Relationship between structure and antiproliferative, proapoptotic, and differentiation effects of flavonoids on chronic myeloid leukemia cells

Tanya V. Ramanouskaya[a], Victoria V. Smolnykova[b] and Vasily V. Grinev[a]

The aim of this study was to reveal the relationships between structure elements of flavonoids and their antileukemic activity. The human leukemia cell line K562 as a model of blast crisis of chronic myeloid leukemia and a set of 18 different flavonoids from four flavonoid subfamilies were used for researching these relationships. Relationships between structure and antiproliferative, proapoptotic, and differentiation activities of flavonoids were estimated by pairwise comparative analysis of selected flavonoids that differ in one structure element. We found that C4 carbonyl and C2–C3 double bonds are critical structure elements for antileukemic activity of flavonoids. We also observed that the ortho-position of hydroxyl groups in the B ring of the flavonoid molecule has an advantage over other variants of B ring hydroxylation patterns. At the same time, flavonoids with a nonhydroxylated B ring were more effective. In the A ring, hydroxylation status of C5 is not critical for antileukemic activity of the flavonoids, whereas the appearance of the hydroxyl group in the C6 position of the flavonoid molecule significantly decreased the IC_{50} (inhibition concentration required for 50% cytotoxic effect) value. Glycosylation of flavonoids was associated with dramatically decreased activity. These data may help in the rational design of semisynthetic flavonoids with improved antileukemic activity. *Anti-Cancer Drugs* 20:573–583 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Flavonoids are low molecular weight plant compounds that are found in vegetables, fruits, nuts, seeds, spices, stems, flowers, and in tea and red wine, and are consumed regularly as a part of the human diet [1]. Primarily recognized as the plant pigments responsible for the autumnal burst of hues and the many shades of yellow, orange, and red in flowers and food [1], they were also found to act as cell cycle, cell death, and cell differentiation regulators of cancer cells *in vitro* and *in vivo* [1–3]. Flavonoids were shown to carry out their anticancer abilities through different mechanisms including inhibition of expression and activity of some tyrosine [4], serine/threonine [5] and phosphatidyl-inositol 3 kinases [5], heat shock proteins [6], topoisomerase [7], proteasome compounds [8], and promotion of p53 protein accumulation [9] or downregulation of ki-ras and c-myc proteins [10]. Moreover, it was proposed that the anticancer activity of flavonoids is dependent not only on the type of target cells, but also mainly on the flavonoid structure [11].

Flavonoids are phenylbenzo-pyrones with a variety of structures based on a common three-ring nucleus: the basic structure of flavonoids is composed of two benzene rings A and B linked through a heterocyclic pyran or pyrone (with a double bond) and ring C in the middle. They are divided into subfamilies such as flavones, flavonols, flavanones, flavanols (catechines), anthocyanidins, and chalcones [1]. This division is primarily based on differences in the structure of the heterocyclic ring C. Most of them may exist in aglycone or glycone form. Over 4000 structurally unique flavonoids have been identified [1]. Therefore, taking into account the diversity of flavonoid structures and multiple mechanisms of their antiproliferative and proapoptotic activity, the investigation of structure–activity relationships is important for the prediction of the cytotoxic potential of a given flavonoid and may facilitate the search for effective candidates for cancer therapy.

In this study, we examined the relationships between the structure of some flavonoids and their effect on the proliferation, apoptosis, and differentiation of K562 human chronic myeloid leukemia (erythroblast crisis) cells. We used 18 different flavonoids (molecular structures are shown in Fig. 1) and applied a set of methods to determine their antiproliferative, proapoptotic, and differentiation activities. A pairwise comparison of structurally similar flavonoids enabled us to find the structural requisites involved in the antileukemic effects of these compounds.

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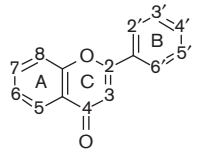
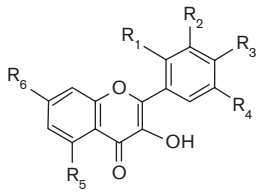
Fig. 1

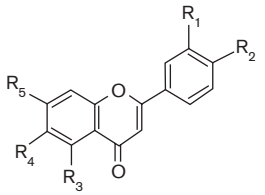


Flavonoid core structure



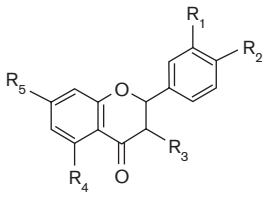
Flavonols

	R1	R2	R3	R4	R5	R6
Flavonol	H	H	H	H	H	H
Galangin	H	H	H	H	OH	OH
Kaempferol	H	H	OH	H	OH	OH
Fisetin	H	OH	OH	H	H	OH
Quercetin	H	OH	OH	H	OH	OH
Morin	OH	H	OH	H	OH	OH
Myricetin	H	OH	OH	OH	OH	OH



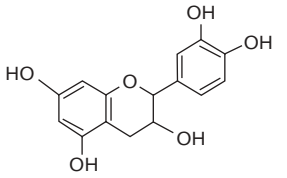
Flavones

	R1	R2	R3	R4	R5
Flavone	H	H	H	H	H
Chrysin	H	H	OH	H	OH
Baicalein	H	H	OH	OH	OH
Apigenin	H	OH	OH	H	OH
Luteolin	OH	OH	OH	H	OH



Flavanones

	R1	R2	R3	R4	R5
Flavanone	H	H	H	H	H
Taxifolin	OH	OH	OH	OH	OH
Naringenin	H	OH	H	OH	OH



Catechines
(–)-Epicatechin

Molecular structure of flavonoids used in this study.

