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Cytotoxic and Apoptotic Effect of Structurally Similar Flavonoids on Parental and Drug-Resistant Cells of a Human Cervical Carcinoma

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Summary

Flavonoids are phytochemicals characterized by a wide range of biological activities, including antioxidant activity, the ability to modulate enzyme or cell receptor activity patterns, and to interfere with essential biochemical pathways. Using HeLa cells of a human cervical carcinoma, and their drug-resistant HeLa CK subline, the effects of three structurally related flavonoids (quercetin, fisetin and luteolin) have been examined, in terms of their: (*i*) cytotoxicity, (*ii*) influence on intracellular glutathione (GSH) level, (*iii*) influence on glutathione S-transferase (GST) activity, and (*iv*) influence on the expression of apoptosis-related genes (PARP, Bcl-2, survivin). Fisetin was more toxic to resistant HeLa CK cell line than to parental cell line, causing decreased expression of survivin in the same cell line. Concentrations of 5 μM of the examined flavonoids caused PARP degradation in parental cell line, leading HeLa cell line into apoptotic cell death. The same event was not determined in the resistant cell line. Fisetin and luteolin induce glutathione and GST in the resistant cell line, pointing to complex cellular effects which could be responsible for higher sensitivity of the resistant cell line in comparison with the parental cell line. Prooxidative nature of the investigated flavonoids was not detected, so free radical formation is not responsible for the induction of GSH, GST and proapoptotic enzymes.

Key words: apoptosis, drug-resistant cells, flavonoids, glutathione, glutathione S-transferase

Introduction

Flavonoids represent a large family of polyphenolic compounds synthesized by plants. The feature they have in common is their chemical structure, characterised by one or more condensed aromatic rings. Due to such structure, flavonoids have specific colour, smell and taste. They exert a wide range of biological activities (*1*). In addition to their antioxidant activity, which is one of the most important features of their functioning, flavonoids can modulate the activity of enzymes or cell receptors, and interfere with the essential biochemical pathways, suggesting their involvement in biochemical and physiological processes not only in plants, but also in humans (*2–4*). Beneficial health effects of fruits and vegetables have been attributed, in part, to flavonoids they contain. Flavonoids can inhibit mutagen uptake or endogenous mutagen formation, activate or modulate cellular detoxifying mechanisms, protect DNA nucleophilic sites, act as reactive oxygen species scavengers, *etc*. (*5–9*). Lee-Hilz *et*

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al. (*10*) showed that flavonoid conjugates induce EpRE-mediated gene expression and that induction of this gene involves deconjugation of quercetin glucuronides. Also, there is plenty of evidence of the prooxidative nature of flavonoids depending on concentration, time of incubation, presence of detoxifying enzymes and transitional metals (*9–12*). Prooxidative nature of flavonoids is related to their cytotoxicity, but antioxidant and prooxidant behaviour of polyphenols strongly depends upon their concentration and free radical source (*13*). However, in spite of abundant data on their biological effects, structural prerequisites and mechanisms underlying these effects are not fully understood yet. Previous investigations have shown that the determination of mechanism of action of compounds characterized by such properties is not a simple, but, on the contrary, even an impossible task (*9–13*). The notion that more than 5000 distinct flavonoids have been identified in plants (*1*), and the fact that in commonly consumed foodstuffs several hundred flavonoids may occur, makes the issue even more complex.

In this study, modulatory effects of three structurally related flavonoids are examined: quercetin, fisetin and luteolin, using the following outcome measures: (*i*) cytotoxicity, (*ii*) intracellular glutathione level, (*iii*) glutathione S-transferase activity, and (*iv*) expression of apoptosis-related genes (PARP, Bcl-2 and survivin). In the present study, cervical carcinoma cell lines, parental HeLa cells and their drug-resistant HeLa CK subline are used in order to determine if drug-resistant HeLa CK cells are more sensitive to flavonoids than their parental HeLa cells.

