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Antiproliferative and pro-apoptotic effects of quercetin on human pancreatic carcinoma cell lines EPP85-181P and EPP85-181RDB

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Abstract: Polyphenols are present in several edible plants and for many years induce high interest mainly due to their antioxidative and anti-inflammatory influence. At present, numerous studies are conducted on antineoplastic effects of the compounds. One of most effective biopolyphenols involves the flavonol quercetin. Our studies aimed at evaluation of antiproliferative and pro-apoptotic effects of quercetin alone and in combinations with daunorubicin on cells of human pancreatic carcinoma lines. The experiments were conducted on two cell lines, sensitive to daunorubicin EPP85-181P line, and its resistant variant EPP85-181RDB. Effect of studied substances on cell proliferation was detected using sulphorhodamine B (SRB) protein staining method. Apoptotic damage was estimated using comet and TUNEL techniques. Our data demonstrated that quercetin exerted cytotoxic action on cells of the both neoplastic cell lines in concentration-dependent manner. In the case of EPP85-181RDB cell line, quercetin seemed to sensitize resistant cells to daunorubicin. In parallel, the effect of both substances on the sensitive cell line was synergistic. Results of the studies confirmed that quercetin may probably break resistance of neoplastic cells to chemotherapy. On the other side, studied flavonol augmented action of cytostatic drug in case of sensitive tumour cells what suggest, that it might allow to decrease dosage of cytostatic drugs and reduce negative side effects of the treatment.

Key words: quercetin, daunorubicin, antiproliferative effect, apoptosis, human pancreatic carcinoma cell lines

Introduction

Quercetin (Q) belongs to the vast group of flavonoids present in several edible plants, particularly in onion, Italian cabbage, apples, and grapes. For the last decades Q has been known as a strong antioxidant, an anti-inflammatory agent, a vaso-dilatatory compound, and an agent which decreases blood pressure and inhibits aggregation of blood platelets [1-3]. Also on multiple occasions Q was found to exert antineoplastic effects on all stages of carcinogenesis [1,2,4,5]. However, mechanisms of Q action have not been fully recognised and continue to provide topic for intense *in vivo* and *in vitro* studies. Several of them showed that Q inhibit proliferation and induce apoptosis in cells of many tumours, including cancers of breast, lungs, colon or gliomas [2,5-11]. In contrast, in normal cells Q was noted to exert an anti-apoptotic effect [12,13].

Anthracyclins, including daunorubicin (DB), belong to cytostatic antibiotics frequently applied in chemotherapy. Cytotoxicity of DB reflects mainly its direct effect on DNA and enzymes such as topoisomerase II (Topo II), generation of high amounts of free radicals and damage to cell membrane. This results in a disturbed replication, transcription and cellular homeostasis, leading to apoptotic death of the cell. Despite its high efficacy, application of DB is restricted due to the pronounced cardio-, nephro- and myelotoxicity. Moreover, the frequently appearing multidrug resistance (MDR) causes that the therapy remains without the expected result [14,15].

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The antineoplastic properties of Q are linked mainly with its effect on expression of some oncogens, cell cycle-suppressory genes and their products. Q affects directly various type kinases, *e.g.*, protein kinase C, PI 3K, MAPK, ERK, CDK and their respective metabolic pathways, which play a significant role in carcinogenesis [16-21]. The studies on effects of carcinogens on normal cells in presence of Q, demonstrated also its antioxidant properties and a protective role against mutagenesis in healthy tissues [2,22-24]. Q exerts influence on expression of several genes and proteins, engaged in control of cell cycle, cell differentiation, programmed cell death, processes of cells adhesion and interactions [7,8,25]. Apart from the direct pro-apoptotic effect on neoplastic cells, administration of some polyphenols might be useful in overcoming MDR phenomenon acting as chemo- and photosensitizers. Results of such studies are very promising [26-29].

Considering the above data, our study aimed at examining if Q is effective cytotoxic agent toward selected cell lines of pancreatic carcinoma and if it supports pro-apoptotic action of DB.

