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Effects of Structurally Related Flavonoids on hsp Gene Expression in Human Promyeloid Leukaemia Cells

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Summary

Quercetin is a known specific inhibitor of hsp70 synthesis and thus might be a potent agent for enhancing the selective cytotoxicity of heat on tumour cells. A comparative analysis of the effects of quercetin and five structurally related flavonoids on hsp90α, hsp70A, hsp60 and hsp27 gene expression was carried out using human myeloid leukaemia cells (HL-60). The cells were preincubated with 50 μM quercetin, kaempferol, myricetin, taxifolin, isorhamnetin, methylquercetagetin or 0.1 % DMSO (controls) for 24 h at 37 °C before heat shock treatment (43 °C for 30 min). Total RNA was isolated from heat-stressed and unstressed cells and analysed by RT-PCR. Hsp27 gene expression was inhibited by flavonoids more strongly than other hsp genes investigated in heat stressed as well as in unstressed cells. Among the hsp genes tested, only hsp60 was expressed above control level under the influence of taxifolin. Members of the hsp70 and hsp27 families are highly expressed in breast and lung cancer and leukaemias and they play a role in the acquired resistance to chemotherapy or radiation therapy combined with hyperthermia. Therefore, hsps present potential targets for cancer diagnosis and treatment. The present structure/activity study indicates that position, number and substitution of hydroxyl groups of the B ring and saturation of the C2-C3 bond are important factors affecting flavonoid activity on hsp gene expression. This study could help provide a basis for further design of specific inhibitors of hsp gene expression.

*Key words***:** hsp genes, flavonoids, leukaemia, HL-60, dietary supplements

Introduction

Flavonoids are a group of about 4000 naturally occurring compounds that are ubiquitous in all vascular plants and important constituents of the human diet. On the average the daily diet contains approximately 1 g of different flavonoids but the uptake of specific compounds may vary greatly depending on the food source. These low weight phenylbenzopyranes are found in fruits, vegetables, nuts, seed as well as in beverages like red wine, tea, coffee and beer. Flavonoids have probably existed in the plant kingdom for over one billion years and hence have a long history of co-evolution with the animal kingdom. Interesting biological activities of plant flavonoids have prompted intensive research on physiological properties of these compounds and their effects on human health.

Flavonoids are capable of modulating the activity of many enzymes and possess a remarkable spectrum of biochemical and pharmacological activities, some of

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which interfere with control processes in carcinogenesis (for review see *1, 2*). These include antioxidant activities (*3*), the scavenging effect on activated carcinogens and mutagens (*4*), the action on cell cycle progression (*5*) and altered gene expression (*6*). One of the best investigated flavonoids with antitumour activity is quercetin, which is abundant in onions, apples, broccoli and berries. Several effects of quercetin have been documented in different human cell types. Quercetin may interfere with regulatory pathways of apoptosis (*7*), it causes cell cycle arrest (*8*) and suppresses heat shock gene expression (*9*). Heat shock proteins function as molecular chaperones, guiding the transport, assembly and degradation of intracellular polypeptides. Under the influence of non-physiological conditions heat shock protein synthesis is accelerated to aid cell survival. Elevated expression of the members of the hsp70 family has been reported in high-grade malignant tumours. Quercetin is a known specific inhibitor of hsp70 synthesis and thus might be a potent agent for enhancing the selective cytotoxic action by heat on tumour cells. Overexpression of either hsp27 or hsp70 protects malignantly transformed cells from apoptotic cell death and fosters resistance to chemotherapy or radiation therapy combined with hyperthermia (*10*), increases the tumourgenic potential of rodent cells (*11*) and the metastatic potential of human breast cancer (*12*). In addition, hsp27 has been of interest in cancer research and oncology because 27-kDa heat shock protein facilitates basic fibroblast growth factor release from endothelial cells (*13*), which in turn promotes tumour vascularisation and its subsequent growth (*14*). The level of basic fibroblast growth factor is increased in sera of patients with malignant tumours (*15*). Hsp90α and hsp90β are also overexpressed in breast tumours, lung cancer, leukaemias and Hodgkin's disease (for review see *10*). Yano *et al. (16)* reported that 90α-kDa heat shock protein may play a role in cell proliferation in breast cancer. Therefore, hsp genes present potential targets for cancer diagnosis and treatment.