Materials and Methods

Human cell lines

HeLa cells of a human cervical carcinoma were grown as monolayer cultures in Dulbecco's Modified Eagle Medium (DMEM; GIBCO, USA), supplemented with 10 % of the foetal bovine serum (GIBCO, USA), 4500 mg/L of glucose and 1 % of penicillin/streptomycin. Drug-resistant HeLa CK subline was developed in the Laboratory of Genotoxic Agents at the Ruđer Bošković Institute (Zagreb, Croatia), *via* treatments utilising stepwise-increased concentrations of cisplatin (*14*). By virtue of the aforementioned, HeLa CK cells became resistant to cisplatin and cross-resistant to vincristine and methotrexate (*14*). In these cells, activation of stress-activated protein kinases/c-Jun-N-terminal kinases (SAPK/JNK) and p38 kinase, as well as Fas L expression, induced by cisplatin, were attenuated as compared to the parental cells (*15*). The expression of heat shock protein (HSP) 70, induced by anticancer drugs and hyperthermia, was altered, too (*16*), while the levels of markers pointing towards cancer invasion and metastasizing (cathepsin D and plasminogen activator inhibitor type 1) were increased (*17*).

Chemicals

Flavonoids (quercetin, fisetin and luteolin), Neutral Red (NR), Ellman reagent, NADPH, glutathione, glutathione reductase, 1-chloro-2,4-dinitrobenzene, thiobarbituric acid (TBA) and malondyaldehyde (MDA) were purchased from Sigma Chemicals (Germany). Primary antibody anti-PARP (mouse monoclonal C2-10) was purchased from Pharmingen, USA, anti-bcl-2 (mouse monoclonal Ab-1) was purchased from Oncogene USA, anti-survivin (rabbit polyclonal FL-142) and anti-actin (mouse monoclonal C-2) were purchased from Santa Cruz USA. HRP-labelled secondary antibodies were purchased from Amersham Pharmacia Biotech, Sweden, sheep anti-mouse NA 931 and donkey anti-rabbit NA 934 ECL systems were purchased from Amersham Pharmacia, UK. The chemicals used in the experiments were of analytical grade.

Flavonoids

Prior to each experiment, DMSO stock solutions for each flavonoid were prepared. Each freshly prepared stock solution served as a source for the preparation of the dilutions in growth medium (DMEM). The cytotoxic effects of flavonoids were investigated in dilution series ranging from 100 mM to zero. Flavonoids were dissolved in DMSO at a concentration 1000 times higher than the highest concentration used in the experiment. All concentrations of diluted flavonoids used in the experiment were checked for the formation of microscopically apparent crystals to be sure that all flavonoids are in soluble form after dilution of stock solutions in DMEM (*18*). In all experiments, final concentration of DMSO present in the cell culture medium was below 0.1 % at all times.

Cytotoxicity assay

Cytotoxicity of selected flavonoids was determined by Neutral Red (NR) assay. HeLa and HeLa CK cells were seeded in wells ($4.5 \cdot 10^3$ per well) of 24 well plates. After a 24-hour incubation, cells were treated with different flavonoid concentration ranges (0.01–100 μM of each tested flavonoid) for 72 h, subsequent to which the NR assay was carried out, as described by Babich and Borenfreund (*19*). The intensity of absorbance was measured at 540 nm in Cecil spectrophotometer (Cecil Instruments Ltd, Technical Centre Cambridge, UK). The effect of appropriate dilutions of DMSO as a solvent had been taken into account as well. Each flavonoid concentration was tested in quadruplicate, and each experiment was repeated three times.

Determination of gluthatione level

Intracellular glutathione (GSH) content was examined spectrophotometrically, according to the procedure developed by Tietze (*20*). Cells ($4 \cdot 10^5$) were seeded in 10-centimetre Petri dishes. After a 24-hour incubation, cells were incubated with 5-μM concentrations of the tested flavonoids and incubated for the next 72 h (for each experiment, 3 replicates of each cell line for each flavonoid were used). Thereafter, cells were collected, counted, lysed and centrifuged at 12 000 rpm for 15 min. GSH was determined in supernatants following the reaction with 5,5′-dithio-bis-(2-nitrobenzoic acid). The formation of 2-nitro-5-thiobenzoic acid, which absorbs at 412 nm, was monitored. The results are expressed as GSH concentrations (μM per mg of protein). The measurements of GSH concentrations were performed in

triplicates for each treated Petri dish. Total amount of proteins present in the supernatants was determined by Bradford assay (*21*).

Determination of glutathione S-transferase activity

Glutathione S-transferase (GST) activity was measured as described by Habig and Jacoby (*22*). The activity of this enzyme was determined in the same supernatant in which the GSH level was determined, using 1-chloro-2,4-dinitrobenzene as electrophylic substrate. GST activity was expressed in terms of the amount of conjugate formed (nmol/(min·mg protein)). The measurements of GSH concentrations were performed in triplicates for each treated Petri dish.