Material and methods

Cell lines and conditions of culture. The *in vitro* studies were conducted on cell lines of human pancreatic carcinoma sensitive and resistant to DB, EPP85-181P and EPP85-181RDB respectively. Both cell lines were obtained from Institute of Pathology, University Hospital Charite in Berlin where they had been established by *in vitro* exposure to DB (Farmitalia Carlo Erba, Freiburg, Germany). The cell cultures were grown in modified Leibovitz L15 medium in a humidifed incubator at 37°C in an atmosphere of 5% CO_2. The cell culture followed the description of Lage *et al*. [30].

Proliferation tests. All proliferation tests were based on a colorimetric technique using sulphorhodamine B (SRB) dye, as described by Skehan *et al*. [31]. Substances added to the cultures, Q and DB, and the remaining reagents were purchased from SIGMA (Germany). The absorbance was read at the wavelength of 564 nm using a microplate-reader (ELX-800, BIO-TEK, USA).

On the basis of preliminary tests (calibration tests for individual lines and a cytotoxic test for the two examined compounds) the final experiment was performed, aimed to detect cytotoxic effect of DB and Q, used separately or together in various combinations of concentrations, on EPP85-181P and EPP85-181RDB cell lines. The cells were transferred to 96-well plates, 800 cells per well. Following 24 h of culture, the drugs were added in various concentrations and combinations (12 repetitions) as follow:
1) Q: Q3 (3 μM), Q6 (6 μM) and Q12 (12 μM);
2) DB: K2 (0.43 μM, the concentration corresponding to the therapeutic dose of DB in patient's blood 2h after administration of the drug, for DB it amounted to 0.25 mg/ml), K1 (0.043 μM) and K3 (4.3 μM);
3) combinations: Q3/K1, Q3/K2, Q3/K3, Q6/K1, Q6/K2, Q6/K3, Q12/K1, Q12/K2, Q12/K3 (the cytostatic drug was added to the culture 2 h after addition of Q);
4) control (with no drugs added).

Absorbance (optical density values, OD) was read after 72h drugs exposure.

Evaluation of apoptosis. Cells of both lines were transferred to 24-well plates, at 2×10^4 cells/ml, the tested substances were added, as in the case of proliferation tests, following 24 h, omitting the combination with the K3 dose of a cytostatic drug. After 72h damage to cell nuclei was detected using comet assay with neutral electrophoresis, according to the procedure of Yasuhara et al. [32]. The slides were silver-stained, as described by Cerda et al. and then analyzed under a light microscope (Olympus BX41, Japan) [33]. The proportion of damaged cell nuclei was appraised per visual field, taking into account a mean score following screening 5 fields in every slide (magnification of 200×). In the neutral technique, routinely intact cells (lack of the typical tail, normal size of the head) were marked as 0 grade, and 4 grades of cell nucleus damage are distinguished, taking into account size of the head, length of the tail and their reciprocal ratio [32]. The obtained results were collected and categorized to 3 ranges of cell nucleus damage:
1) 0 – intact cell nuclei
2) grades 1 and 2 – intermediate damage (size of the tail <50%)
3) grades 3 and 4 – apoptotic nuclei (size of the tail >50%).

In addition, the apoptotic cells were detected by the TUNEL technique. Following 72h of incubation, cells cultured and prepared as before the comet assay tests were placed on diagnostic slides, fixed in the cold (-20°C) methanol:acetone (1:1) mixture for 15 min at the temperature of 4°C and dried with a cold air. The apoptotic cell nuclei were detected using Apop Tag Peroxidase in situ Apoptosis Detection kit (INTERGEN, USA), as recommended by the manufacturer. At the terminal stage of the reaction, DAB was used as a dye and cell nuclei were counterstained with hematoxylin. The preparations were analyzed under a light microscope (Olympus BX41, Japan) using a software for computer-assisted image analysis (Analysis 3.2, Germany).

Percentage of apoptotic cell nuclei was appraised per visual field, taking into account a mean score from 3 fields for every well (magnification ×200) in every slide for 3 wells/repetitions.

Statistical analysis. The obtained results were subjected to statistical analysis using Student's t-test and taking adventage of SPSS 14 software (USA). The results were regarded statistically significant at $p<0.05$.

