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Structure–activity relationships of flavonoid-induced cytotoxicity on human leukemia cells

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Abstract

The aim of this study was to identify structure elements in flavonoids that are associated with enhanced cytotoxic activity. We determined the cytotoxicity (EC_{50}) of 23 different flavonoids, including *O*-methylated and glucuronidated metabolites, on the human leukemia cell line Jurkat E6-1 by analyzing cell death triggered after 24 and 48 h. By comparing the cytotoxicity of selected molecules that differ in only one structure element, we identified several structure–function relationships associated with enhanced cytotoxicity, including the presence of a 2–3 double bond, the presence of a 4-carbonyl group and *ortho*- compared to *meta*-hydroxylation in the B ring. Molecules with a 3-hydroxyl group exhibited significantly lower cytotoxicity than their non-hydroxylated counterparts. *O*-Methylation and glucuronidation were associated with a significant increase in cytotoxicity, suggesting that metabolites found *in vivo* are more active than unmodified flavonoids. We identified the solubility maximum of the tested flavonoids in culture medium and found a negative correlation between maximum solubility and cytotoxicity. The results of our study may help to identify novel flavonoid structures with optimized cytotoxic activity to be tested for anti-cancer treatment.

Keywords: Flavonoids; Metabolites; Structure–activity relationships; Cytotoxicity; Lymphocytes; Anticancer; EC_{50}; Solubility maximum

Flavonoids consist of a group of polyphenolic compounds derived from 2-phenylchromane. They are found in considerable quantities in fruits, vegetables, seed, peel and tubers [1,2]. Flavonoids, together with some antioxidative vitamins, carotenoids and others, belong to a chemically heterogeneous group of small molecules with chemopreventive activity [3]. Moreover, flavonoids exert specific cytotoxic activity towards cancer cells which has generated large interest in developing flavonoid-based cytostatics for anti-cancer therapy (reviewed in [4]) and flavonoids have been shown to possess anticancer activity in a mouse xenograft model [5].

Different mechanisms have been linked to flavonoid-mediated cytotoxicity, including proteasome inhibition [6,7], inhibition of fatty acid synthesis [8], topoisomerase inhibition [9], inhibition of phosphatidyl-inositol 3-kinase [10], induction of cell cycle arrest [11], accumulation of p53 [12] or enhanced expression of c-fos and c-myc [13]. As multiple mechanisms account for flavonoid-induced cytotoxicity, the development of structure–activity relationships to predict the cytotoxic potential of a given compound may facilitate the search for effective candidates for cancer therapy.

In this study we investigated the toxicity of different flavonoids on the human leukemic T cell line Jurkat E6-1 in order to reveal structure elements associated with cytotoxic activity. We used a library of 23 different flavonoids and determined the EC_{50} (the concentration at which 50% of the cells die) of each substance. Structure–activity relationships

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were analyzed by comparing the EC_{50}'s of several substance pairs that differ in only one structure element.