Measurement of lipid peroxidation

Cells ($4.5 \cdot 10^5$) were seeded in 10-centimetre Petri dishes. After a 24-hour incubation, they were treated for 72 h with 5-μM flavonoids either solely or in combination with hydrogen peroxide (8 μM). As a positive control, cells were treated either with 8 or 80 μM hydrogen peroxide. Thereafter, the cells were scraped, washed twice with PBS, lysed in 520 μL of potassium chloride (1.15 %) for 30 min, and centrifuged at 5000 rpm. A volume of 500 μL of the supernatant was incubated with 2 mL of TBA (100 g/L) for 15 min at 100 °C, cooled at room temperature, and centrifuged at 1000 rpm for 10 min. The volume of 2.5 mL of the supernatant, obtained in the previously described manner, was mixed with 1 mL of 0.8 % trichloroacetic acid (TCA) and incubated for 15 min at 100 °C. After cooling with tap water, the absorbance of the samples was determined both at 532 and 600 nm (the latter concerns non-specific absorbance). The concentration of MDA-TBA complex, as an indicator of lipid peroxidation, was calculated from standard curves (*23,24*). The concentration of cellular proteins was determined according to the Bradford method (*21*). The results are expressed as concentrations of TBA-MDA complex (μM per mg of protein). Each experiment was repeated three times.

Western blot analysis

Cells ($4.5 \cdot 10^5$) were seeded in 10-centimetre Petri dishes, incubated for 24 h and then treated for 72 h with 5 μM of the tested flavonoids. Protein extracts were prepared *via* cytolysis in RIPA buffer (150 mM NaCl, 1 % NP-40, 0.5 % sodium deoxycholate, 0.1 % SDS, 50 mM Tris HCl, pH=8) containing 10 mM EDTA and 1 mM PMSF (30 min at 4 °C). After centrifugation (15 min, 15 000×*g*), the concentration of proteins in the supernatant was determined according to the Lowry method (*25*). For each sample, 25 μg of protein were loaded on 12.5 % SDS-polyacrylamide gel, electrophoresed, and transferred to nitrocellulose membrane (0.22 μm, Protran, Canada). Non-specific binding membrane sites were blocked by incubation (1 h at room temperature) in the blocking buffer (Tris-buffered saline (TBS) buffer containing 0.1 % Tween 20 (by mass per volume) and 5 % milk (by mass per volume) (Sirela, Croatia). Subsequently, the membranes were incubated (1 h at room temperature, or overnight at 4 °C) with the following primary antibodies: anti-PARP, anti-bcl-2, anti-survivin and anti-actin. Except for anti-actin, which was diluted to 1:500 (by volume), all other target antibodies were diluted to 1:1000 (by volume). Thereafter, the membranes were incubated for 1 h with the following HRP-labelled secondary antibodies: sheep anti-mouse (diluted to 1:2500, by volume), and donkey anti-rabbit (diluted to 1:5000, by volume). Membranes were developed using the ECL system, according to the manufacturer's instructions.

Statistical analysis

Statistical analyses were performed using the SPSS v. 8.0 (SPSS Inc., Chicago, IL, USA). The one-way analysis of variance (ANOVA) was employed to determine whether the means obtained with various groups differ significantly from each other. The significance was established using the Dunnett post-hoc test. The probability level of $p<0.05$ was considered significant. All data are expressed as means±standard deviations (SD) of the values obtained by three independent measurements.

For cytotoxicity testing, data sets were analyzed by linear regression, and EC_{10} and EC_{50} were determined from the direction of the equation (*18*).

Results and Discussion

Cytotoxicity

The last decades have witnessed an increasing interest in investigations of biological effects of secondary plant metabolites, which represent normal constituents of a human diet. Dietary flavonoid intakes and food sources have been based mostly on the content of only three flavonols (quercetin, myricetin and kaempherol) and two flavones (apigenin and luteolin). Using different cell lines and animal models, it has been shown that flavonoids affect cellular signalling pathways, inhibit cellular proliferation, induce cell cycle arrest and apoptosis and stimulate angiogenesis (*26*). Quercetin is one of the most investigated flavonoids, while fisetin is almost uninvestigated (*1*). Therefore, quercetin, fisetin and luteolin were chosen for our investigation (Fig. 1).

