Drug treatment

Cisplatin (Sigma) at a final concentration of 10 μg/ml and quercetin (Sigma) at a final concentration 15 μg/ml were used in the experiments on the basis of our earlier experiments and the observations of other authors [37–42]. The drugs were dissolved in dimethyl sulfoxide (DMSO, Sigma), but 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.

Three variants of drug treatment were performed. In the first one HeLa cells were incubated only with quercetin or only with cisplatin for 8 or 24 h. In the second cisplatin and quercetin were added to the culture medium at the same time and incubated for 8 or 24 h. In the third variant:

- the cells were preincubated with quercetin for 4 or 12 h, followed by cisplatin addition and incubated for the next 4 or 12 h;
- the cells were preincubated with cisplatin for 4 or 12 h, followed by quercetin addition and incubated for the next 4 or 12 h.

As control cells were incubated with 0.1% DMSO.

2.3. Hsp72 detection by indirect immunofluorescence

After drug treatment, the cells were washed three times with PBS, fixed in 3.7% paraformaldehyde in PBS for 10 min, washed three times with PBS, treated with 0.2% Triton X-100 for 7 min, 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 anti Hsp72 monoclonal antibody (SPA 810, StressGen) diluted 1:200. Anti Hsp72 labeled cells were detected with FITC-conjugated goat anti mouse antibody (Sigma) at a 1:30 dilution.

Cells were analyzed using scanning head PASCAL5 (Zeiss). The data were rejestrated in fluorescent channel ($\lambda = 488$ nm).

As controls, cells were incubated in absence of primary antibody. Such omission resulted in no disposition of reaction product (not shown).

2.4. Hsp72, MRP and procaspase-3 detection by immunobloting

After drug treatment HeLa cells 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 supernatant was collected. The protein concentration (average 14 mg/ml) was determined by the Bradford method [43] and samples of supernatants containing exactly 80 μg of proteins were

separated by 10% SDS-polyacrylamide gel electrophoresis [44] and subsequently transferred onto Immmobilon P membrane (Sigma). Following transfer, the membrane was blocked with 3% low fat milk in PBS for 1 h, and incubated overnight with mouse monoclonal antibodies against Hsp72 (SPA 810, StressGen) diluted 1:1000 and anti-MRP (QCRL-1) antibodies (Calbiochem) diluted 1:100 or rabbit antibodies anti-procaspase-3 (Sigma) diluted 1:2000. The membrane was washed three times for 10 min with PBS containing 0.05% TRITON X-100 (Sigma) and incubated for 2 h with a 1:30 000 dilution of alkaline phosphatase-conjugated goat anti-mouse IgG or anti-rabbit IgG (Sigma). The membrane was visualized with alkaline phosphatase substrate (5-bromo-4-chloro-3-indolyphosphte and nitro-blue tetrazolium, Sigma) in a colour development buffer (DMF, Sigma). Two independent experiments were performed.

Quantitative heat shock protein, MRP and procaspase-3 levels were assessed using Bio-Profil Bio-1D Windows Application V.99.03 program.

Significance levels were calculated using one-way ANOVA test.

2.5. Apoptosis detection

For apoptosis identification, the cells were stained with fluorescent dye Hoechst 33342 (Sigma) [45]. Morphological analysis was performed under fluorescence microscope (NIKON E-800). The cells exhibiting blue fluorescent nuclei (intact or fragmented) were interpreted as apoptotic. At least 1000 cells in randomly selected microscopic fields were counted under microscope. Two independent experiments were performed. The obtained results were analyzed for significance by one-way ANOVA test.

3. Results

3.1. Quercetin and cisplatin-induced apoptosis of HeLa cells

Quercetin and cisplatin-induced apoptosis was examined by staining HeLa cells with fluorochrome Hoechst 33342 (Table 1). Both quercetin and cisplatin added to the culture medium separately or in combination induced apoptosis. The highest level of apoptosis was observed after 24 h of incubation when flavonoid treatment preceded cisplatin addition and it was time dependant. In 8 h long variant, after preincubation with quercetin 27.33% of apoptotic cells were noticed and it was higher by 7.8% in 24 h long variant. Strong (about 25.5%) time-independent induction of apoptosis was also observed when both studied drugs were added to the culture medium at the same time. Incubation of HeLa cells only with quercetin, only with cisplatin and preincubation with cisplatin before quercetin treatment were less effective.