Materials and methods

Flavonoids

Quercetin (3′,4′,3,5,7-pentahydroxyflavone)-dihydrate, isorhamnetin (4′,3,5,7-tetrahydroxy-3′methoxy-flavone), phloroglucin (1,3,5-trihydroxybenzene), myricetin (3′,4′,5′,3,5,7-hexahydroxyflavone), eriodictyol ((+)-(2S)-3′,4′,5,7-tetrahydroxyflavanone), and apigenin (4′,5,7-trihydroxyflavone) were purchased from Fluka (Deisenhofen, Germany). Phloretin (2′,4′,6′-trihydroxy-3-(4-hydroxyphenyl)propiophenone), taxifolin (3,5,7,3′,4′-pentahydroxyflavanone), morin (2′,4′,3,5,7-pentahydroxyflavone)-dihydrate, kaempferol (4′,3,5,7-tetrahydroxyflavone), naringenin ((−)-(2S)-4′,5,7-trihydroxyflavanone), and hesperetin ((−)-(2S)-3′,5,7-trihydroxy-4′-methoxyflavanone) were purchased from Roth (Karlsruhe, Germany). Scutellarein (4′,5,6,7-tetrahydroxyflavone) was purchased from Extrasynthese S. A. (Genay Cedex, France). Baicalein (5,6,7-trihydroxyflavone), baicalein-7-glucuronide (5,6-dihydroxyflavone-7-glucuronide) and chrysin (5,7-dihydroxyflavone) were purchased from Aldrich (Steinheim, Germany) product. (+)-Catechin ((+)-(2R:3S)-5,7,3′,4′-tetrahydroxyflavan-3-ol) was purchased from Roth (Germany). Hispidulin (4′,5,7-trihydroxy-6-methoxyflavone) and cirsimaritin (4′,5-dihydroxy-6,7-dimethoxyflavone) were isolated as published elsewhere [14]. Xanthohumol (2-propen-1-one, 1-[2,4-dihydroxy-6-methoxy-3-(3-methyl-2-butenyl)phenyl]-3-(4-hydroxyphenyl)-, (2E)- (9CI) was kindly provided by Dr. Hans Becker (Saarland University, Saarbruecken, Germany). Luteolin (3′,4′,5,7-tetrahydroxyflavone), phloroacetophenon (2,4,6-trihydroxyactophenone), 7-methoxy-baicalein (5,6-dihydroxy-7-methoxyflavone), and 7-methoxy-apigenin (4′,5-dihydroxy-7-methoxyflavone) were purchased from Alfa Aesar (Karlsruhe, Germany); 5,7-dihydroxy-2-methylchromone and 5-hydroxy-7-methoxy-2-methylchromone were purchased from Specs (Netherlands); Naringeninchalcon (*trans*-2,4′,6′,4-tetrahydrochalcone) was purchased from TimeTec (USA). All chemicals and solvents were of analytical grade.

Semiquantitative determination of the solubility maximum (S_{max})

To determine the solubility maximum S_{max} in culture medium, a dilution series of each substance was prepared in DMSO, starting at 200–1000 mM. Solved substance from each DMSO-dilution step was then transferred into 37 °C pre-warmed RPMI in a ratio of 1:200. The highest concentration at which the flavonoid remained in solution, as defined by the absence of microscopically apparent crystals, was identified as S_{max}.

Cell culture

Human T lymphoblasts (Jurkat Clone E6-1) were cultured in RPMI-1640 (Invitrogen, Karlsruhe, Germany) supplemented with 10% heat-inactivated fetal calf serum (FCS)[1], streptomycin (100 μg/mL), penicillin (100 IU/mL) and L-glutamine (100 mg/mL). All substances were purchased from Sigma (Germany). Cultures were maintained at 37 °C in a humified 5% CO_2 atmosphere.

Semiquantitative determination of S_{max}

Due to their hydrophobic backbone, flavonoids have a much higher solubility in DMSO than in aqueous solution. In order to be able to obtain aqueous solutions in a reasonable volume and accuracy, we prepared DMSO stock-solutions for each flavonoid that served as a source for the preparation of dilutions in RPMI. To determine S_{max} in RPMI, we prepared dilution series of the flavonoids in DMSO and transferred the dissolved substance from each DMSO-dilution step into 37 °C prewarmed RPMI in a ratio of 1:200. The highest concentration at which the flavonoid remained in solution was identified as S_{max}.

Cytotoxicity experiments

The cytotoxic effects of flavonoids were investigated in dilution series ranging from S_{max} to zero. To facilitate solution of the flavonoids in culture medium, flavonoids were dissolved in DMSO at a concentration 200 times higher than $S_{max(RPMI)}$. This solution was subsequently diluted with 37 °C pre-warmed culture medium in a ratio of 1:200, yielding to a final DMSO-concentration of 0.5% (which was not cytotoxic over 72 h). Starting from this solution, dilution series in RPMI (containing 0.5% DMSO) were prepared. Jurkat cells (10^5 cells per well) were cultured in triplicates in 96 well flat bottom culture plates (BD Falcon) in a total volume of 200 μL at different flavonoid concentrations for 24 and 48 h.