Results

Effect of DB and Q on cells proliferation

EPP85-181P line

Effect of DB and Q applied separately, as compared to the control. The line of EPP85-181P was found to be sensitive to action of DB in cases of all tested doses. K1 reduced cell number by 32%, K2 by 89%, while K3 by 94%, as compared to the control. Sensitivity of cells to Q was also evident in every applied concentration of the polyphenol. In comparison with the control, Q3 concentration decreased OD by 22%, Q6 by 36%, while Q12 by as much as 74%. All differences proved to be statistically significant ($p<0.001$).

Effect of DB/Q combinations in comparison with the action of Q alone (Fig. 1). Administration of Q in parallel to DB, as compared to application of the same concentration of Q alone, decreased OD in all tested cases. The combinations with DB demonstrated inhibition of proliferation dependent on the dose of the cytostatic drug and the differences were statistically significant ($p<0.001$).

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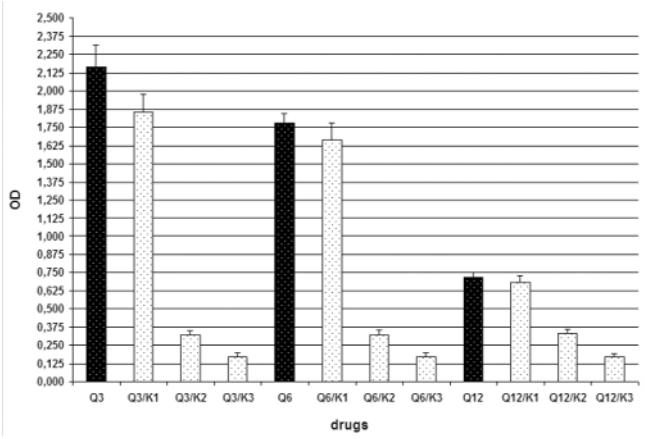

Fig. 1. Effect of administering various concentrations of Q and DB on optical density (OD) for cells of EPP85-181P line, p<0.001 for all drug combinations as compared to effect of Q alone.

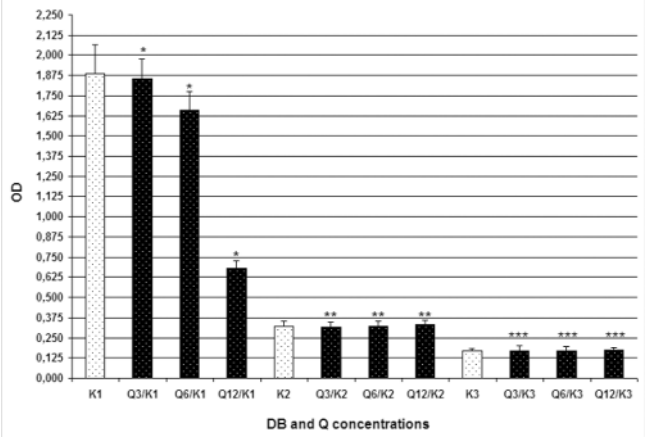

Fig. 2. Effect of administering various concentrations of Q and DB on optical density (OD) for cells of EPP85-181P line, *p<0.001 for combinations K1/Q *vs* K1, **p<0.05 for combinations K2/Q *vs* K2, ***p>0.05 for combinations K3/Q vs K3.

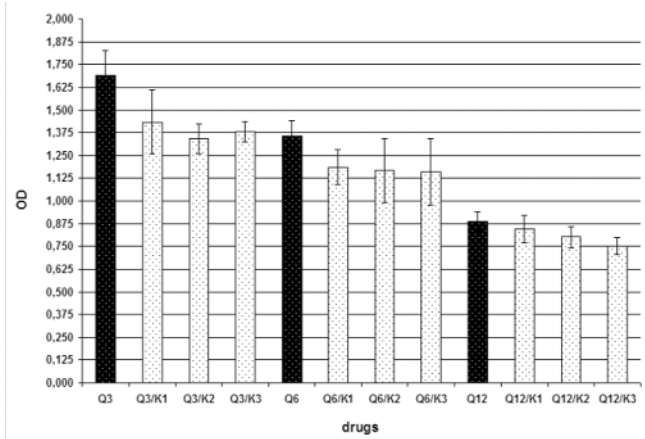

Fig. 3. Effect of administering various concentrations of Q and DB on optical density (OD) for cells of EPP85-181RDB line, p<0.001 for all drug combinations as compared to effect of Q alone.