Table 1
Apoptosis induction in HeLa cells incubated with drugs for 8 or 24 h

	Apoptotic cells (%)	
	8 h	24 h
Control	0.65 ± 0.07	0.95 ± 0.07
C	6.06 ± 3.1*	11.27 ± 1.7***
Q	9 ± 0.0***	13.57 ± 1.4***
CQ	11.43 ± 1.94***	14.65 ± 0.07***
QC	27.33 ± 2.7***	35.15 ± 1.54***
C+Q	25.7 ± 3.2***	25.27 ± 3.1***

Values are means (±S.D.) of two independent experiments. C, cisplatin; Q, quercetin; CQ, preincubation with cisplatin followed by quercetin treatment; QC, preincubation with quercetin followed by cisplatin addition; C + Q, simultaneous cisplatin and quercetin treatment.
* $p < 0.05$.
*** $p < 0.001$.

3.2. Effect of cisplatin and quercetin on the level of procaspase-3

Quercetin and cisplatin used both and separately appeared to diminish the level of inactive form of caspase-3 (procaspase-3).

Preincubation of HeLa cells with quercetin followed by cisplatin treatment resulted in 75% inhibition after 8 h long and 60% inhibition after 24 h long incubations (Fig. 1). Strong inhibition was also noticed in variant when cells were pretreated with cisplatin and after that incubated with quercetin (70% after 8 h of incubation and 45% after 24 h of incubation). Separate and simultaneous incubation of studied cells with drugs did not result in significant lowering of procaspase-3 level.

3.3. Effect of quercetin and cisplatin on Hsp72 expression

Western blot analysis revealed that 8 h long HeLa cells incubation with quercetin in combinations with cisplatin inhibited Hsp72 expression. Cisplatin itself did not change Hsp expression. Quercetin alone also inhibited Hsp72 expression but this did not reach significance. Preincubation of studied cells with quercetin before cisplatin treatment was the most effective.

After 24 h long incubation of HeLa cells with studied drugs inhibition of Hsp72 expression was observed when HeLa cells were preincubated with quercetin before cisplatin treatment and reverse (cisplatin preincubation followed by quercetin addition). In contrast to that, after separate or simultaneous cisplatin and quercetin treatment induction of Hsp72 expression was observed (Fig. 2).

3.4. Effect of quercetin and cisplatin on Hsp72 localization

Indirect immunofluorescence showed that in non-treated HeLa cells Hsp72 was located mainly in cytoplasm. Immunological reaction toward detecting studied protein in nucleus was weak. (Fig. 3a). Similar distribution was