Materials and methods

Flavonoids

Flavone, flavonol (3-hydroxyflavone), flavanone, luteolin (3′,4′,5,7-tetrahydroxyflavon), chrysin (5,7-dihydroxyflavon), baicalein (5,6,7-trihydroxyflavon), apigenin (4′,5,7-trihydroxyflavon), galangin (3,5,7-trihydroxyflavon), fisetin (3,3′,4′,7-tetrahydroxyflavon), quercetin (3′,4′,3,5,7-pentahydroxyflavone)-dihydrate, myricetin (3′,4′,5′,3,5,7-hexahydroxyflavon), morin (2′,4′,3,5,7-pentahydroxyflavon)-dihydrate, kaempferol (4′,3,5,7-tetrahydroxyflavon), taxifolin (3,5,7,3′,4′-pentahydroxyflavanone), naringenin (4′,5,7-trihydroxyflavanone), epicatechin (3,3′,4′,5,7-pentahydroxyflavan), rutin (quercetin-3-rhamnoglucoside), and naringin (naringenin-7-rhamnoglucoside) were purchased from Sigma-Aldrich Co. (St. Louis, Missouri, USA). All chemicals and solvents were of analytical grade. Flavonoids were dissolved in dimethyl sulfoxide (DMSO) and stored at −20°C. In experiments, the stock solutions were diluted by RPMI 1640 medium up to DMSO concentration not more than 0.2% that is nontoxic for cells.

Cell lines

Human erythroblast crises of chronic myelogenous leukemia K562 cell line was cultured in RPMI 1640 medium containing 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mmol/l L-glutamine, and 10% heat-inactivated fetal bovine serum, at 37°C in a humidified atmosphere with 5% CO_2. Human normal mononuclear peripheral blood (MPB) cells were separated from the healthy donors' peripheral blood by Ficoll-Hypaque density gradient centrifugation (SERVA, Heidelberg, Germany) and cultured at the same conditions in RPMI 1640 medium in the absence (nonstimulated MPB cells) or presence of 5 μg/ml of phytohemagglutinin (PHA) (stimulated MPB cells).

MTT test

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (purchased from Sigma-Aldrich Co.) test was carried out as described by Niks and Otto [12]. Briefly, 20 μl of MTT stock solution (5 mg/ml) was added to each well of flat-bottom 96-well culture plate with target cells, and cells were incubated for additional 4 h at 37°C in humidity atmosphere with 5% CO_2. Then, supernatant was carefully removed and cell pellets were dissolved in 200 μl of DMSO. Optical density was measured by Multiscan Ascent Photometer Plate Reader (Varian Inc., Palo Alto, California, USA) at $\lambda = 492$ nm.

Trypan blue exclusion test

Cell suspension was mixed with the same volume of 0.4% trypan blue solution (Sigma-Aldrich Co.). Thereafter, the number of viable (unstained) cells and the number of dead (stained) cells were counted using a hemocytometer. Cell viability (%) was counted as the number of viable cells divided by the total number of dead and viable cells.

DNA fragmentation assay

DNA extraction was carried out as described by Gong *et al.* [13]. In brief, 2×10^6 cells were fixed in 70% ethanol at −20°C for 24 h. Fixed cells were resuspended in the extraction buffer containing 192 parts of 0.2 mol/l Na_2HPO_4 and 8 parts of 0.1 mol/l citric acid, incubated and centrifuged. Supernatant with low-weight DNA fragments was collected and consecutively cleaned by Triton-X100, RNase A, and proteinase K treatment. The solution was concentrated in the vacuum concentrator up to residual volume of 10 μl. DNA fragments were separated by standard electrophoresis in 2% agarose gel with 4 V/cm voltage.

Cell cycle analysis

The method of Gong *et al.* [13] was used to analyze cell cycle distribution. The percentage of cells in G_0/G_1,

S, and G_2/M phases, and in the sub-G_1 fraction was determined by FACScan flow cytometry (Becton Dickenson, Franklin Lakes, New Jersey, USA).

Clonogenic assay

Cells were suspended at a concentration of 1.5×10^3 cells/ml in semisolid RPMI 1640 medium containing 0.8% methylcellulose, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mmol/l L-glutamine, and 20% heat-inactivated fetal bovine serum. Cell suspension (0.5 ml) was added to each well of a 24-well culture plate and cells were incubated with or without flavonoids for the next 10 days at 37°C in a humidified atmosphere with 5% CO_2. At the end of incubation, clones with at least 20 cells were analyzed and counted.

Estimation of erythroid differentiation by hemoglobin production

Leukemia cells (10^6) were lysed in 40 µl of 2% solution of NP-40 (Nonidet P 40; Fluka, Buchs, Switzerland) in PBS and centrifuged for 30 min at 20 000*g* and 4°C. The 20-µl aliquots of supernatant were transferred into new Eppendorf tubes and mixed with 100 µl of 2% solution of benzidine in 90% acetic acid and 100 µl of 1% hydrogen peroxide solution. The reaction was stopped after 20 min by adding 1.1 ml of 10% acetic acid. Optical density of the solution was measured by a spectrophotometer Cary 50 (Varian) at $\lambda = 510$ nm, and the hemoglobin concentration was calculated. A standard curve was plotted using bovine hemoglobin solutions with known protein concentrations.

Mathematical data processing

The inhibition concentration required for 50% cytotoxic effect (IC_{50}) for each flavonoid was calculated according to the method of Khafif *et al.* [14] using results from the MTT test. Regression analysis was performed by OriginPro 7.0 program package (OriginLab, Northampton, Massachusetts, USA).

Statistical data analysis

Results were presented as a mean ± standard error of mean. Comparative statistic analysis for two data sets was performed using standard Student's *t*-test. A *P* value of less than 0.05 was required for the determination of statistical significance. The descriptive and comparative statistics were carried out using OriginPro 7.0 program package.