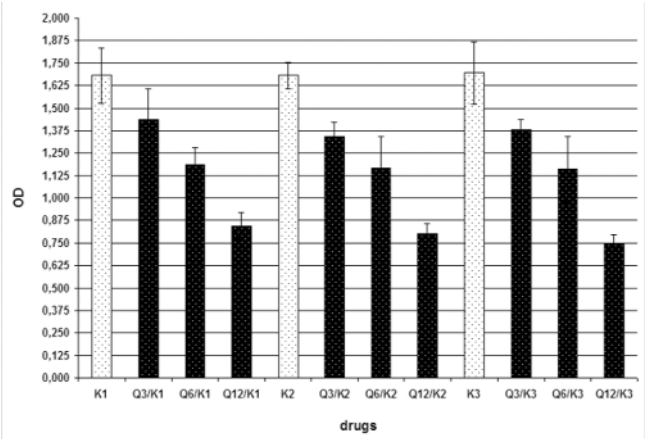

Fig. 4. Effect of administering various concentrations of Q and DB on optical density (OD) for cells of EPP85-181P line, p<0.001 for all drug combinations as compared to effect of Q alone.

Effect of DB/Q combinations in comparison with the action of DB alone (Fig. 2). Only the combinations of Q6/K1 and Q12/K1 induced a statistically significant decrease in the cell number (p<0.001) as compared to the effect of the cytostatic drug alone (K1).

EPP85-181RDB line

Effect of tested substances, as compared to the control. No effect of DB on cells of the resistant line was detected, the differences in absorbance were insignificant (p>0,05). On the other hand, Q exerted effect on the studied cells in concentrations of Q6 and Q12. Following administration of Q6 OD decreased by 24%, and following Q12 by 50%. The differences between effects of individual doses and also between Q6 or Q12 and the control were statistically significant (p<0,001). The concentration of Q3 exerted no significant effect on the proliferation (p>0.05).

Effect of DB/Q combinations in comparison with the action of Q alone (Fig. 3). Combinations of DB and Q in comparison with the influence of Q alone decreased OD in a statistically significant manner (p<0.001). Only in the case of Q12/K1 the difference was insignificant (p>0.05).

Effect of DB/Q combinations in comparison with the action of DB alone (Fig. 4). As compared to the effect of DB, supplementation with Q resulted in an additional inhibition of proliferation in cases of any applied concentration of Q. The differences between individual drug combinations were also statically significant (p<0.001).

Apoptotic damage under effect of quercetin and daunorubicin

EPP85-181P line. As demonstrated by results of the comet technique, pro-apoptotic action of DB, as

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Table 1. Effect [%] of Q and DB on cells of EPP85-181P (P) and EPP85-181RDB (RDB) lines, examined by the TUNEL technique.

Sample	P	RDB
	% apoptotic cell nuclei	
Control	3	0
K1	15	0
K2	81	0
K3	90	0
Q3	13	0
Q3/K1	64	1
Q3/K2	80	2
Q6	38	0
Q6/K1	72	1
Q6/K2	82	3
Q12	50	15
Q12/K1	82	21
Q12/K2	90	30

compared to the control, was noted in K2 and K3 concentrations of the cytostatic drug: in both cases the apoptotic cell nuclei comprised around 80%. The remaining cell nuclei (15-18%) also demonstrated injuries, which were classified as intermediate ones.

Cell cultures supplemented with Q alone contained around 20% of typical apoptotic cell nuclei for Q3 and Q6, while for Q12 their number increased to 30%, as compared to the control. In case of intermediate damage of cell nuclei for Q3 comprised the same fraction of apoptotic nuclei (20% each, summing up to 40% of affected cell nuclei). For Q6 the intermediate damage was slightly more frequent, accounting for the total fraction of 50% affected nuclei. In case of Q12 only 40% cell nuclei remained without injury, with the intermediate damage comprising 30%.