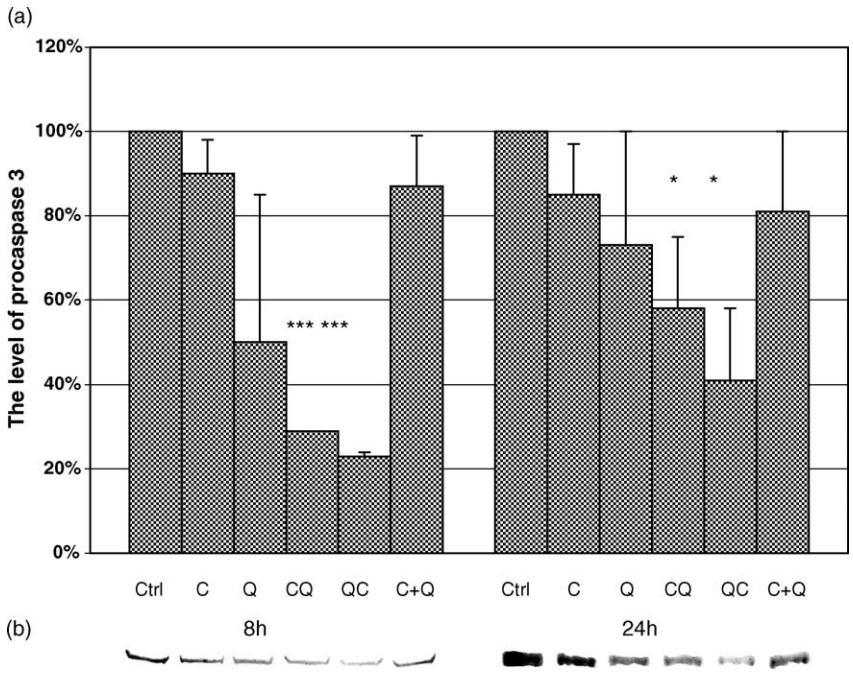

Fig. 1. Quantitative (a) and qualitative (b) analysis of the effect of quercetin (Q) and cisplatin (C) on the level of procaspase-3 in HeLa cells after 8 and 24 h long incubation. Values are means (±S.D.) of two independent experiments carried out in duplicate. $^{*}p < 0.05$, $^{***}p < 0.001$. CQ, preincubation with cisplatin followed by quercetin treatment; QC, preincubation with quercetin followed by cisplatin addition; C + Q, simultaneous cisplatin and quercetin treatment.

observed in cells after quercetin treatment, when quercetin incubation was followed by cisplatin addition and after simultaneous quercetin and cisplatin treatment (Fig. 3b, e and f). After incubation of HeLa cells only with cisplatin and in variant of experiment when cells were incubated with cisplatin and after this with quercetin cytoplasmic but also strong nuclear localization of Hsp72 was noticed (Fig. 3c and d).

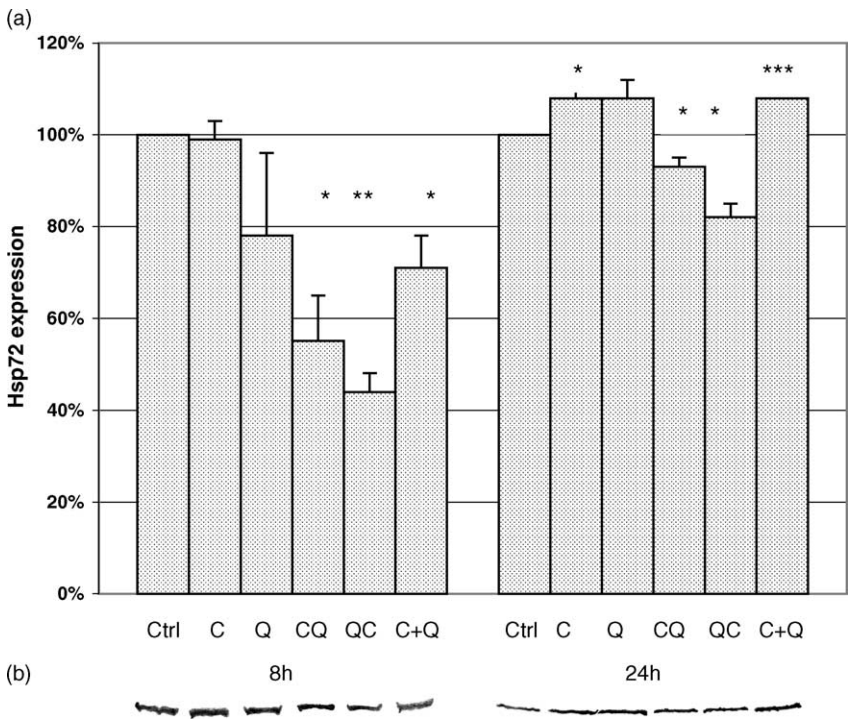

Fig. 2. Quantitative (a) and qualitative (b) analysis of the effect of quercetin (Q) and cisplatin (C) on the level of Hsp72 in HeLa cells after 8 and 24 h long incubation. Values are means (±S.D.) of two independent experiments carried out in duplicate. $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.001$. CQ, preincubation with cisplatin followed by quercetin treatment; QC, preincubation with quercetin followed by cisplatin addition; C + Q, simultaneous cisplatin and quercetin treatment.