Flow cytometry

Cells were stained with annexin-V-fluoroscein isothiocyanate (Annexin-V-FITC; BD Biosciences, Heidelberg, Germany) and 7-aminoactinomycin D (7-AAD; BD Biosciences, Germany) according to the instructions of the manufacturer. Ten thousand cells per sample were analyzed by flow cytometry using a FACScan flow cytometer (Becton–Dickinson, Germany) with CellQuest Pro Software (BD) running on an Apple Macintosh. Markers were set according to untreated cells. In order to exclude any staining effects by flavonoid-mediated fluorescence, markers were double checked with cells that were short-time incubated (i.e., "stained") with the respective flavonoid. Annexin-V-negative/7-AAD-negative events were scored as living cells. Single or double positive events were scored as dead cells. To quantify accumulated cell death in treated cell cultures, 50 μL of cell suspension (equivalent to about 2.5×10^4 cells) were mixed with Annexin-V/7-AAD staining solution and 50 μL of phycoerythrin (PE)-labeled calibrite microbeads (Becton–Dickinson; adjusted to about 0.25×10^4 beads per 50 μL). Beads (PE-positive) and living cells (Annexin-V-FITC negative/7-AAD-negative) were quantified. Living cell numbers in each measurement were normalized to numbers of co-measured beads.

Determination of EC_{50} and statistical analysis

All statistical analyses were performed with a curve fit program (GraphPad Prism for Mac OSX). The relative number of living cells in each treatment condition derives from triplicates, expressed as mean plus standard deviation. The mean of living cells in untreated cells (control) was set to 100% and the corresponding standard deviation was adjusted accordingly. The number of living cells in one experiment was normalized to the number of cells in the control and the corresponding standard deviations were adjusted accordingly.

Relative living cell numbers of each experiment were analyzed using logarithmic transformation of the flavonoid concentrations and subsequent non-linear regression applying a best-fit algorithm (sigmoidal dose–response curve, top value set to 100, bottom value set to 0, variable slope) to determine the EC_{50}. Statistical comparison of cytotoxicities of two different data sets was performed by application of an *F*-test.

Correlation between EC_{50} and S_{max} was done applying a nonparametric test (two-tailed *P* with a 95% confidence interval). Correlation was graphically visualized by linear regression without restrictions for the position of the regression line.

Results

Solubility maximum of flavonoids

In this study we wanted to analyze the effects of different flavonoids (Fig. 1) on cell viability over a wide range of concentrations, reaching from zero to the solubility

[1] *Abbreviations used:* FCS, fetal calf serum; PE, phycoerythrin; P-gp, P-glycoprotein.