In comparison with action of DB alone, supplementation with Q increased the fraction of damaged cell nuclei. In combinations Q with K1 high levels of intermediate damage could be noted (80%). In combinations of Q3/K2, Q6/K2 the fraction of typical apoptotic cells increased (up to 80%) as compared to K2. Frequency of intermediate damage was lower than in combinations with K1.

The combinations DB/Q were more effective than action of Q alone, particularly a marked increase was noted in the number of apoptotic cell nuclei (Fig. 5).

On the other hand, the TUNEL technique (Table 1) demonstrated that both DB and Q exerted pro-apoptotic effect on cells of the EPP85-181P line. Following

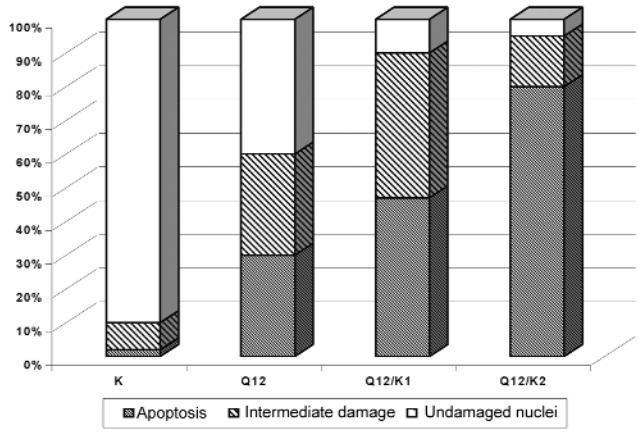

Fig. 5. Effect of Q12 administration and its supplementation with DB on proportion of damaged cell nuclei [%] in EPP85-181P cell line.

administration of Q/DB combinations the action was synergistic. Q intensified effects of DB, particularly in presence of the lowest DB concentration of K1. Administration of Q12/K1 yielded the same effect as the tenfold higher, therapeutic dose of DB (K2) did. K2 exerted such a high effect alone that supplementation with the polyphenol yielded not so pronounced changes.

EPP85-181RDB line. In the case of resistant cell line of pancreatic carcinoma DB induced apoptotic injury only in a low percentage of cell nuclei, examined using the comet technique.

Q induced damage in cell nuclei in all applied concentrations. In cases of Q3 and Q6 the fraction of damaged cell nuclei was low (around 6 – 6.5%), but for Q12 it increased to 17%. In every case the intermediate damage was rare. The highest percentage of all damaged cell nuclei, 20%, was observed for Q12.

Administration of Q/DB combination increased frequency of damaged cell nuclei as compared to action of DB alone. In the cases of combinations with K1 significant alterations and increased number of cell nuclei with intermediate damage in particular could be noted only for Q12/K1. More pronounced differences could be observed in all K2 combinations with Q (Fig. 5).

On the other hand, comparison between influence of Q concentration alone and combinations DB/Q has shown rising fraction of apoptotic cells in the case of suplementation with Q12. The combinations with cytostatic drug significantly augmented frequency of apoptotic changes, particularly of intermediate damage (Table 2).

TUNEL technique (Table 1) demonstrated no significant apoptotic alterations in the resistant cells following administration of DB alone, Q3 or Q6 concentrations or combinations of these polyphenol concentrations with the cytostatic drug. Only in the case of Q12 concentra-

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Table 2. Effect [%] of various Q and DB combinations on nuclear damage in cells of 85-181RDB cell line, as compared to effect of Q alone, examined by the comet technique, C – control.