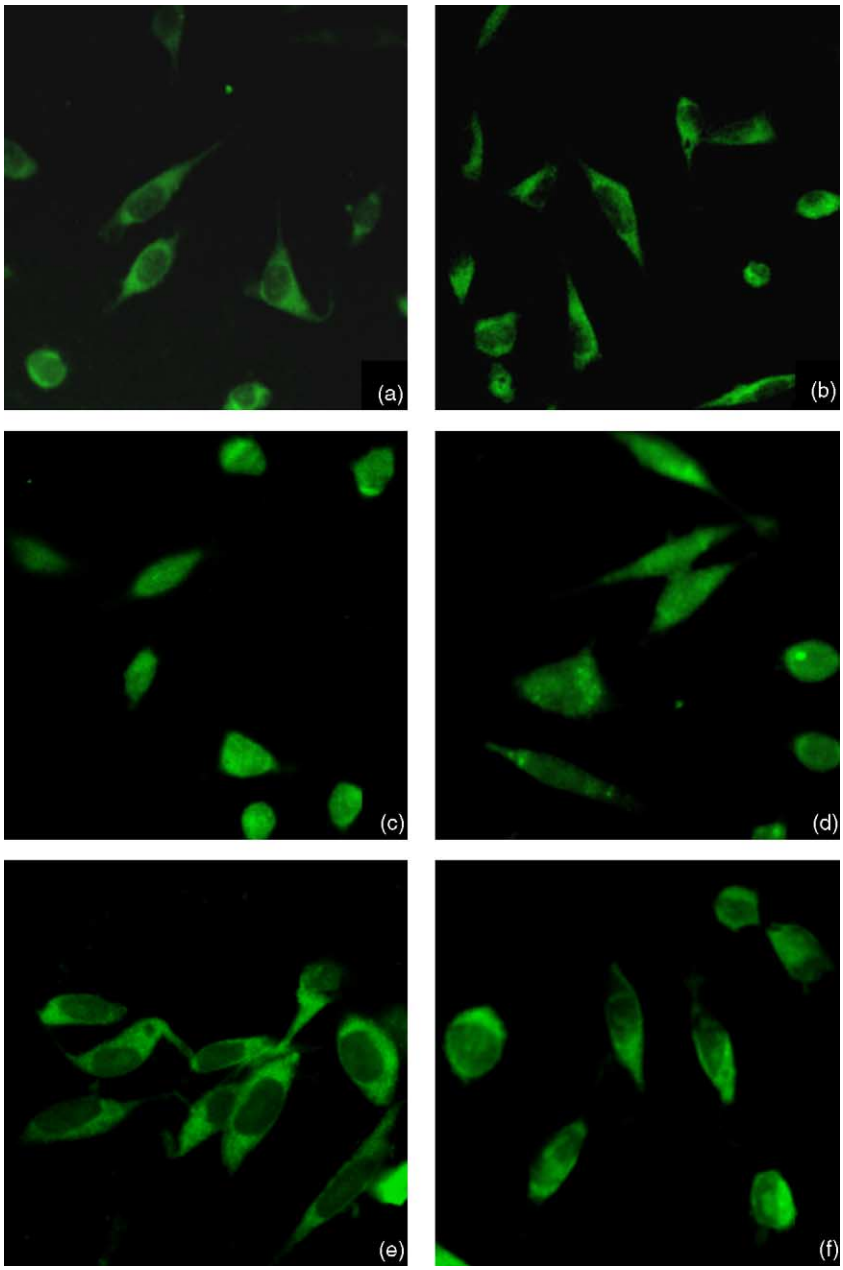

Fig. 3. Distribution of Hsp72 in HeLa cells after 24 h long cisplatin and quercetin treatment under fluorescent microscope (magnification 126×). (a) Control cells; (b) cells incubated with quercetin; (c) cells incubated with cisplatin; (d) cells preincubated with cisplatin followed by quercetin treatment; (e) cells preincubated with quercetin followed by cisplatin treatment; (f) simultaneous quercetin and cisplatin treatment.

3.5. Effect of quercetin and cisplatin on MRP expression

It is known that overexpression of multi-drug resistance proteins (MRP) results in elevated drug resistance of tumour cells. In our experiments, high level of MRP was observed in HeLa cells treated only with cisplatin (Fig. 4). Quercetin appeared to inhibit MRP expression. Preincubation of cells with flavonoid, simultaneous quercetin and cisplatin treatment for 8 and 24 h and 24 h long incubation only with quercetin were the most effective.