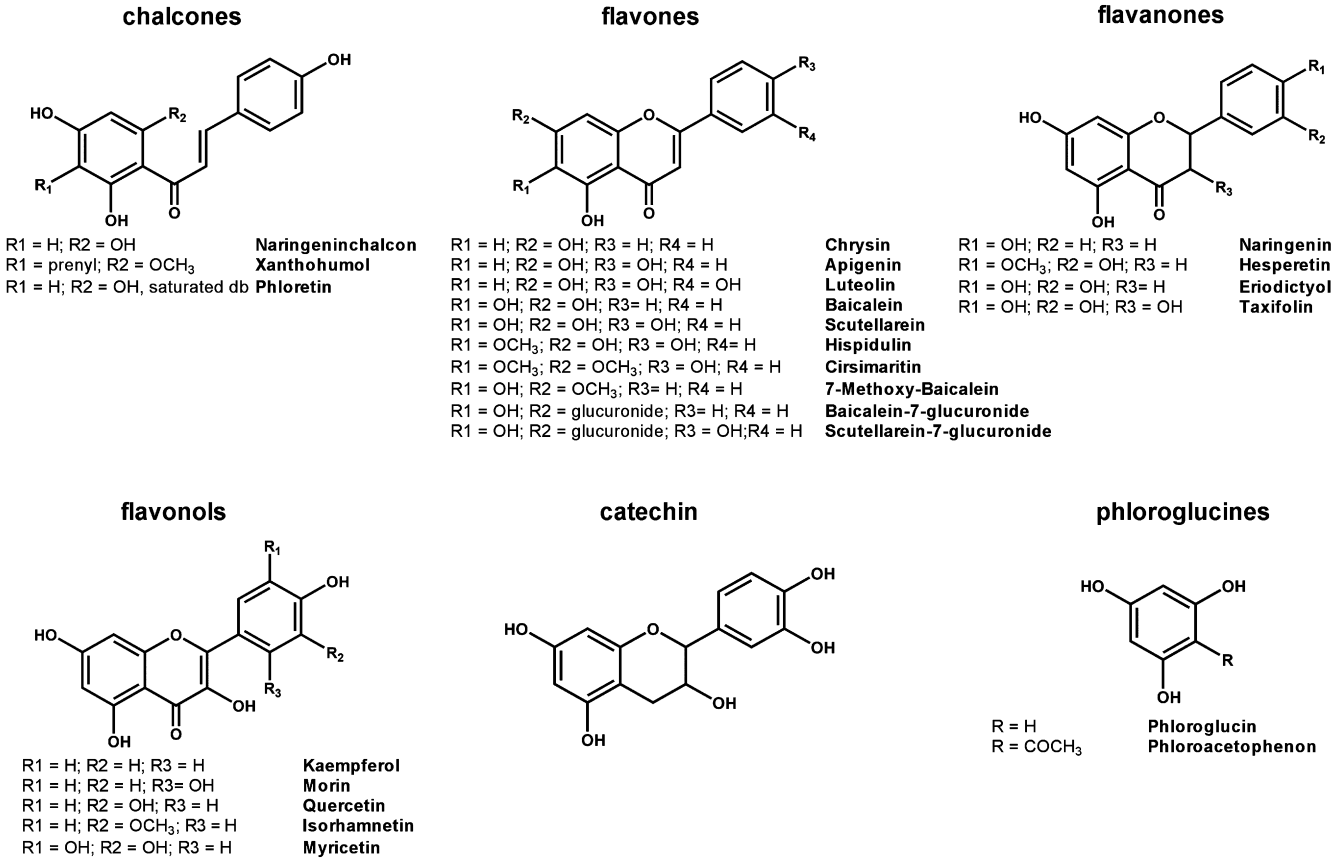

Fig. 1. Structural formulae of polyphenolic compounds used in this study.

maximum in cell culture medium. Since systematic analyses of maximum solubilities (S_{max}) of different flavonoids in aqueous solutions are missing, we determined the solubility of the substances used in this study in a semiquantitative manner (Table 1). Maximum solubility of flavonoids in RPMI was in the range of 60–300 μM for flavones, 200–500 μM for chalcones, 250–5000 μM for flavonols and dihydroflavonols and 1250–1400 μM for flavanones.

Flavonoids are cytotoxic

We have studied the effects of different flavonoids on cell viability of cultured Jurkat T lymphoblasts. Cells were incubated with dilution series of different flavonoids ranging from S_{max} to zero for 24 and 48 h. Cell viability was analyzed by Annexin-V/7-AAD flow cytometry and living cell numbers were normalized to co-quantified PE-labeled microbeads, allowing direct comparison of living cell numbers between different concentrations of flavonoids. All studied flavonoids exhibited cytotoxic activity, although marked differences in the degree of toxicity between studied substances have been observed (Fig. 2). The most toxic substances in this study had an EC_{50} in the range of 20–100 μM after 24 h and belonged to the subclass of flavones or chalcones (see EC_{50}/24 h in Table 1). At 48 h, the EC_{50} of these substances further declined to 10–50 μM (see EC_{50}/48 h in Table 1). On the other hand, flavanones and flavanols displayed the weakest toxicity of all tested substances, ranging from 200–4000 μM after 24 h. This suggests that structure–function relationships could be deduced that allow the prediction of the cytotoxic activity of a given flavonoid.

The presence of a C2–C3 double bond is associated with cytotoxicity

In order to determine structure–activity relationships as predictors of cytotoxicity, we compared the cytotoxic effects of closely related compounds that differ in only one functional group. Applying an *F*-test, we compared the toxicities of three molecule pairs (quercetin versus taxifolin, apigenin versus naringenin, luteolin versus eriodictyol) that have identical chemical structures except for the presence or absence of the C2–C3 double bond. As shown in Fig. 3a–c, structures that exhibit the C2–C3 double bond (quercetin, apigenin, luteolin) show increased toxicity compared to their molecular counterparts, which exhibit a single bond in C2–C3 (taxifolin, naringenin, eriodictyol). Differences in EC_{50}s were statistically significant for all substance pairs ($P < 0.0001$). For the tested molecules, the presence of a C2–C3 double bond correlates with a 3- to 10-fold increase of cytotoxicity (EC_{50}) compared to an identical molecule that lacks this structure element.