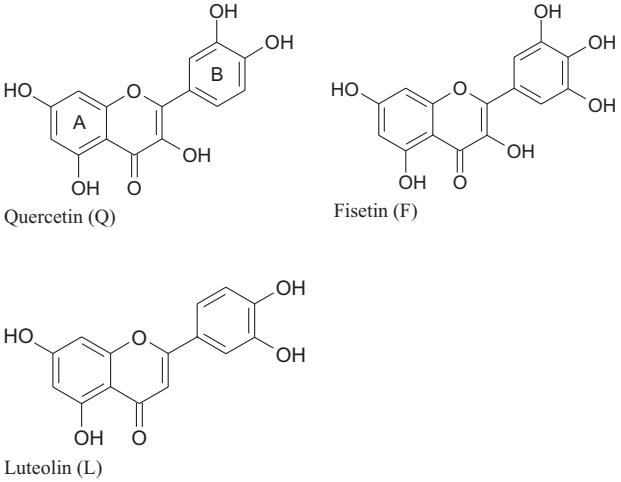

Fig. 1. Chemical structure of the investigated flavonoids

In this study, survival curves and the highest non-toxic concentrations of the investigated flavonoids were determined using Neutral Red Test, subsequent to 72-hour cell treatment with a particular flavonoid (Fig. 2). According to the literature data, in this period of time, flavonoids accomplish their biological effects (*2,27,28*). Quercetin proved to be more toxic to HeLa (EC_{50}=7.3 μM) than HeLa CK cells (EC_{50}=9.8 μM). Fisetin was more toxic to HeLa CK cells (EC_{50}=3.8 μM) in comparison with parental HeLa cell line (EC_{50}=10.5 μM). Toxic effect of luteolin was observed at the concentration of 13.4 μM on parental cell line and it was more toxic to resistant HeLa CK cell line (EC_{50}=7.4 μM). DMSO, which was used as a solvent, when tested at the highest concentration applied (0.1 %), failed to affect cell viability in any significant manner.

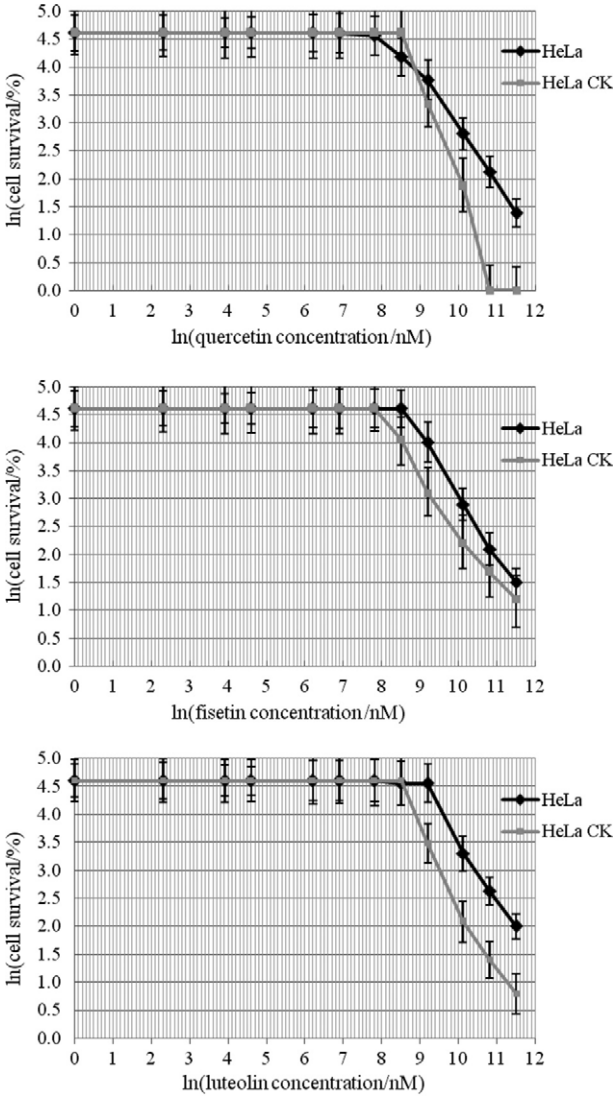

Fig. 2. Survival of human cervical carcinoma HeLa and HeLaCK cells following 72-hour treatment with flavonoids. Results are shown as ln values (±S.D.) obtained within three experiments. Estimated cytotoxicity EC_{50} values of three flavonoids in parental HeLa cells are: quercetin 7.3 μM, fisetin 10.5 μM and luteolin 13.4 μM. Estimated cytotoxicity EC_{50} values of three flavonoids in HeLa CK subline are: quercetin 9.8 μM, fisetin 3.8 μM and luteolin 7.4 μM