Results

Common cytotoxicity of flavonoids and structure–activity relationships

To evaluate and compare cytotoxic action of flavonoids on human K562 chronic myelogenous leukemia cells, we carried out the MTT test using a 96-h incubation period and ranges of flavonoid concentrations from 5 to 160 µmol/l (or to 640 µmol/l, if the flavonoid had low efficacy). We calculated the IC_{50} for each investigated flavonoid using dose–effect curves and the Michaelis–Menten equation as described in Ref. [14]. We investigated the cytotoxicity of 16 aglycone flavonoids, such as flavone, flavonole, flavanones, and their hydroxyl groups substituted derivatives and epicathehin (Fig. 1) and two glycosylated forms of flavonoids – rutin (quercetin-3-rhamnoglucoside) and naringin (naringenin-7-rhamnoglucoside) as well. Flavones and most flavonols exhibit the strongest cytotoxicity. The flavanone subfamily members (except flavanone itself) were less effective and the flavan epicatechin had the weakest cytotoxicity among nonglycosylated flavonoids, indicating the important role of the C2–C3 double bond and C4 carbonyl for the cytotoxic properties of flavonoids. We also observed that the ortho-position of hydroxyl groups in the B ring of the flavonoid molecule has an advantage over other variants of B ring hydroxylation patterns. At the same time, flavonoids with the nonhydroxylated B ring were effective as well. In the A ring, hydroxylation status of C5 is not critical for the antileukemic activity of the flavonoids, whereas appearance of the hydroxyl group under the C6 position of flavonoid molecule significantly decreased the IC_{50} value. Glycosylation of flavonoids was associated with dramatically decreased activity (Table 1). Finally, 11 flavonoids of 18 seemed to be the most effective cytostatics (with IC_{50} values of approximately 80 µmol/l or less), which include five flavones (flavone, chrysin, baicalein, apigenin, luteolin), five flavonols (flavonol, galangin, fisetin, quercetin, myricetin), and flavanone.

To examine whether the tested flavonoids are cytotoxic toward normal human cells, we also carried out an analogous MTT test with human MPB cells from healthy donors. We used two variants of the MPB cells in the study. The first variant, nonstimulated MPB cells, representing nondividing cells, and the second, MPB cells stimulated with PHA, representing dividing cells. As is evident from the Table 1, not all of the tested flavonoids are specific in their antileukemic action. Nevertheless, most flavonoids showed stronger cytotoxicity toward leukemic cells as compared with normal MPB cells. At the same time the PHA-stimulated normal cells were more sensitive to flavonoid action as compared with unstimulated normal cells. Baicalein and myricetin were shown to exert the most specific antileukemic action.

Effect of flavonoids on proliferation of human K562 chronic myelogenous leukemia cells

To evaluate the effect of flavonoids on proliferation of the human K562 chronic myelogenous leukemia cells, we estimated the concentration of viable leukemia cells, their clonogenic potency, and cell cycle distribution. In this series of experiments, leukemia cells were incubated with flavonoid concentrations of 5, 20, 40,

Table 1 Cytotoxic effect (IC_{50}) of flavonoids on human K562 chronic myelogenous leukemia cells and on normal human MPB cells

		IC_{50} (μmol/l)		
Flavonoid	Systematic name	K562	MPB cells, nonstimulated	MPB cells, stimulated with PHA
Flavones				
Baicalein	5,6,7-trihydroxyflavone	24.3 ± 2.5	319.6 ± 36.6[a]	160.2 ± 12.6[a]
Luteolin	3′,4′,5,7-tetrahydroxyflavone	30.7 ± 2.0	38.2 ± 6.9	20.7 ± 3.5
Apigenin	4′,5,7-trihydroxyflavone	36.5 ± 4.9	29.2 ± 4.8	23.5 ± 1.7
Chrysin	5,7-dihydroxyflavone	59.4 ± 5.5	84.4 ± 9.2	67.4 ± 8.9
Flavone	2-phenyl-4H-1-benzopyran-4-one	80.1 ± 1.3	114.8 ± 18.8	136.5 ± 17.8
Flavonoles				
Galangin	3,5,7-trihydroxyflavone	44.3 ± 2.0	137.7 ± 20.2[a]	82.8 ± 12.9[a]
Flavonol	3-hydroxyflavone	49.8 ± 8.0	260.8 ± 53.4[a]	130.2 ± 20.3[a]
Fisetin	3,3′,4′,7-tetrahydroxyflavone	62.9 ± 3.4	122.2 ± 24.6[a]	66.9 ± 6.1
Quercetin	3,3′,4′,5,7-pentahydroxyflavone	64.1 ± 5.1	212.9 ± 22.8[a]	139.1 ± 13.8[a]
Myricetin	3,3′,4′,5,5′,7-hexahydroxyflavone	78.2 ± 4.8	1192.2 ± 456.6[a]	310.8 ± 35.8[a]
Kaempferol	3,4′,5,7-tetrahydroxyflavone	98.7 ± 11.2	NM	NM
Morin	2′,3,4′,5,7-pentahydroxyflavone	>320	NM	NM
Flavanones				
Flavanone	2,3-dihydroflavone	55.1 ± 4.0	102.8 ± 7.3[a]	51 ± 7.6
Taxifolin	(2R, 3R)-3,3′,4′,5,7-pentahydroxyflavanone	212.5 ± 12.3	NM	NM
Naringenin	4′,5,7-trihydroxyflavanone	291.9 ± 8.2	NM	NM
Catechines				
(−)-Epicatechin	(−)-cis-3,3′,4′,5,7-pentahydroxyflavane	>320	NM	NM
Flavonoid glycosides				
Rutin	Quercetin-3-rhamnoglucoside	>640	NM	NM
Naringin	Naringinin-7-rhamnoglucoside	>640	NM	NM

For determination of IC_{50} (inhibition concentration required for 50% cytotoxic effect), data set from 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) tests with each individual flavonoid were analyzed by best-fit nonlinear regression algorithm. Average results (as mean ± standard error of mean) of three independent experiments are shown.
MPB, mononuclear peripheral blood; NM, not measured; PHA, phytohemagglutinin.
[a]Data are distinct as compared with K562 cells, $P<0.05$.

and 80 μmol/l for 96 h. We have found that the viability of the flavonoid-treated cells decreases in dose-dependent manner starting from 20 μmol/l for all flavonoids (Fig. 2; in this figure and in all subsequent figures, results for the most active flavonoids are represented). Baicalein and galangin most effectively reduced cell viability, whereas flavone, chrysin, luteolin, flavonols, and myricetin were far less active. We observed analogous dynamics in cell concentration data (Fig. 3), but the steeper fall of this parameter for all flavonoids gave reason to consider that not only cell death but also proliferation rate reduction under the influence of flavonoids was the cause of the obtained results. The steepest dose-dependent lowering in cell concentration was observed during incubation of cells with baicalein, luteolin, apigenin, galangin, whereas flavone, flavonol, and myricetin had more gentle antiproliferative action.