4. Discussion

Since it was discovered that quercetin as a component of human diet, posses several biological activities [24,46–48], many studies were performed to understand the mechanisms of its action. Our previous experiments indicated that quercetin inhibited Hsp72 expression in tumour cells and this phenomenon was correlated with increased sensitivity for apoptosis. The flavonoid also appeared as good apoptotic inducer in cells in which Hsp72 expression was blocked by antisense oligonucleotides [37–39].

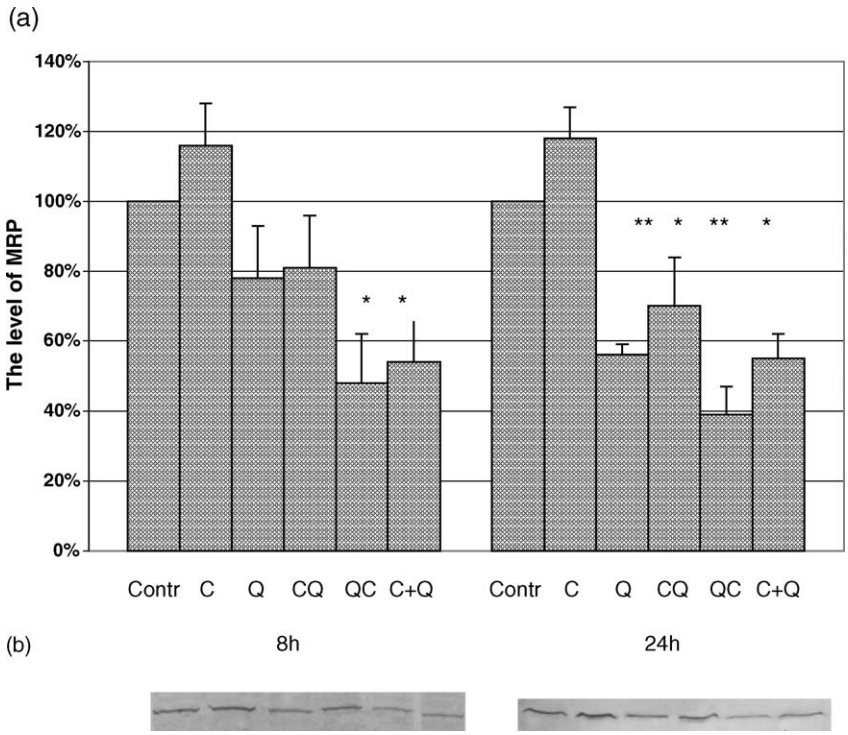

Fig. 4. Quantitative (a) and qualitative (b) analysis of the effect of quercetin (Q) and cisplatin (C) on the level of multi-drug resistance proteins (MRP) in HeLa cells after 8 and 24 h long incubation. Values are means (±S.D.) of two independent experiments carried out in duplicate. $^{*}p < 0.05$; $^{**}p < 0.005$. CQ, preincubation with cisplatin followed by quercetin treatment; QC, preincubation with quercetin followed by cisplatin addition; C + Q, simultaneous cisplatin and quercetin treatment.

Many studies demonstrated that some flavonoids exhibited a synergistic antitumour effect with chemotherapeutics [49]. For example pretreatment with quercetin of HL-60 and L1211 leukemia cells enhanced cisplatin-induced DNA fragmentation [21]. Thus, we decided to examine the effect of quercetin on the cisplatin-induced apoptosis of HeLa cells. We measured apoptosis of cells pretreated with quercetin and after that incubated with cisplatin and compared the effect with apoptosis induced by cisplatin or quercetin used separately or simultaneously. As we expected quercetin sensitised HeLa cells for cisplatin and increased the level of apoptosis.