Sample	C	Q3	Q3/K1	Q3/K2	Q6	Q6/K1	Q6/K2	Q12	Q12/K1	Q12/K2
Apoptosis	0.3	6.5	2	10	6	3.5	9	17	18	20
Intermadiate damage	2.9	0	2	2	1	4.4	0	3	0	13
Intact nuclei	96.8	93.5	96	88	93	92.1	91	80	82	67

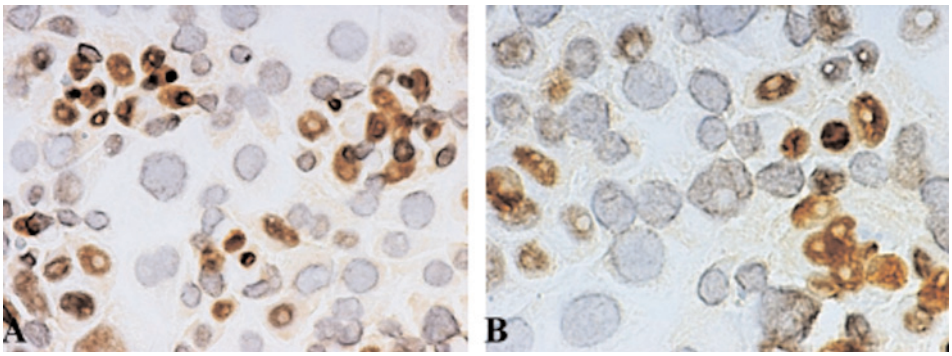

Fig. 6. DNA damage, apoptotic nuclei stained brown. TUNEL technique, original magnification ×400. (**A**) Cells of EPP85-181RDB line subjected to action of Q12. (**B**) Cells of EPP85-181RDB line subjected to action of Q12/K1.

tion (Fig. 6A), in combinations of Q12/K1 (Fig. 6B) and Q12/K2 an evident pro-apoptotic effect could be noted.

Discussion

A flavonol quercetin (Q), the *in vitro* activity of which on selected neoplastic cell lines provided topic for this study, is commonly present in vegetables and fruits [2]. Two cell lines of human pancreatic cancer, a sensitive and a resistant to DB, provided the cell models.

Tests of cytotoxicity showed that Q inhibits cell divisions in the both studied neoplastic cell lines in a time- and concentration-dependent manner. The subsequent step involved testing if Q accentuates anti-proliferative effect in the sensitive cell line of human pancreatic carcinoma and if it sensitizes cells to action of a cytostatic drug in the case of the resistant cell line. The experiment was conducted using standard doses of DB, the therapeutic one, the tenfold lower and the tenfold higher one. In the case of Q the priority was to select as low as possible doses, as compared to the commonly used ones, to exclude their possible toxic effect on normal cells. Anti-proliferative action on neoplastic cells of various plant polyphenols including Q is commonly known and proven [34,35]. In parallel, its protective role is stressed in respect to normal cells, related to its antioxidative, anti-inflammatory, cardioprotective, neuroprotective and anti-carcinogenic effects [10,36-39]. It has also been suggested that exists possibility of some toxic effect of Q, even in normal cells, particularly following supplementation of high doses of the flavonol. Therefore, results of studies on its interactions with various drugs seems relatively controversial [7,13,40,41].

Our studies showed that both studied compounds exert effect on the sensitive cell line. Administration of the cytostatic drug alone in its therapeutic dose abolished cell proliferation almost completely. A strong anti-proliferative effect was manifested also by Q alone, particularly its Q6 and Q12 concentrations. Applied together, Q and DB acted synergistically on P cells, which could have been observed on the example of the lowest concentration of K1. The result suggests that in cases of cells sensitive to cytostatics administration of Q might allow to significantly decrease doses of the drug. This effect might be linked to strong inhibition of topo II by both compounds [18,42]. The decrease in therapeutic dose of DB may prove important due to the chance for prevention of negative side effects induced by the drug, assuming that Q acts selectively: it inhibits proliferation only in neoplastic cells in parallel protecting normal tissues [13,21,36].

In the case of the studied resistant cell line, administration of polyphenol alone significantly inhibited proliferation, particularly in its Q12 concentration. Application of Q in combination with DB evidently intensified the effect. Most probably, Q sensitizes neoplastic cells resistant to anti-proliferative action of DB in the manner dependent on Q concentration and, to a slightly lower extent, on dose of the cytostatic drug. The results corroborate data obtained by other investigation centres [9,43,44].