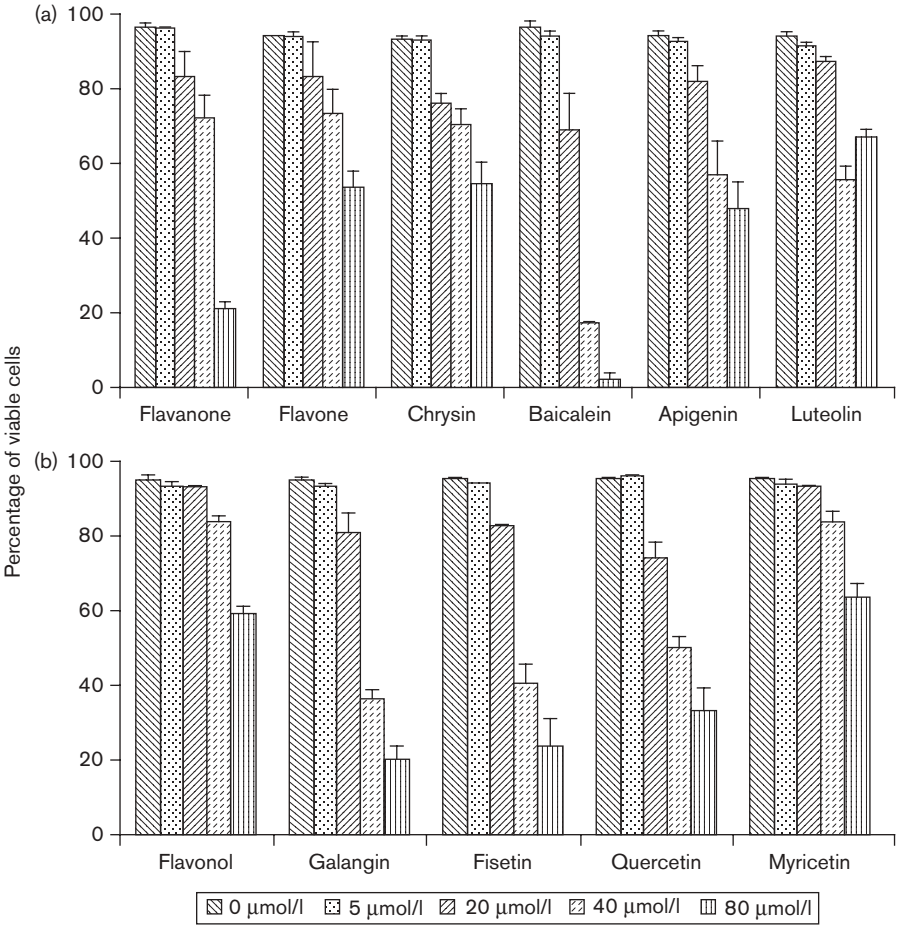
Clonogenic assay enabled us to evaluate how flavonoids influence the clonogenic potency of K562 leukemia cells. Cells were incubated in semisolid RPMI 1640 medium with same flavonoid concentrations of 5, 20, 40, and 80 μmol/l for 10 days, needed for colonies to be formed. Cells were inoculated at a density of 750 cells per well of the 24-well cell culture plate. In our clonogenic experiments, only 485 ± 14 colonies were generated in control wells (without any flavonoid); this may be because of population heterogeneity and low clonogenicity of K562 leukemia cells. While flavonoids were being added, a dose-dependent decrease in the number of colonies being formed was observed. For six flavonoids, no colonies were found when they were taken in the dose of 80 μmol/l (Fig. 4). The strongest effect was observed when cells were incubated with baicalein. Surprisingly, galangin had a rather weak effect in these series of experiments. Therefore, these findings also indicate high antiproliferative potential of the flavonoids in the study.

In conclusion, to determine whether flavonoids influence the cell cycle distribution of K562 leukemia cells, flow cytometry analysis was performed. Cells were treated with flavonoids in concentrations of 5, 20, 40, and 80 μmol/l for 96 h and the percentages of cells in G_0/G_1, S, and G_2/M phases were determined as described earlier [13]. We found that significant changes in cell cycle distribution of K562 leukemia cells were induced by apigenin (in concentrations 20 μmol/l and more), luteolin, fisetin, quercetin (in concentrations 20 and 40 μmol/l for each of indicated flavonoids), flavone, and flavanone (in concentration 80 μmol/l for each of indicated flavonoids). In these cases, accumulation of leukemia cells in G_2/M phase of cell cycle with simultaneous decrease of G_0/G_1-phase and/or S-phase cells was observed (Fig. 5).

Effect of flavonoids on the apoptosis of human K562 chronic myelogenous leukemia cells

We used two different approaches to determine whether flavonoids influence the programmed cell death of K562 leukemia cells: flow cytometry detection of cells with hypodiploid DNA content (apoptotic cells) and analysis

Fig. 2



Effect of C ring nonhydroxylated (a) and hydroxylated (b) flavonoids on viability of human K562 chronic myelogenous leukemia cells. Viability of leukemia cells was determined by the trypan blue exclusion test after 96-h incubation of target cells with indicated flavonoids. Average results (as mean ± standard error of mean) of three independent experiments are shown.

of internucleosomal DNA fragmentation by agarose gel electrophoresis. As shown in Fig. 5, all selected flavonoids increase the number of apoptotic hypodiploid cells in dose-dependent manner. Baicalein was the strongest inducer of apoptosis in K562 cells. Myricetin showed the lowest effectiveness. These flow cytometry data were successfully confirmed by the results from DNA gel electrophoresis experiments. We also observed a strong dose-dependent internucleosomal DNA fragmentation in K562 leukemia cells after 96-h incubation with flavonoids (Fig. 6). Therefore, our results indicate the pro-apoptotic effect of flavonoids on K562 leukemia cells.