Because the biochemical activities are dependent on the individual chemical structure, each compound needs to be studied systematically to assess its individual biological potency. The aim of this study was to elucidate the effect of structurally related flavonoids on hsp gene expression and to provide new information on the structure/activity relationship of flavonoids. To this end, the effects of quercetin, kaempferol, methylquercetagetin, myricetin, taxifolin and isorhamnetin (Fig. 1) on hsp90α, hsp70A, hsp60 and hsp27 gene expression in heat stressed and heat unstressed human promyeloid leukemia HL-60 cells were investigated.

Material and Methods

Flavonoids

Quercetin, myricetin, kaempferol and taxifolin were purchased from Sigma (Germany) and isorhamnetin from Fluka (Switzerland). Methylquercetagetin was isolated in its glycosylated form (quercetagetin 3'-methyl ether 7-O-β-D glucopyranoside) from *Centaurea rupestris* L. *(17)*. The isolated glucopyranoside was dissolved in Sörensen's phosphate buffer (pH=5) and β-glucosidase (Sigma, Germany) was added to obtain methylquercetagetin (quercetagetin 3'-methyl ether) in the aglycone form. The enzymatic reaction was shown by reversed phase HPLC on C_{18} using water-methanol gradient from 35–100 % methanol for over 30 minutes. The final separation of aglycone from the traces of glucoside was achieved by reversed phase HPLC under the same conditions.

All flavonoids were dissolved in DMSO as 50 mM stock solutions and keept at –20 °C.

Cell culture

Human promyeloid leukemia cells HL-60 were cultured in RPMI 1640 medium (Biochrom) supplemented with 10 % FCS (Biochrom) at 37 °C in a 5 % CO_2 humidified atmosphere.

Reverse transcription and polymerase chain reaction (RT-PCR)

In each well of a 6–well plate 10^5 cells were cultured for 72 h and then flavonoid stock solution was added to yield a final concentration of 50 μM and a concentration of 0.1 % DMSO in all samples including the controls. Cells were incubated for further 24 h and then total RNA was isolated using the Invisorb RNA Kit II (Invitek GmbH, Germany). In a parallel experiment, after 24-hour-incubation with flavonoids, cells were heat-stressed at 43 °C for 30 min. The heat shock genes were allowed to tran-

Table 1. Primers sequences used for reverse transcription and RT-PCR

Gene	Sense and antisense primers	Location	mRNA size[a]	GenBank
HSP27	5´-ATGGCGTGGTGGAGATCACC-3´	451-470	347 bp	XM_004991
	5´-CAAAAGAACACACAGGTGGC-3´	797-778		
HSP60	5´-ATTCCAGCAATGACCATTGC-3´	1444-1463	306 bp	NM_002156
	5´-GAGTTAGAACATGCCACCTC-3´	1749-1730		
HSP70A	5´- TGTTCCGTTTCCAGCCCCCAA-3'	433-453	359 bp	M11717
	5´- GGGCTTGTCTCCGTCGTTGAT-3'	791-771		
HSP90α	5´-AAAAGTTGAAAAGGTGGTTG-3	1803-1822	624 bp	X15183
	5´-TATCACAGCATCACTTAGTA-3´	2426-2406		
β-actin	5´CAGCTCACCATGGATGATGAT3´	1084-1104	626 bp[a]	M10277
	5´CTCGGCCGTGGTGGTGAAGCT3´	2280-2260		

[a] Expected size of RT-PCR product if mRNA is amplified

scribe for 45 min at 37 °C to reach maximal value *(9)*. After standard spectrophotometric quantification (Ultrospec 2000, Pharmacia Biotech, UK) 500 ng RNA was reverse transcribed using 200 U of SuperScript reverse transcriptase (Qiagen, Germany), 40 U of RNase OUT (Promega, Germany), dNTPs (final concentration 500 μmol/L), 50 pmol oligo-dT_{15} primers (Life Technologies Inc., Germany), and buffer as recommended by the supplier. The cDNA synthesis was allowed by incubation of samples at 37 °C for 1 h followed by incubation at 60 °C for 20 min. Amplification of cDNA (1 μL) was carried out by PCR in a 15-μL reaction mixture containing 0.4 U Taq-DNA-polymerase (Peqlab Biotechnologie GmbH, Germany), 100 μM dNTPs and 1 μM of each primer pair specific for hsp27, hsp60, hsp70A or hsp90a (Table 1). Amplification (Biometra UNO-Thermoblock, Biometra, Germany) was set to 45 sec at 94 °C, 30 sec at 58 °C, followed by 90 sec at 72 °C (30 cycles). Finally, primer extension was allowed for 10 min at 72 °C. RT-PCR products were resolved by electrophoresis at 200 V through 1 % agarose in 1xTAE (0.04 Tris-acetate, 0.001 M EDTA, pH=8.0) and visualized with ethidium bromide (0.01 % in 1xTAE buffer). Data were calculated using the β-actin signal as internal control.