According to their EC_{50} values, flavonoids can be ranked as follows: quercetin>fisetin>luteolin for HeLa and fisetin>luteolin>quercetin for HeLa CK cell line. This is in line with the literature data generally referring to the relationship between flavonoid chemical structure (Fig. 1) and their cytotoxicity (*2,12,28*). However, it should be pointed out that in the present study, cytotoxic effect of three different flavonoids was examined *in vitro*, on cultivated cell lines. In this work, the cytotoxicity of flavonoids was proven to be cell type-specific (*28*). Parental HeLa cells were more resistant to toxic effects of fisetin and luteolin than were the cells of drug-resistant cell line. Since prooxidative activities of the investigated flavonoids were not detected when they were applied at 5 μM concentrations (see below), these results are in agreement with the findings of Sergediené *et al.* (*13*), who pointed out that prooxidative nature of polyphenols depends upon concentration that is applied. They proved that quercetin caused strong cytotoxic effect accompanied by prooxidant events at the concentration of 300 μM. Since this concentration is 60 times higher than the concentrations of flavonoids applied in this work, no prooxidative effect was observed at 5-μM concentrations of the investigated flavonoids.

In all investigations of biological effects of flavonoids carried out in this study, 5-μM concentrations of each tested flavonoid were applied. There was no reason to investigate biological effects of higher concentrations of flavonoids because they could not be obtained in systemic circulation. According to Van der Woude *et al.* (*29*), blood serum levels in humans after oral quercetin ingestion from onions vary around 1 μM, while higher quercetin concentrations are found in the intestine. After ingestion of a standard quercetin supplement (250–500 mg), free quercetin concentration inside the intestine lumen may vary between 0 and 100 μM (*29*). This fact explains why the influence of nonmetabolised flavonoid aglycones was examined here. In fact, deconjugation of conjugated flavonoids can occur in *in vivo* conditions resulting in production of aglycones (*30–32*).

Van der Woude *et al.* (*29*) reported that quercetin precipitated in aqueous medium at a higher concentration (80 μM). Concentrations of flavonoids used for testing biological effects in this work were more than 10 times lower than the concentration of flavonoids that was shown to precipitate in the aqueous medium.

Level of total cellular glutathione

The effects of flavonoids on basal (constitutive) level of gluthatione hosted by HeLa and HeLa CK cells are shown in Fig. 3. We found similar constitutive levels of GSH both in parental HeLa and in drug-resistant HeLa CK cells. None of the examined flavonoids altered GSH level encountered in the parental HeLa cells. On the contrary, all flavonoids increased GSH level encountered in the drug-resistant HeLa CK cells. Since the investigated flavonoids did not alter GSH level in HeLa cells, but increased it in HeLa CK cells, this effect should also be viewed as a cell type-specific. The increase of GSH level, observed in HeLa CK cells following the treatment with examined flavonoids, might suggest that these compounds increase an antioxidative-protective capacity of

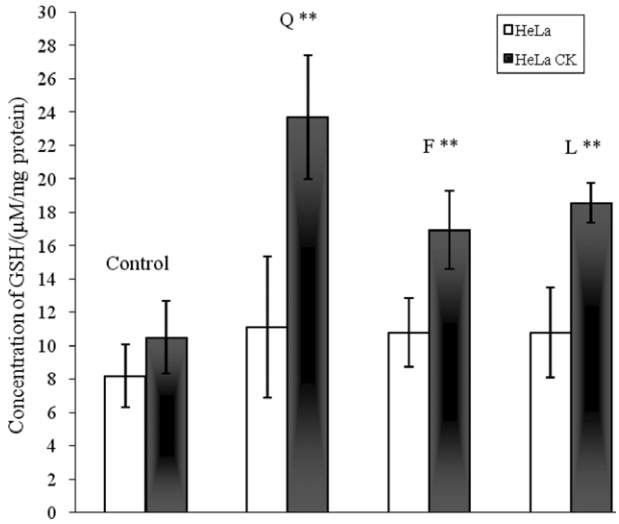

Fig. 3. The concentration of glutathione in HeLa and HeLa CK cells, obtained after 72-hour treatment with 5-μM concentrations of flavonoids: quercetin (Q), fisetin (F), luteolin (L), and untreated control (C). Pooled data obtained from three experiments (the mean at the point±S.D.). Statistically significant increase (*) was not observed in GSH levels of HeLa cells (white bars) as compared to the control, while significant increase (**) in GSH content in HeLa CK cells (gray bars) as compared to the control was detected after the treatment of cells with the tested flavonoids. Basal levels of GSH in untreated HeLa and HeLa CK cell lines were not statistically different