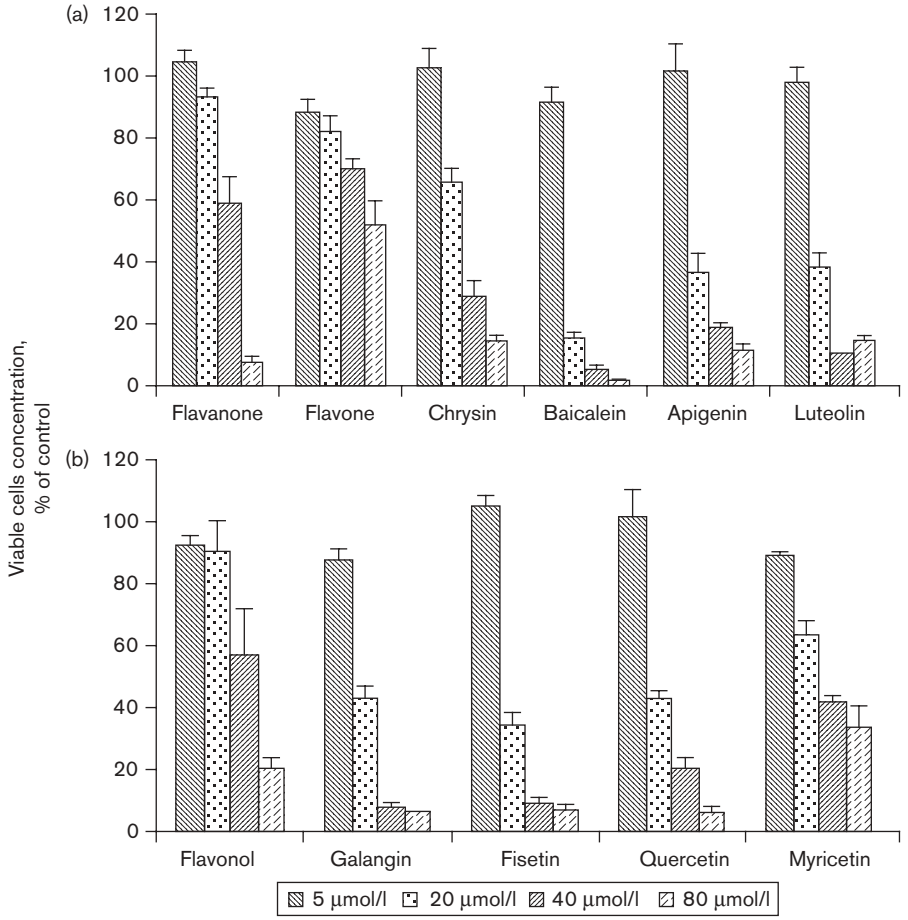
Effect of flavonoids on the differentiation of human K562 chronic myelogenous leukemia cells

The K562 cell line is a culture of erythroblast-like cells of chronic myelogenous leukemia. Two different ways of inductor-stimulated differentiation of K562 leukemia cells have been described in literature: erythroid (under the influence of inductors, such as hemin) or megakaryocytic (under the influence of inductors, such as forbol ester). In our investigation, we hypothesized that flavonoids can induce differentiation of K562 leukemia cells.

We studied the changes in the production of hemoglobin in K562 leukemia cells after 96-h incubation with flavonoids in concentrations of 5, 20, 40, and 80 μmol/l. As it is shown in Fig. 7, only galangin in the concentration of 40 μmol/l (but not in concentrations of 5, 20, or 80 μmol/l) was capable of inducing relatively large and statistically significant increase (1.8-fold) in hemoglobin production. All remaining flavonoids did not increase the hemoglobin production. Moreover, some flavonoids substantially decreased the hemoglobin production in K562 leukemia cells, especially when they were taken in highly toxic doses.

We examined whether flavonoids induce megacariocytic differentiation of K562 cells by morphology observation and flow cytometric determination of poliploid cell

Fig. 3



Effect of C ring nonhydroxylated (a) and hydroxylated (b) flavonoids on proliferation of human K562 chronic myelogenous leukemia cells. The concentration of viable cells was determined by standard approach after 96-h incubation of leukemia cells with indicated flavonoids. Concentration of cells that were incubated without any flavonoid served as a control. Average results (as mean ± standard error of mean) of three independent experiments are shown.

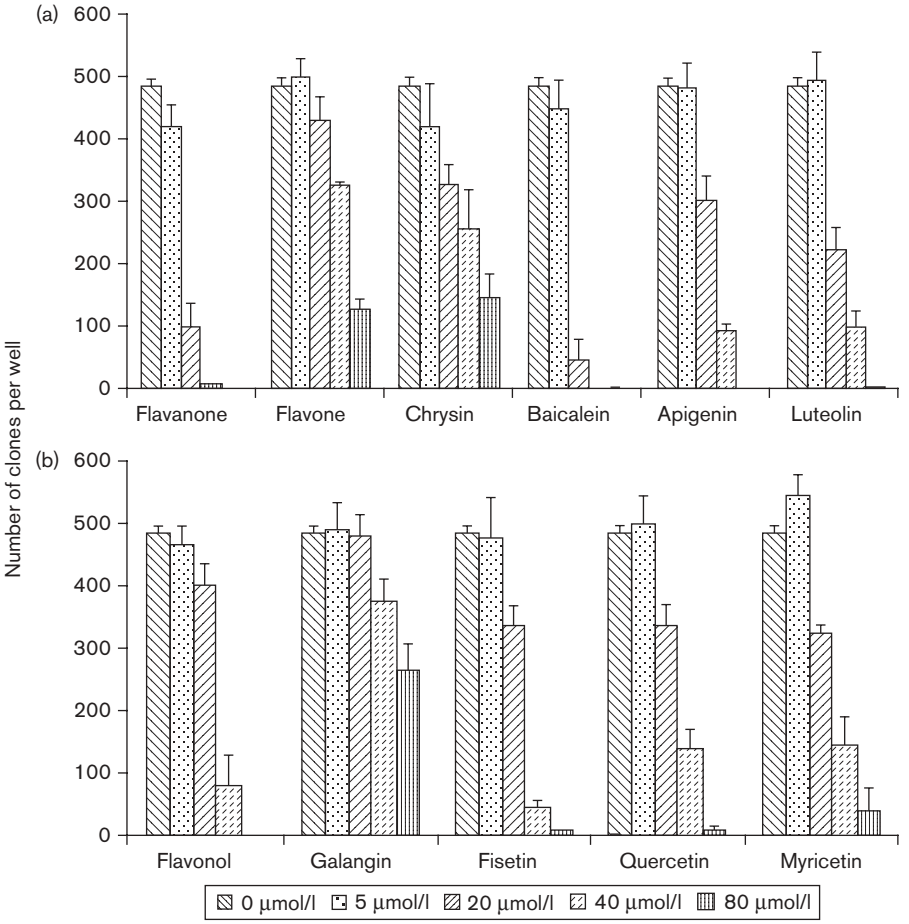
percentage. We found no evidence of megacariocytic differentiation in flavonoid-treated K562 leukemia cells (data not shown). Taken together, these data indicate that the flavonoids studied have no or little effect on the differentiation of K562 cells.