Quantitative evaluation of RT-PCR products was performed by area morphometry analysis using a digital imaging system (Biometra, Germany) and the appropriate software (Optimas Co., DC, USA). Each experiment was repeated at least three times and data are expressed as means ±SD. Significance of differences between control and treated cells were determined by Student's *t* test. Significance levels were set at $P < 0.05$.

Results

The effect of various flavonoids (50 μM) on hsp90α, hsp70A, hsp60 and hsp27 gene expression in heat-stressed and non heat-stressed HL-60 cells was investigated. The chemical structures of the chosen flavonoids are shown in Fig. 1.

Quercetin and taxifolin inhibit hsp90α gene expression significantly, causing suppression by 30 and 25 %,

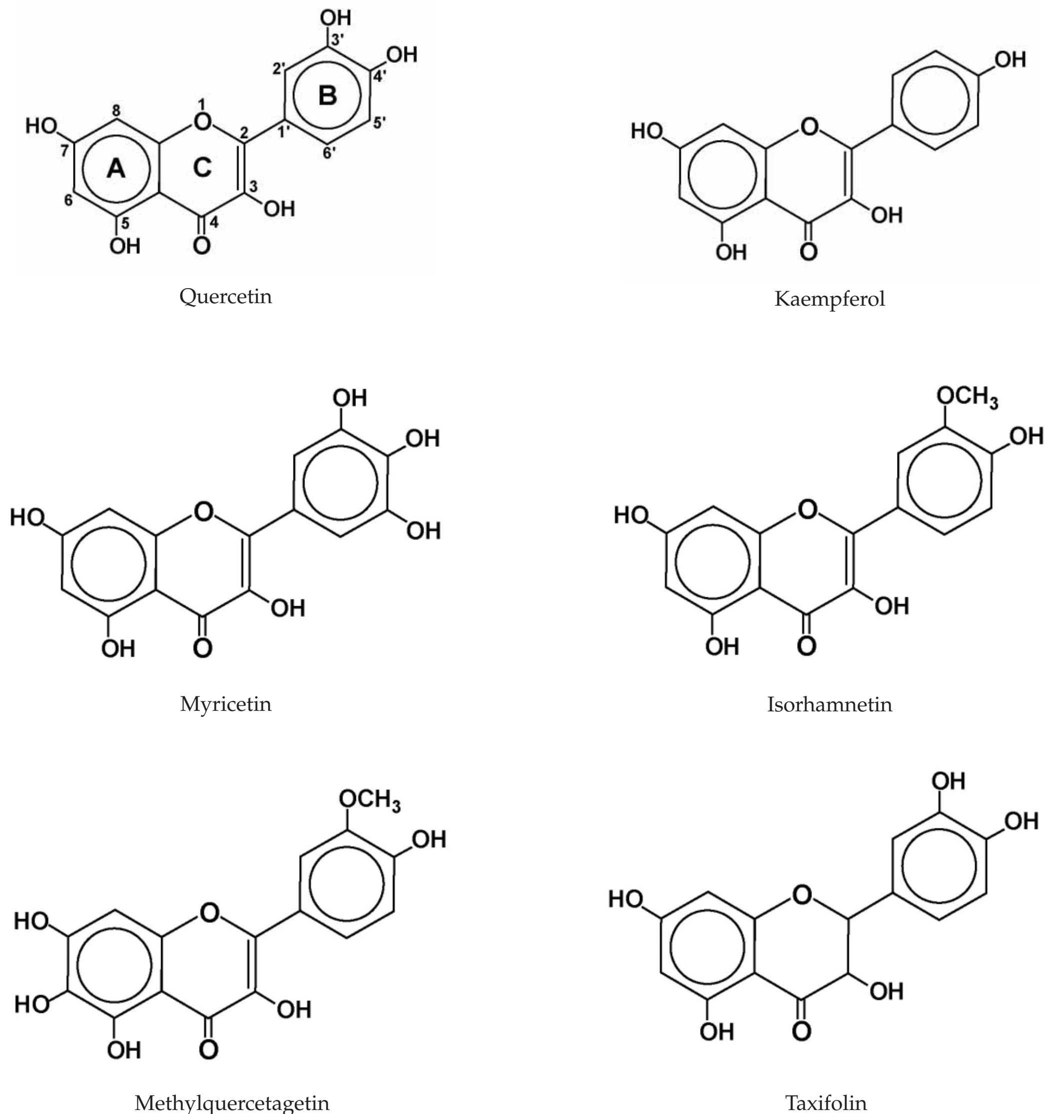

Fig. 1. Chemical structure of investigated flavonoids

respectively. Inhibition induced by isorhamnetin and myricetin was not significant, whereas methylquercetagetin and kaempferol were ineffective (Fig. 2).

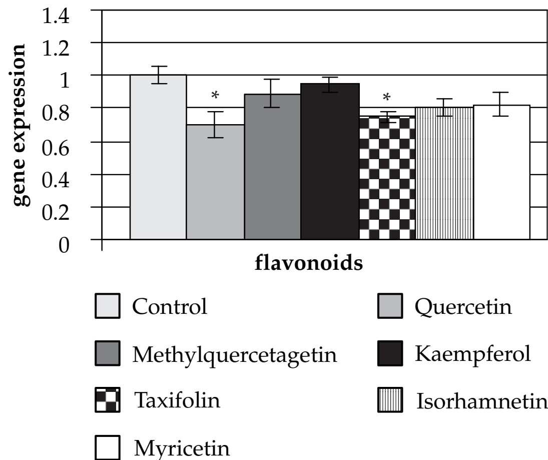

Fig. 2. Influence of structurally related flavonoids on hsp90α gene expression in heat-stressed HL-60 cells; expressed relative to control (=1); cells were incubated with flavonoids (50 μM) at 37 °C for 24 h, stressed at 43 °C for 30 min and then RT-PCR was carried out and quantitative evaluation of RT-PCR products was performed; data are expressed as means of three separate experiments.*P<0.05.