The subsequent step involved examination if the reduced number of cells reflects only more pro-

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nounced inhibition of cell divisions or Q sensitizes the resistant cells to action of DB, inducing their death by apoptosis and, also, if Q may amplify the effect in cells of pancreatic carcinoma cells line sensitive to DB.

The vast literature devoted to Q shows that it manifests pro-apoptotic properties, as documented in cases of multiple types of tumours [45-47]. Most probably, this involves the late type apoptosis, developing along the mitochondrial pathway, sometimes independent of death receptors (through effects on MAPK, ERK kinases, protein kinase C, proteins of Bcl family or caspases), which was proven on the example of melanoma cells [48]. Additionally, quercetin augments TRAIL-induced apoptosis by affecting the ERK signal transduction pathway [20]. Q may affect also transcription factors, products of suppressor genes in the cell cycle, *e.g.* p-53. In cases of mutations in this type regulatory proteins, Q mobilizes apoptosis along other pathways, independent of p-53 protein, which was documented i.e. for cells of prostate cancer [9,17,49]. It is also known that Q may trigger apoptosis in cells of several types of tumours, including pancreatic carcinoma. Additional analysis of apoptosis-linked genes in several of them demonstrated increased expression in presence of as low as 5 μM concentration of Q [7,48-50]. Moreover, Q does not induce apoptosis in normal cells in concentrations around 10 μM and, reciprocally, in such a situation it may protect cells from oxidative stress, inhibit apoptosis and supress neoplastic transformation [13,37,40,51,52].

In case of the sensitive cells, the comet technique proved pro-apoptotic effects of Q and DB, when applied separately. The tendency persisted in the case of Q/DB combination, in which both Q and DB concentrations were important. This points to a synergistic effect of the two substances. It should be accentuated that apoptosis was strongly intensified after treatment with Q12 and its combinations with DB (3 and 4 grade according to Yashuara *et al*.), while the remaining combinations have contained high fraction of intermediate damage (grades 1 and 2 according to Yashuara *et al*.) [32]. Intensity of apoptosis examined using the TUNEL technique proved slightly lower than that documented by the comet technique but the general tendencies were confirmed.

In the resistant cell line pro-apoptotic effects could have been demonstrated by the comet assay only for Q12 concentration and its combinations with individual doses of DB. On this basis it might be assumed that Q concentrations lower than 12 μM inhibit cell divisions (cell number lower than in the control) but they do not mobilize the apoptotic process. This was also evident that the action of Q12 with combination with DB is augmented in comparison with the effects of the Q and DB given separately. These tendencies were confirmed using TUNEL technique.

The above data are consistent with results which suggested that Q it is able to act synergistically with other pro-apoptotic drugs and, in the cases of cells resistant to cytostatic drugs, it may sensitize such cells to action of the cytostatics [25,50].

On the basis of multi-annual studies Q, even in its high concentrations, is known to protect normal cells from toxins and oxidative stress, rather than exert negative side effects [2,53]. Q acts as a strong anti-oxidant in cases of cells such as epithelium of renal tubuli, cardiomyocytes or bone marrow cells and, therefore, it may protect against cardio-, myelo- or nephrotoxicity of the cytostatic drugs, including DB [13,44,53-55]. The described by some research teams mutagenic action of Q on normal cells was probably linked to administration of very high doses, most frequently exceeding 50 μM. In cases of low concentrations (<20 μM) Q also affects genes but it is not a genotoxic or carcinogenic effect. Analysis of various investigations performed during several years, including *in vivo*, *in vitro* and clinical studies, confirmed by detailed analysis of several genes expression, corroborate the thesis [56-58].

Results of our studies confirmed that Q sensitizes cells of human pancreatic carcinoma line resistant to DB. It seems promising for the therapy of tumours manifesting drug resistance. Moreover, Q amplifies action of the cytostatic drug on cells of the sensitive line, which provides perspective for decreasing dosage of the drug. This might be particularly valuable that the synergistic effects of Q and DB in their antiproliferative and pro-apoptotic actions take place only in cases of neoplastic cells, while normal cells in parallel are protected, also against toxic action of cytostatic drugs [3,16,51].

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