Discussion

Recent studies have shown that the anticancer properties of flavonoids are realized through diverse mechanisms, including oxidative processes, alteration of enzyme activity, and influence on the expression of some genes [1–10]. In contrast, the observed effects significantly depend on the bioavailability of flavonoids for the target cells. These determine rather complicated relationships between flavonoid structure and biological activity. That is why there are many discrepancies between studies regarding this [11]. Kuntz *et al.* [15] found no obvious structure–activity relationships for cytotoxic action of flavonoids of different subfamilies in their study. To investigate the structure–activity relationships for cytotoxic action of flavonoids on K562 leukemia cells, we calculated and compared the IC_{50} for 18 flavonoids representing four flavonoid subfamilies. They include three simple synthetic flavonoids with nonhydroxylated A and B rings – flavone, flavon-3-ol, and flavanone, and their hydroxylated analogs.

Most authors, having investigated the anticancer properties of flavonoids, postulated that the necessary requisites for high effectiveness are the carbonyl group at C4 and the unsaturated C2–C3 double bond [16–19]. The same requisites were shown to be essential for inhibiting the activity of flavonoids toward phosphatidylinositol 3 kinase and protein kinase C, enzymes strongly associated with cell cycle regulation [5]. In our study, it was shown (in the pair taxifolin–epicathehin) that the presence of C4 carbonyl is indeed critical for proapoptotic and antiproliferative effects on K562 leukemic cells. The C2–C3 double bond is also important

Fig. 4

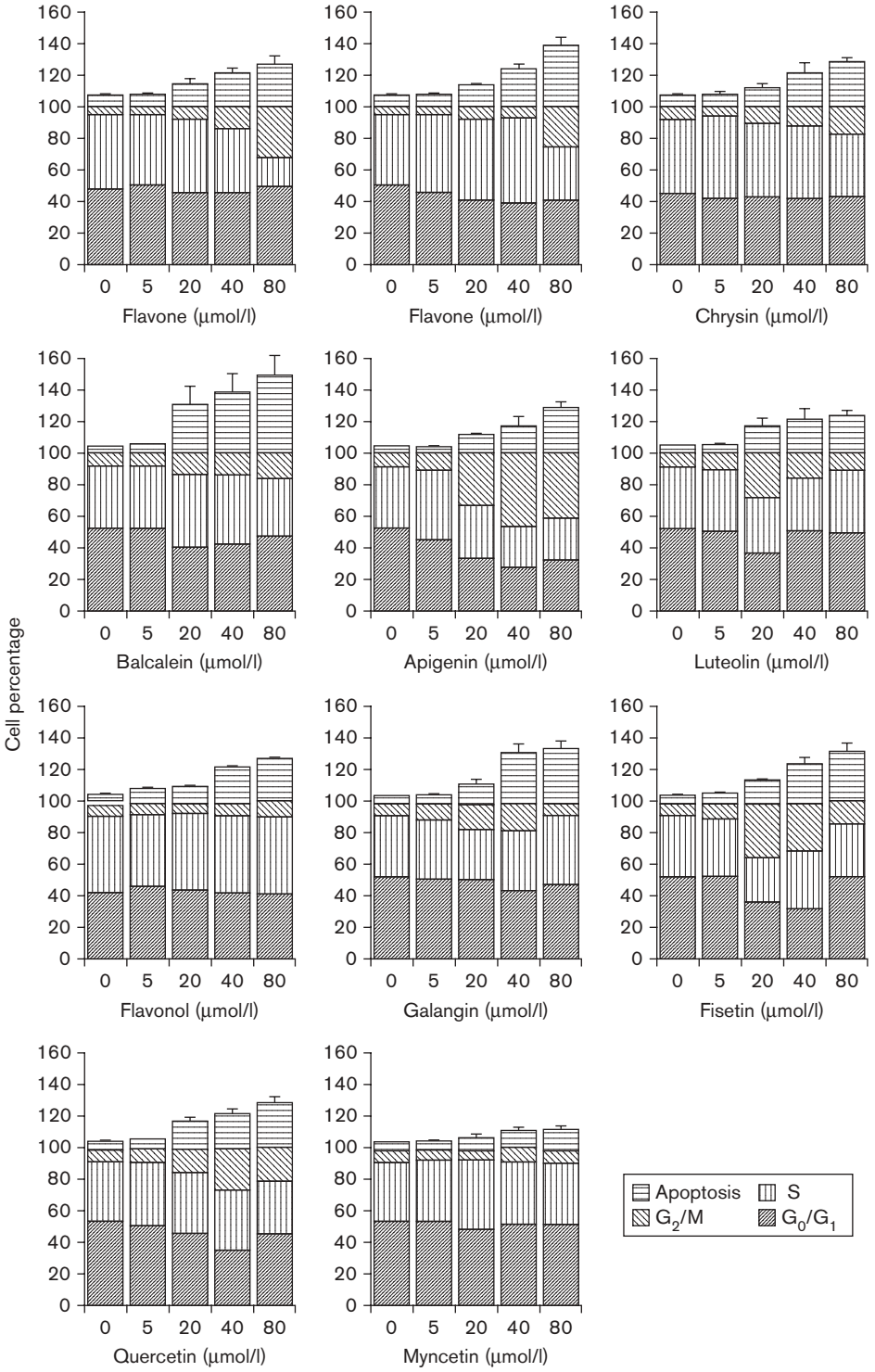


Effect of C ring non-hydroxylated (a) and hydroxylated (b) flavonoids on clonogenicity of human K562 chronic myelogenous leukemia cells. Clonogenic assay was carried out in 24-well cell culture plate format as described in the Materials and methods. Average results (as mean ± standard error of mean) of three independent experiments are shown.

for high cytotoxicity as this follows from the analysis of quercetin–taxifolin and apigenin–naringenin pairs, confirming the work of other authors. Meanwhile, in the pair flavone–flavanone, cytotoxicity was the strongest for flavanone with the saturated C2–C3 bond. Interesting results were obtained with regard to the influence of C3 hydroxylation on the cytotoxic activity of flavonoids. In the pairs luteolin–quercetin and apigenin–kaempferol, C3 nonhydroxylated forms were more potent. This is in agreement with the results of Kawaii *et al.* [16] and Plochmann *et al.* [17], having shown that hydroxylation at C3 of flavones reduced their activity. At the same time, inverse relationships were observed in our study in the pairs chrysin–galangin and flavone–flavonol. It can be concluded from the above-mentioned facts that apart from the C ring structure, the hydroxylation pattern in A and B aromatic rings may have substantial influence on the antileukemic activity of individual flavonoids. Some illusory contradictions may arise from the existence of several intracellular targets of flavonoids and different structure–activity relationships with regard to each of them [11,18].