Amongst the flavonoids tested quercetin was the most potent inhibitor of hsp70 gene expression causing inhibition of approximately 35 %. Incubation of cells with taxifolin, kaempferol or isorhamnetin prior to heat shock inhibited hsp70 gene induction by 25–30 %, whereas myricetin and methylquercetagetin did not show any significant effect (Fig. 3).

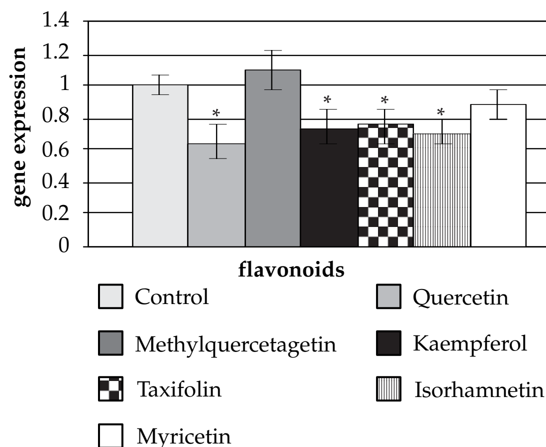

Fig. 3. Influence of structurally related flavonoids on hsp70A gene expression in heat-stressed HL-60 cells; expressed relative to control (=1); cells were incubated with flavonoids (50 μM) at 37 °C for 24 h, stressed at 43 °C for 30 min and then RT-PCR was carried out and quantitative evaluation of RT-PCR products was performed; data are expressed as means of three separate experiments ±SD. *P<0.05.

Taxifolin was found to be an inducer of hsp60 gene expression in heat stressed cells causing more than 55 % induction of gene expression compared to control levels. Overexpression of hsp60 gene induced by isorhamnetin and methylquercetagetin was not significant, whereas quercetin, kaempferol and myricetin were ineffective (Fig. 4).