Table 1
Overview of maximum solubilities (S_{max}) and cytotoxicities (EC_{50}) of different flavonoids

Substance	S_{max} (mM)	EC_{50} (μM)				Increase of toxicity EC_{24}/EC_{48}
		24 h	95% Confidence interval	48 h	95% Confidence interval	
Xanthohumol	0.200	20.5	19.4–21.5	11.5	10.9–11.9	1.8
Scutellarein-7-glucuronide	2.000	43.86	34.25–56.16	n.d.	n.d.	n.d.
Cirsimaritin	0.175	66.8	43.3–102.9	44.4	25.0–78.6	1.5
Apigenin	0.125	72.7	62.5–84.5	27.0	20.2–36.2	2.7
Luteolin	0.250	78.1	66.0–92.5	18.6	11.3–30.5	4.2
Chrysin	0.125	84.3	76.3–93.2	49.1	45.0–53.1	1.7
7MeO-Baicalein	0.113	91.0	55.22–149.9	54.3	30.75–96.0	1.7
Isorhamnetin	0.225	116.8	55.5–245.8	n.d.	n.d.	n.d.
Baicalein-glucuronide	1.455	137.0	118.9–158.0	n.d.	n.d.	n.d.
Phloretin	0.450	140.6	121.6–162.7	96.1	78.4–117.9	1.5
Hispidulin	0.300	153.1	106.5–220.1	62.7	54.1–72.7	2.4
Kaempferol	0.250	163.1	133.6–199.1	46.4	36.3–59.3	3.5
Naringeninchalcon	0.500	166.0	124.0–222.2	83.9	57.6–122.1	2.0
Baicalein	0.175	213.3	114.7–396.6	48.2	38.7–60.2	4.4
Eriodictyol	1.400	226.2	142.6–358.9	34.1	23.1–50.3	6.6
Myricetin	0.700	349.3	291.6–418.5	202.0	150.7–270.7	1.7
Quercetin	0.450	354.7	268.0–469.4	83.3	70.6–98.4	4.3
Scutellarein	0.300	501.9	276.8–910.1	176.0	113.1–273.8	2.9
Hesperetin	1.300	591.6	449.2–779.1	274.5	238.2–316.4	2.2
Naringenin	1.250	617.7	578.3–659.9	131.6	103.8–166.9	4.7
Morin	0.450	680.3	297.7–1555	230.2	180.8–293.1	3.0
Taxifolin	5.000	2247	1584–3188	65.9	25.3–171.9	34.1
Catechin	5.000	4410	2196–8855	1834	1093–3072	2.4
Phloroacetophenon	11,000	6297	3111–12,746	428.2	120.9–1517	14.7
Phloroglucin	25,000	18,676	6125–56,949	4228	1558–11,472	4.4

S_{max} was determined in 37 °C pre-warmed RPMI containing 10% FCS. For determination of EC_{50}, data sets from cytotoxicity experiments were analyzed by non-linear regression. EC_{50} was determined by a best-fit algorithm for each individual substance and for each time point. Substances displayed in the table were sorted according to decreasing toxicity after 24 h. S_{max} data derived from single analysis, EC_{50} data derived from triplicate analyses.

The presence of a 4-carbonyl-group is associated with cytotoxicity

We then analyzed the effects of the presence of the 4-carbonyl group, a molecular characteristic found in all flavonoids except for flavanols and flavans. We compared the toxicities of the molecule pair taxifolin (with a C4 carbonyl group) and catechin (without a carbonyl group in C4) and found an approximately 30-fold, statistically significant ($P = 0.0022$) higher toxicity of the C4-carbonylated molecule after 48 h (Fig. 3d). At earlier time points (24 h), this difference was much less pronounced (factor 2) and statistically not significant ($P = 0.5274$, graph not shown). Although one has to be very cautious to draw conclusions from only one pair of molecules, the importance of the C4 carbonyl group for cytotoxicity is further underlined by the fact that catechin—the only substance in our study without a carbonyl group in C4—exhibited the lowest cytotoxicity of all investigated flavonoids at 24 and 48 h (Table 1).