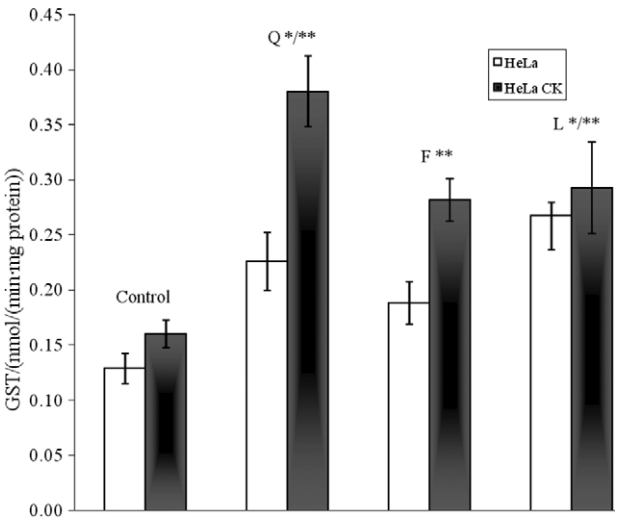

Fig. 4. Activities of glutathione S-transferase (GST) in HeLa and HeLa CK cell lines after 72-hour treatment with 5-μM concentrations of flavonoids: quercetin (Q), fisetin (F), luteolin (L), and untreated control (C). Pooled data obtained after three experiments (the mean at the point±S.D.). Statistically significant difference of GST activity obtained after the treatment of HeLa (*) and HeLa CK cells (**) with the tested flavonoids, as compared to untreated controls. Basal levels of GST in untreated HeLa and HeLa CK cell lines were not statistically different

drug-resistant cells through the induction of EpRE (electrophile responsive element) as was suggested by Lee-Hilz *et al.* (*10*). Recent findings suggest that low concentrations of quercetin lead to small increase of cellular thiols and total antioxidative activity (TAC), but higher concentrations lead to a progressive decrease in the TAC (*11*). In our previous study (*33*,*34*), it was shown that constitutive levels of GSH are higher in cisplatin-resistant CK2 cells of laryngeal carcinoma than in the parental HEp2 cells, but also that quercetin induces a significant increase in the GSH levels of both cell lines, again pointing towards the importance of cellular status wherever the response to flavonoid exposure is an issue.

The activity of cellular glutathione S-transferase

Quercetin and luteolin significantly increased the activity of glutathione S-transferase in parental HeLa cells, and the same effect of all investigated flavonoids was observed after the treatments of drug-resistant HeLa CK cell line (Fig. 4). Similar to other enzymes involved in the metabolism of foreign compounds, glutathione S-transferases can be either induced or inhibited by xenobiotics or by their metabolic intermediates. These enzymes catalyse the conjugation of GSH and xenobiotics, participating in this manner in the formation of non-toxic conjugate that can be excreted from the cell. However, the role of this enzyme, according to published data, extends also to cell signalling pathways and cellular maintenance of the redox state (*35*,*36*). HeLa and HeLa CK cell lines have similar basal GSTs activities.

High activity of GSTs in HeLa CK line, observed following the treatment with flavonoids, could protect these cells from xenobiotics. Lee-Hilz *et al.* (*37*) determined that flavonoids with higher intristic potential to cause oxidative stress are the most potent inducers of EpRE-mediated gene expression, which is responsible for GSTs expression. The phenomenon of GSTs inhibition, induced by phenolic compounds and recorded by several authors (*12*,*38*,*39*), failed to be confirmed within the framework of our study. According to Robaszkiewicz *et al.* (*11*), decrease of GSTs can be noticed at higher concentrations of flavonoids.

Lipid peroxidation

In order to determine the ability of the investigated flavonoids to induce the formation of free radicals which could be responsible for the induction of GSH and GSTs as well as for the induction of apoptotic cell death, the formation of malondialdehyde (MDA), occurring after the prolonged exposures of HeLa and HeLa CK cells to 5-μM flavonoid concentrations, was examined. Free radicals attack unsaturated lipids contained in the cell. Final products of this reaction are lipid breakdown products, lipid alcohols, aldehydes and MDA (*6*). Therefore, in *in vitro* experiments, measurement of MDA concentration is a commonly used method for the determination of primary toxic effects caused by free radicals (*40*). Decker (*7*) proved that flavonoids express their prooxidative nature in systems that use redox-active metals. In the presence of oxygen, copper and iron catalyze redox reactions between phenolic compounds and oxygen, causing formation of phenoxyl radicals and reactive oxygen species, which can initiate chain reaction involving DNA, as well as oxidation of cellular lipids and proteins (*7*). Furthermore, in the presence of transitional metals, flavonols can be autooxidized, and the products of their autooxidation may cause ROS formation (*41*). Another mechanism responsible for the formation of phenoxyl

radicals and ROS is the metabolism of flavonoids mediated by cytochrome P450 system (*12*).