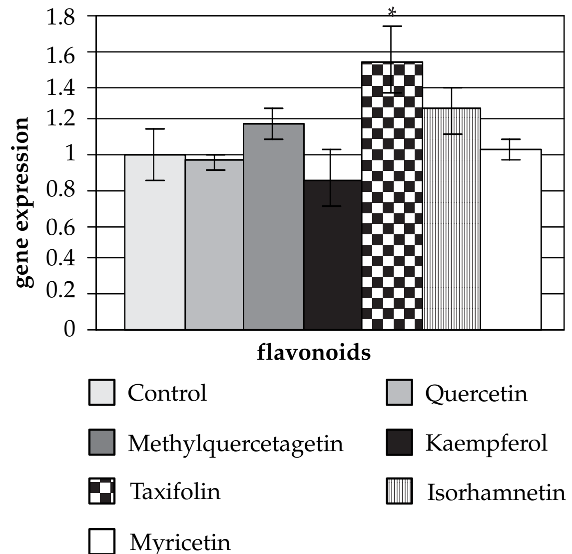

Fig. 4. Influence of structurally related flavonoids on hsp60 gene expression in heat-stressed HL-60 cells; expressed relative to control (=1); cells were incubated with flavonoids (50 μM) at 37 °C for 24 h, stressed at 43 °C for 30 min and then RT-PCR was carried out and quantitative evaluation of RT-PCR products was performed; data are expressed as means of three separate experiments ±SD.*P<0.05.

Hsp27 gene expression was affected much more strongly by the presence of flavonoids than the other hsp gene investigated. Moreover, quercetin and kaempferol were found to be significant inhibitors of hsp27 gene expression in heat stressed as well as in unstressed HL-60 cells (Figs. 5A,B), whereas hsp90α hsp70A and hsp60 were not affected by investigated flavonoids in non heat-stressed cells (data not shown). Quercetin, kaempferol and myricetin suppressed hsp27 gene expression significantly in unstressed HL-60 cells by about 55 % (Fig. 5A). Quercetin and kaempferol showed the same inhibitory effect in heat shocked cells whereas suppression of hsp27 gene in stressed cells induced by myricetin was not significant (Fig. 5B). Hsp27 gene was not affected by methylquercetagetin and isorhamnetin neither in stressed nor unstressed cells (Figs. 5A,B).

The flavonoids we chose for our analysis are structurally closely related (Fig. 1) yet some differences in their biological activity were apparent in our analysis. As a result, some preliminary conclusions can be drawn concerning structure/function relationships of the compounds.

The fact that taxifolin and quercetin showed inhibitory effect on hsp90α gene expression, whereas methylquercetagetin and kaempferol were ineffective, indicated that double bond between C-2 and C-3 is not essential for this activity.

Quercetin, taxifolin, kaempferol and isorhamnetin suppressed hsp70 gene expression after heat shock, whereas myricetin and methylquercetagetin did not

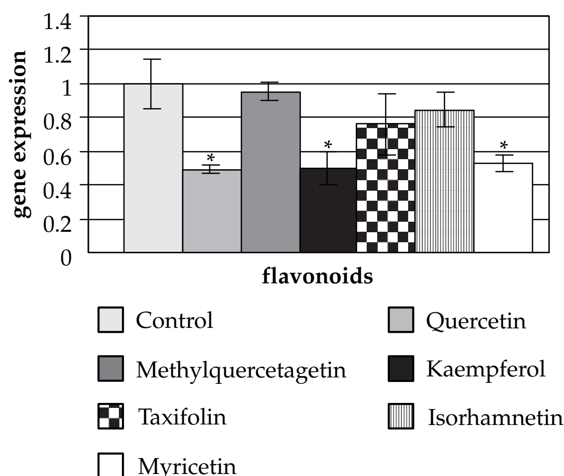

Fig. 5A. Influence of structurally related flavonoids on hsp27 gene expression in not stressed HL-60 cells; expressed relative to control (=1); cells were incubated with flavonoids (50 μM) at 37 °C for 24 h and then RT-PCR was carried out and quantitative evaluation of RT-PCR products was performed; data are expressed as means of three separate experiments ±SD.*P<0.05.