The presence of a C3-hydroxyl-group is associated with reduced cytotoxicity

Some flavonoids, i.e., flavonols and flavanols, carry a hydroxyl residue position C3. We analyzed three different pairs of substances (apigenin versus kaempferol, luteolin versus quercetin, eriodictyol versus taxifolin) that differ only in the presence of hydroxylation in C3. As depicted (Fig. 3e–g), the presence of a hydroxyl group in C3 (kaempferol, quercetin, taxifolin) was associated with a 2- to 10-fold lower cytotoxicity compared to molecules that do not possess this residue (apigenin, luteolin, eriodictyol). The observed differences were statistically significant ($P < 0.0001$).

Ortho- versus meta-hydroxylation in the B ring is associated with enhanced cytotoxicity

The B ring of flavonoids exhibits various hydroxylation patterns that may have an influence on flavonoid-induced cytotoxicity. We compared quercetin (3′,4′-hydroxyl) and morin (2′,4′-hydroxyl) and observed significant ($P < 0.0001$) changes in cytotoxicity. The *ortho*-hydroxylated quercetin was about three times more cytotoxic than the *meta*-hydroxylated morin (Fig. 3h), indicating that the hydroxylation pattern of the B ring is involved in cytotoxic activity of flavonoids.

O-methylation and glucuronidation are associated with enhanced cytotoxicity

Methoxylation of hydroxyl residues located in the A ring is found in several flavonoids, such as 7-methoxy-baicalein, cirsimaritin, hispidulin or isorhamnetin and represents a typical modification found in flavonoid metabolites. We determined the effects of methoxylation on cytotoxicity by