It was shown in the pairs quercetin–morin, quercetin–myricetin, and quercetin–kaempferol that the orthoposition of hydroxyl groups in the B ring of the flavonoid molecule has an advantage over other variants of B ring hydroxylation pattern for flavonoid cytostatic activity. These results are in agreement with most other similar investigations [16]. It is interesting that C4′ hydroxylation in the pairs galangin–kaempferol and chrysin–apigenin plays an opposite role in cytotoxicity of the flavonoids. Therefore, flavonoids with a nonhydroxylated B ring, such as baicalein, chrysin, galangin, flavonol, and flavanone, were approximately equal or even stronger cytostatics than flavonoids with 3′,4′–OH groups (for the pairs galangin–quercetin and chrysin–luteolin, see Fig. 1).

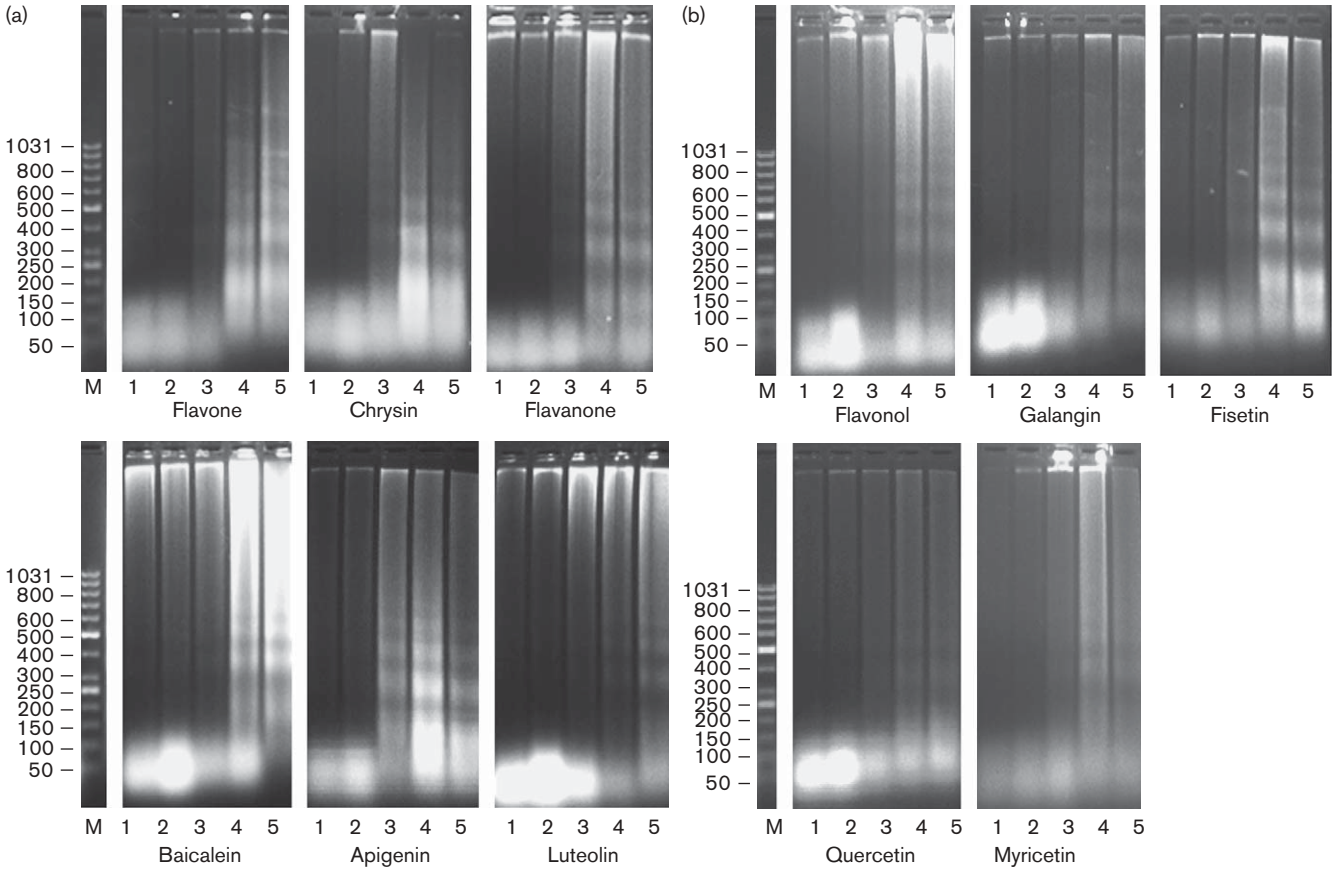
Fig. 5



Cell cycle distribution of human K562 chronic myelogenous leukemia cells after 96-h incubation with flavonoids. Percentage of cells in G_0/G_1, S, and G_2/M phases and in the sub-G_1 fraction was determined by FACScan flow cytometry. Average results of three independent experiments are shown (standard error of mean is indicated just for sub-G_1 fraction of cells).

Hydroxylation status of C5 in the A ring of flavonoid molecules in quercetin–fisetin did not significantly influence the IC_{50} values of the flavonoids. At the same time, the presence of the hydroxyl group in C6 position in chrysin–baicalein resulted in a significantly lower IC_{50} value.