In this study, lipid peroxidation caused by flavonoids was examined and compared with lipid peroxidation induced by hydrogen peroxide. In addition, oxidative damage of the cells treated with the mixture of hydrogen peroxide and flavonoids was determined. In HeLa CK cell line (Fig. 5), basal lipid peroxidation was twofold higher than in the parental HeLa cell line (Fig. 6). In the tested cell lines, no oxidative damage of lipids was induced either by flavonoids or by the mixture of hydrogen peroxide and flavonoids. The decrease in basal lipid peroxidation, observed with HeLa CK cells caused by quercetin and fisetin, failed to reach statistical significance.

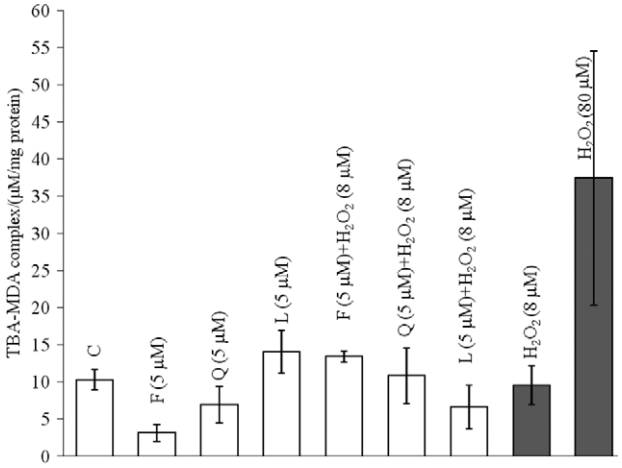

Fig. 5. Concentration of TBA-MDA complex in HeLa CK cells after 72-hour treatment with 5-μM concentrations of flavonoids and with the mixture of flavonoids and hydrogen peroxide (white columns): quercetin (Q), fisetin (F), luteolin (L) and untreated control (C). There was no statistically significant difference in the level of lipid peroxidation in treated cells compared to nontreated cells, control (C). Gray columns: positive controls, cells treated with hydrogen peroxide only

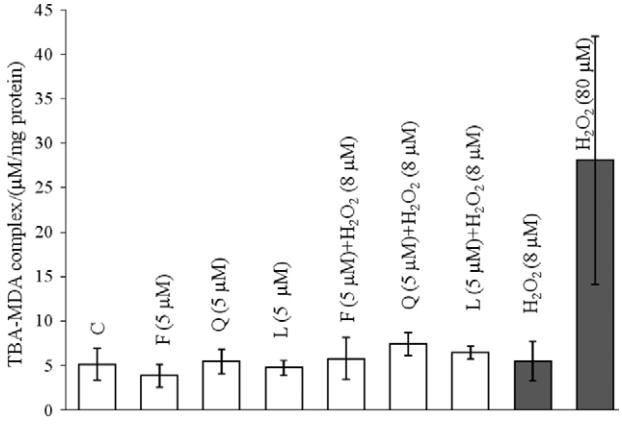

Fig. 6. Concentration of TBA-MDA complex in HeLa cells after 72-hour treatment with 5-μM concentrations of flavonoids and with the mixture of flavonoids and hydrogen peroxide (white columns): quercetin (Q), fisetin (F), luteolin (L) and untreated control (C). There was no statistically significant difference in the level of lipid peroxidation in treated cells compared to nontreated cells, control (C). Gray columns: positive controls, cells treated with hydrogen peroxide only

Influence of flavonoids on the expression of apoptosis-related proteins

It is well known that compounds of dual, prooxidative and antioxidative nature may induce apoptotic cell death (*42*). They can activate pro-apoptotic signals, inducing the release of cytochrome c from mitochondria, or cause necrotic death (*42*). De Flora *et al.* (*6*) pointed out that it is difficult to determine whether a certain compound triggers either a protective mechanism, or a number of undesirable events. One of the important characteristics of flavonoids is their influence on gene expression (*28*) and the activity of enzymes involved in the cell cycle regulation and apoptosis (*12*). Therefore, the effects of flavonoids on PARP cleavage (the latter being a marker of apoptosis), as well as their influence on anti-apoptotic enzymes Bcl-2 and surviving were examined here.