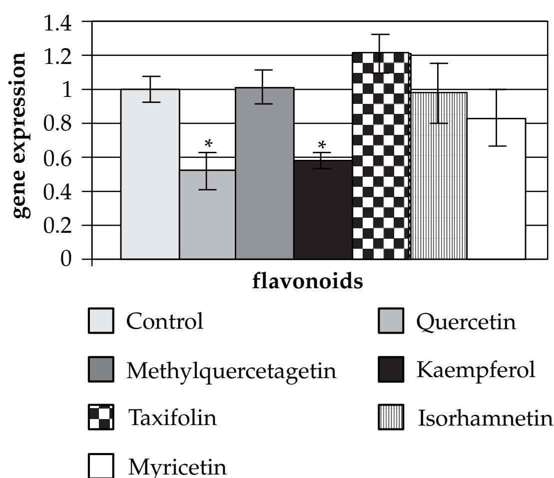

Fig. 5B. Influence of structurally related flavonoids on hsp27 gene expression in heat-stressed HL-60 cells; expressed relative to control (=1); cells were incubated with flavonoids (50 μM) at 37 °C for 24 h, stressed at 43 °C for 30 min and then RT-PCR was carried out and quantitative evaluation of RT-PCR products was performed; data are expressed as means of three separate experiments ±SD. *P<0.05.

show any significant effect (Fig.3). These results suggest that C-3' hydroxyl group of flavonoids and a double bond between C-2 and C-3 are not essential for this activity. An additional C-5' or C-6 hydroxyl group seems to inhibit this activity.

Among the flavonoids tested only taxifolin was found to be significant inducer of hsp60 gene expression (Fig. 4). Therefore, a single bond between C-2 and C-3 is believed to be essential for this induction.

Quercetin and kaempferol were potent inhibitors of HSP27 gene expression in heat-stressed cells (Fig 5B). The lack of suppressive activity of taxifolin in heat-stressed cells indicated that double bond between C-2 and C-3 is required for suppression of hsp27 gene expression by flavonoids after heat shock. Ineffectiveness of methyquercetagetin and isorhamnetin indicated that methyl group at C-3 inhibits this activity.

Discussion

Flavonoids, a class of naturally occurring phenolic compounds ubiquitously present in vascular plants, especially fruits and vegetables, are considered semi-essential food components since they are the most common and most active antioxidant substances in our food. It is widely believed that antioxidant micronutrients obtained from fruits and vegetables afford significant protection against cancer and heart diseases, as well as ageing. Therefore, it is not surprising that many papers have been published in the last two decades with the aim of establishing potency of flavonoids to affect gene expression, cell proliferation, differentiation, cell survival or death. In the present study we studied the effects of structurally related flavonoids on hsp gene expression in heat-stressed and non stressed HL-60 cells.

Among the flavonoids tested, quercetin was the most potent inhibitor of hsp90α gene expression in heat stressed cells causing an inhibition by 30 % (Fig. 2). Yano *et al. (16)* reported that the mentioned heat shock protein may play a role in breast cancer cell proliferation.

Our results showed that 50 μM quercetin, taxifolin, kaempferol or isorhamnetin suppress hsp70A gene expression in heat stressed cells by 25–35 % (Fig 3). These results are consistent with previous report (9) which demonstrated dose dependent inhibition of hsp70 gene expression in colon carcinoma cells by treatment with quercetin. The authors showed that hsp70 gene expression was suppressed by 20 % in the presence of 50 μM quercetin, whereas 500 μM quercetin caused suppression by 89 %. Hsps belonging to the 70kDa family play various roles in the cell, including the well-known chaperone function (*18,19*) and the role in the control of cell cycle and of apoptosis (*20*). In addition, hsp70 have been reported to act in cellular protection and repair and were suggested to mediate well-known effect of acquired thermotolerance (*21,22*). Hyperthermia, alone or in combination with radiotherapy or chemotherapy is recognised as an effective form of treatment of certain types of cancer. The development of thermotolerance in tumour cells is one of the major concerns in the treatment of human cancer with hyperthermia. In breast tumours elevated expression of hsp70 is associated with short-term disease-free survival, metastasis and poor prognosis among patients treated with combined chemotherapy, radiation therapy and hyperthermia *(10)*. Therefore, specific inhibitors of hsp70 on gene expression or protein synthesis level may be potent agents in cancer treatment.