Fig. 6



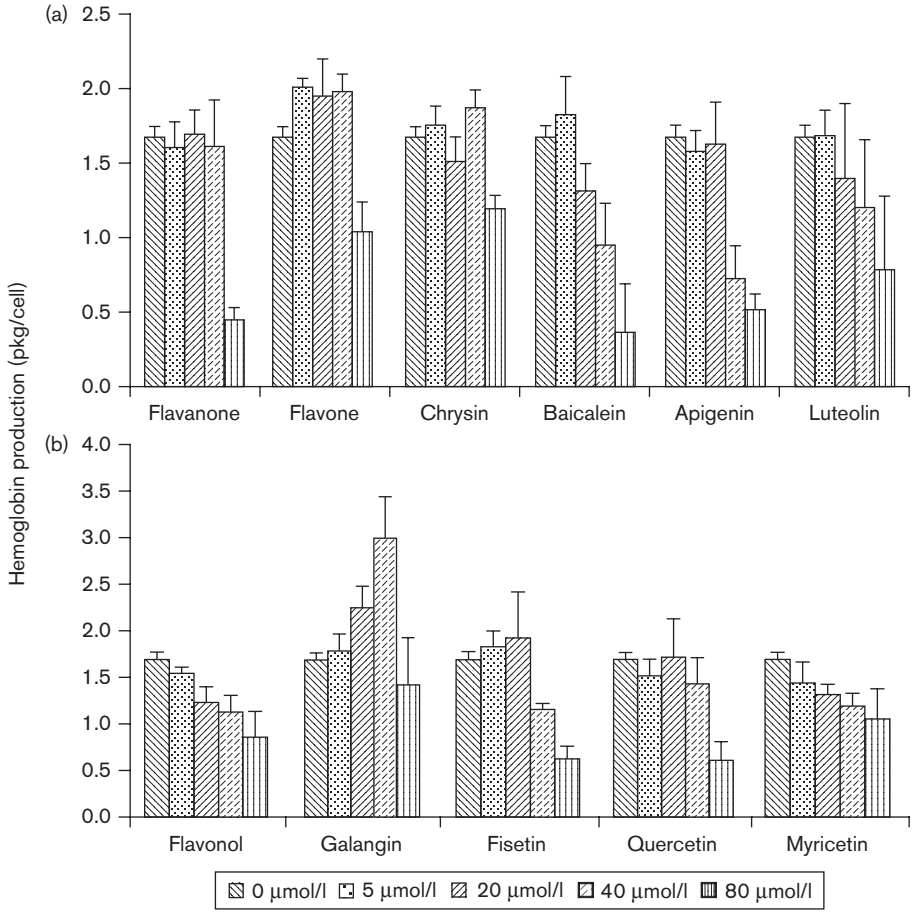
Flavonoid-induced internucleosomal DNA fragmentation in human K562 chronic myelogenous leukemia cells. Leukemia cells were treated with C ring nonhydroxylated (a) or hydroxylated (b) flavonoids for 96 h and low-weight DNA fragments were isolated and separated by standard 2% agarose gel electrophoresis as described in Ref. [13]. Representative pictures from three independent experiments are shown. M, molecular weight markers (Fermentas, Hanover, Maryland, USA)

Results of the cytotoxicity assay in the pairs quercetin–rutin and naringenin–naringin showed critical loss of anticancer potency of glycosylated forms of flavonoids, agreeing with data from other studies [16]. We did not find any correlation between the number of hydroxyl groups in flavonoid molecules and their antileukemic properties.

For 11 selected flavonoids with the lowest IC_{50} values (up to 80 µmol/l), we investigated the manner in which they influenced proliferation, cell cycle, survival/apoptosis, and differentiation of leukemia cells. We obtained conclusive evidence of cell proliferation inhibition and apoptosis induction in K562 leukemia cells under treatment with flavonoids. Some of them were shown to induce cell cycle arrest in G_2/M phase. The influence of flavonoids on the differentiation processes in cancer cells still remains insufficiently investigated. Few flavonoids have been tested for this aspect of their action, and several authors have outlined controversial results [1]. We studied changes in several indicators of erythroid versus megacariocytic differentiation of K562 cells and did not find any evidence of differentiation inducing activity in selected flavonoids.

Very important is the question of whether the cytotoxic action of flavonoids is specific enough toward cancer cells and what is the ratio between the effective doses and maximum tolerable doses of the compounds. There are very few data about tolerable doses of different flavonoids in humans. In phase I of clinical trials of quercetin, Ferry *et al.* [20] have shown that the serum levels achieved immediately after intravenous injection of quercetin were in the range of 200–400 µmol/l at 945 mg/m^2, with serum levels above 1 µmol/l being maintained up to 4 h. They found that several dose-limiting toxicities took place, if the concentration of quercetin exceeded 1700 mg/m^2. One of the reasons may be the DMSO usage as a solvent for quercetin. A possible solution to this problem may be to design water-soluble flavonoid prodrugs. No data of in-vivo toxicities are available for other flavonoids investigated in this study.

Fig. 7



Flavonoid-induced erythroid differentiation of human K562 chronic myelogenous leukemia cells. Leukemia cells were treated with C ring nonhydroxylated (a) or hydroxylated (b) flavonoids for 96 h and erythroid differentiation was assessed by determination of hemoglobin production as described in the Materials and methods. Average results (as mean ± standard error of mean) of three independent experiments are shown.

While experimenting with human normal MPB cells we found that at least several of tested flavonoids (such as baicalein, myricetin, flavonol) had rather low cytotoxicity toward normal blood cells as compared with leukemic cells.

Therefore, in conclusion, we can postulate that flavonoids are proapoptotic and antiproliferative agents toward K562 leukemic cells and that the degree of antileukemic properties is dependent on their molecular structure. Favorable requisites are the presence of carbonyl at C4 (which is absent in catechines), a double bond C2–C3 (all flavones and flavonols), C3′,C4′–OH hydroxylation pattern (such as luteolin and quercetin) for flavones, or flavonols with a hydroxylated B ring, or a C5,C6, C7–OH hydroxylation pattern for flavones with a nonhydroxylated B ring (such as baicalein). C3 hydroxylation may differently influence antileukemic activity of flavonoids. This may be explained by having different intracellular targets involved in achieving the cytostatic effects by molecules with different hydroxylation pattern. Finally, aglycone forms of flavonoids are much more effective than their glycosylated analogs.

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