In parental HeLa cells, nontoxic concentrations of fisetin and luteolin, as well as slightly toxic concentration of quercetin cleaved PARP protein, while none of them caused its cleavage in drug-resistant HeLa CK cells (Fig. 7), suggesting their pro-apoptotic role in parental cell line. Zhang and Zhang (*43*) examined the effects of quercetin on the apoptosis of HeLa cells and determined that increase of quercetin concentration from 20 to 80 μM increased apoptosis rate from 18 to 70 %. Also, they showed that quercetin can decrease invasiveness and adhesion ratio of the same cell line, suggesting that this compound could play an antimetastatic role.

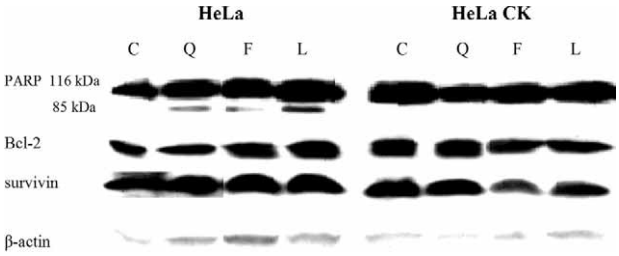

Fig. 7. Western blot analysis of PARP cleavage, and Bcl-2 and survivin expression in HeLa and HeLa CK cells following 72--hour treatment with 5-μM concentrations of flavonoids: quercetin (Q), fisetin (F), luteolin (L), and untreated control (C). β-actin was used as an equal-loading control

These results are in concordance with our results obtained in this study, but it is worth mentioning that quercetin concentrations applied in their work are 4 to 16 times higher than concentrations that were examined in our work. Also, it is important to mention that EC_{50} values determined in this work (Table 1) showed that concentrations that were applied in the work published by Zhang and Zhang (*43*) are cytotoxic (EC_{50} value for quercetin was 7.3 μM). In our work (Fig. 7) we proved that 5-μM concentrations of all examined flavonoids caused PARP degradation in parental, HeLa cells, indicating their proapoptotic nature at concentrations that can be found in systemic circulation in *in vivo* system (*29,30, 31*). The expression of Bcl-2 in parental HeLa cells was not influenced by flavonoids (except for luteolin, where an insignificant increase in Bcl-2 expression was determined in parental HeLa cells). The expression of survi-

Table 1. EC_{10} and EC_{50} values obtained after treatment of HeLa and HeLa CK cells with quercetin (Q), fisetin (F) and luteolin (L). EC_{10} and EC_{50} values were analyzed by linear regression and determined from the direction of equation

Substance/cell line	$c(EC_{10})$/μM	$c(EC_{50})$/μM
Quercetin/HeLa	39.7	7.3
Fisetin/HeLa	45.5	10.5
Luteolin/HeLa	47.7	13.4
Quercetin/HeLa CK	17.8	9.8
Fisetin/HeLa CK	23.8	3.8
Luteolin/HeLa CK	26.6	7.4

vin was decreased after the fisetin treatment of the resistant cell line, so it can be seen that pro-apoptotic action of flavonoids could be useful in eliminating mutant cells.

Conclusion

In conclusion, within the frame of the present study, some effects of three structurally related flavonoids on HeLa cells of a human cervical carcinoma and on their drug-resistant HeLa CK subline were examined. Based on their cytotoxicity, flavonoids can be ranked as follows: quercetin>fisetin>luteolin for HeLa cell line and fisetin>luteolin>quercetin for resistant HeLa CK cell line. Differences in the extent of measured biological effects were observed for the tested flavonoids, although the differences in their determined cytotoxic EC_{50} concentrations are less than 3-fold. Investigated flavonoids increased the level/activity of detoxification proteins, and that effect was more pronounced in HeLa CK cells. None of the investigated flavonoids altered TBA-MDA formation, pointing out that ROS formation was not responsible either for the induction of detoxifying enzymes or for the induction of mechanisms involved in the apoptotic cell death. Concentrations of 5 μM of the investigated flavonoids cause PARP degradation in parental cell line. Further investigation is needed with the aim to determine specificities and the acting mechanism exerted by flavonoids in resistant cell line.

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