Among the hsp genes tested only hsp60 was expressed over control level by influence of taxifolin (more than 55 %) in stressed cells (Fig. 4). Heat shock 60 kDa protein has been recognised as an important molecule in infectious and autoimmune diseases. More recently, some authors *(23)* showed that this protein induces tumour necrosis factor-α in monocyte-derived macrophage.

Hsp27 gene expression was affected more strongly by flavonoid than other hsp genes investigated. Moreover, hsp27 gene was suppressed by quercetin and kaempferol in stressed as well as in unstressed cells by about 55 % (Fig. 5A,B). Increased levels of hsp27 have also been detected in a number of cancers such as breast cancer, endometrial cancer and leukaemia *(10)*, contributing to protection of tumour cells from apoptotic cell death. In addition, hsp27-kDa heat shock protein facilitates basic fibroblast growth factor release from endothelial cells *(13)*, which promotes tumour vascularisation and its subsequent growth *(14)*.

Depending on their structure the investigated flavonoids display more or less potent effects on hsp90α, hsp70A, hsp60 and hsp27 gene expression. Our results suggest that double bond between C2 and C-3 is not essential for the inhibitory effect of flavonoids on hsp90α or hsp70A gene expression but seems to be required for suppression of hsp27 gene expression. The methyl group at C3' inhibits suppressive effect of flavonoids on hsp27 gene expression. An additional C-5' or C-6 hydroxyl group seems to inhibit suppressive effect of flavonoids on hsp70A gene expression. A single bond between C-2 and C-3 could be essential for the induction of hsp60 gene expression. In conclusion, the present structure/activity study indicated that position, number and substitution of hydroxyl groups of the B ring as well as saturation of the C2-C3 bond are important factors affecting flavonoid activity on hsp gene expression. A major obstacle in regulating several hsp gene expressions has been the lack of a specific inhibitor. Since the first report that flavonoids inhibit hsp synthesis *(24)* these compounds have been studied intensively, in the hope that they might be used as sensitising agents in combination with chemotherapy. Apparently, the specificity and strength of the inhibitory activity depend on the chemical structure of the compounds. The studied structure/function relationship will be the basis for further work aimed at designing molecules with the desired biological activity.

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Utjecaj strukturno srodnih flavonoida na ekspresiju hsp gena u ljudskim promieloidnim leukemijskim stanicama

Sažetak

Kvercetin je poznati specifični inhibitor sinteze hsp70 i kao takav mogući čimbenik povećanja selektivnog citotoksičnog učinka topline na tumorske stanice. Provedena je komparativna analiza učinka kvercetina i njemu strukturno srodnih flavonoida na ekspresiju hsp90α, hsp70A, hsp60 i hsp27 gena u stanicama humane mijeloidne leukemije (HL-60).

Stanice su inkubirane s 50 μM kvercetina, kempferola, miricetina, taksifolina, izoramnetina, metilkvercetagetina ili 0,1% DMSO (kontrola) tijekom 24 h na 37 °C prije obrade toplinskim šokom (43 °C tijekom 30 minuta). Izolirana je ukupna RNA iz stanica koje su bile, kao i one koje nisu bile, podvrgnute toplinskom stresu te je provedena RT-PCR analiza. Ekspresija gena hsp27 bila je jače inhibirana flavonoidima nego ostali istraživani hsp geni, i to podjednako u stanicama podvrgnutim kao i u onima koje nisu bile podvrgnute toplinskom stresu. Među istraživanim hsp genima jedino je ekspresija hsp60 bila iznad kontrolne razine pod utjecajem taksifolina. Članovi porodica hsp70 i hsp27 snažno su ekspresirani kod raka dojke, pluća i leukemija te imaju bitnu ulogu u stečenoj rezistenciji stanica pri kemoterapiji ili terapiji zračenjem u kombinaciji s hipertermijom. Stoga bi hsp proteine trebalo istraživati pri dijagnosticiranju i liječenju raka. Prikazana studija strukture i aktivnosti upućuje na to da su položaj, broj i supstitucija hidroksilnih skupina na prstenu B kao i zasićeni vez C2–C3 kod flavonoida bitni čimbenici koji utječu na ekspresiju hsp gena. Ova bi studija mogla poslužiti kao osnova za daljnje strukturiranje specifičnih inhibitora ekspresije hsp gena.