It is known that quercetin can induce apoptosis of cells by several mechanisms. Acting as a prooxidant, it stimulates proteolytic cleavage PARP, induces loss of mitochondrial transmembrane potential and elevates reactive oxygen species (ROS) production. Alterations in mitochondrial membrane permeability and in consequence cytochrome C release is accompanied by accumulation of ROS and depletion of antioxidants, such as glutathione (GSH). Generated oxidative stress in tumours stimulates cell defence system, among others the expression of Hsp70 and Hsp27, which in turn limit cytochrome C release, apoptosome formation and procaspase-9 activation [50]. In our experiments we observed that 8 h long incubation with quercetin not significantly inhibited while 24 h long treatment enhanced Hsp72 expression. However, preincubation of HeLa cells with the flavonoid made tumour cells more volnurable for apoptosis upon cisplatin treatment. In this case quercetin inhibited Hsps expression. The mechanism of Hsp72 inhibition by quercetin is not clearly understood and several possible explanations exist. One is that quercetin inhibits Hsp72 expression at the level of transcription by preventing the Heat shock factors 1 and 2 (Hsf1 and Hsf2) binding to the conserved DNA sequence known as the Heat Shock Element (HSE) in the promoter region of *hsp* genes [51]. Other experiments indicate that quercetin acts on early events before Hsps synthesis, by blocking the additional modifications necessary for activation of Hsf, like post-translational phosphorylation or by causing conformational changes of the factor, and inhibiting its interactions with other DNA-binding proteins in the promoter region [52,53].

We cannot exclude that also localization of Hsp72 in cells may play a supplementary role in pro-apoptotic activity of quercetin. In control and quercetin treated cells Hsp72 was located mainly in cytoplasm. We observed that after cisplatin treatment, Hsp72 was located both in cytoplasm and nucleus. Preincubation of cells with quercetin blocked Hsp72 translocation from cytoplasm to the nucleus. It is known that nuclear localization of Hsp72 is an indicator of cellular stress and is correlated with stress induced alterations in nuclear architecture. Hsp72 has been suggested to participate in repair of nuclear damage, what is correlated with protection of cells against stress condi-

tions and is one of the possible mechanisms for cell protection against cell death [54–57]. Therefore detected by us inhibition of Hsp72 translocation can be considered as additional mechanism of antitumour activity of quercetin. It may be connected with stabilizing and antioxidant properties of quercetin on cell membranes, protecting phospholipids against peroxidation [58]. It can possibly make a barrier for Hsp72 to migrate from cytoplasm to the nucleus upon cisplatin treatment.

Conflicting roles of quercetin acting as antioxidant (phospholipids protection against peroxidation) and as prooxidant (increasing ROS production) depends on the redox state of the cell, concentration and the source of free radicals [59,60].

The mechanism involved in resistance of tumour cells to cisplatin is also not clearly understood. It is supposed that the induction of resistance may be associated with decreased cellular cisplatin accumulation, enhanced DNA repair and increased glutathione (GSH) or increased Hsps levels [61,62]. It seems that there might be a relationship between Hsp72 expression and the level of cisplatin-induced apoptosis of HeLa cells. The significant decrease in Hsp72 expression (after quercetin pretreatment) correlated with the highest sensitivity of HeLa cells to cisplatin-induced apoptosis. However, our results also indicate that such sensitivity may be also connected with inhibition by quercetin of MRPs expression. It is known, that cisplatin can form toxic for cells complexes with GSH which might be removed from cells by MRPs, one of the elusive glutathione-*S*-conjugate (GS-X) pomps [63,64]. In our experiments quercetin, added to the culture medium alone or before cisplatin treatment, significantly diminished the MRP levels and in consequence it sensitized cells to cisplatin-induced apoptosis.

It is known that cisplatin can induce apoptosis in caspase-3-dependent and -independent manner [5,7]. In our experiments we confirmed these observations. The lowest levels of procaspase-3 were observed in cells pretreated with quercetin (before cisplatin treatment) and we suppose that it was probably correlated with increased amount of an active form of the enzyme [65]. Decreased expression of procaspase-3 after quercetin treatment was also observed in 2774 ovarian cancer cells treated with manumycin. It was correlated with the enhancement of specific cleavage of PARP into apoptotic fragments and inhibition of Hsp70 expression [40]. It is known that high level of Hsp70 inhibits procaspae-3 processing into its active form by preventing apoptosome formation and caspase-9 activation [66].

In summary our results indicated that preincubation of cells with quercetin followed by cisplatin treatment was very effective in induction of apoptosis in HeLa cells. The pro-apoptotic activity of quercetin was mediated by several mechanisms: activation of caspase-3, inhibition of expression and nuclear translocation of Hsp72 and inhibition of MRP level.

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