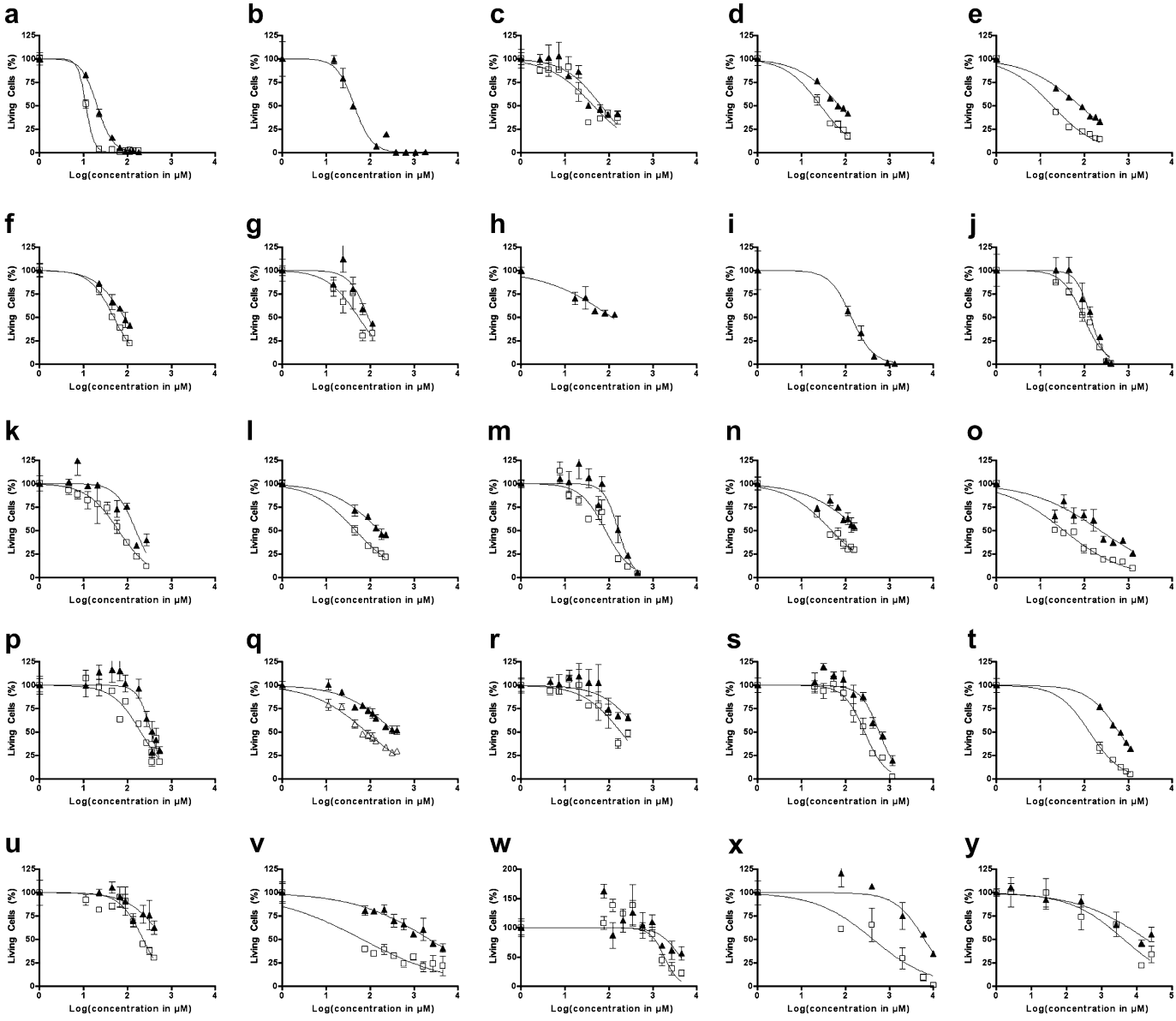

Fig. 2. Overview of cytotoxicity of different flavonoids on Jurkat T lymphoblasts. Jurkat T cells were cultured in the presence of dilution series of different flavonoids for 24 (▲) and 48 (□) hours. Cell viability was analyzed by Annexin-V/7-AAD quantitative flow cytometry. Data sets were analyzed by non-linear regression and EC_{50} (displayed in Table 1) was determined by a best-fit algorithm. Data derived from triplicate analyses. (a) Xanthohumol. (b) Scutellarein-7-glucuronide. (c) Cirsimaritin. (d) Apigenin. (e) Luteolin. (f) Chrysin. (g) 7-MeO-Baicalein. (h) Isorhamnetin. (i) Baicalein-glucuronide. (j) Phloretin. (k) Hispidulin. (l) Kaempferol. (m) Naringeninchalcon. (n) Baicalein. (o) Eriodictyol. (p) Myricetin. (q) Quercetin. (r) Scutellarein. (s) Hesperetin. (t) Naringenin. (u) Morin. (v) Taxifolin. (w) Catechin. (x) Phloroacetophenon. (y) Phloroglucin.

comparing the toxic effects of these compounds with unmethoxylated or less-methoxylated molecules. As depicted in Fig. 4a–d, the higher methoxylated compounds are significantly more toxic than the less methoxylated molecules. 7-Methoxy-baicalein is more cytotoxic than baicalein ($P = 0.0250$) (Fig. 4a), cirsimaritin is more cytotoxic than hispidulin ($P = 0.0103$) (Fig. 4b), hispidulin is more cytotoxic than scutellarein ($P = 0.0111$) (Fig. 4c), and isorhamnetin is more cytotoxic than quercetin ($P = 0.0074$) (Fig. 4d). Similarly, glucuronidation significantly increased flavonoid-triggered cytotoxicity as evidenced by a comparison of baicalein versus baicalein-glucuronide ($P = 0.0327$) (Fig. 4e) and scutellarein versus scutellarein-glucuronide ($P < 0.0001$) (Fig. 4f).

Cytotoxicity inversely correlates with the number of hydroxyl residues and solubility

The overall number of hydroxyl groups negatively correlates with the cytotoxic potential of the molecule, i.e., molecules with 2 hydroxyl groups (cirsimaritin, chrysin, 7-methoxy-baicalein) show a trend towards higher cytotoxicity (i.e., lower EC_{50}) than molecules with 3 hydroxyl groups (apigenin, hispidulin, baicalein, isorhamnetin) or 4 hydroxyl groups (luteolin, kaempferol, scutellarein) (Fig. 5a). Structures with 5 hydroxyl residues (quercetin, myricetin, morin) belong to the least toxic substances in our study. As expected, the number of hydroxyl residues

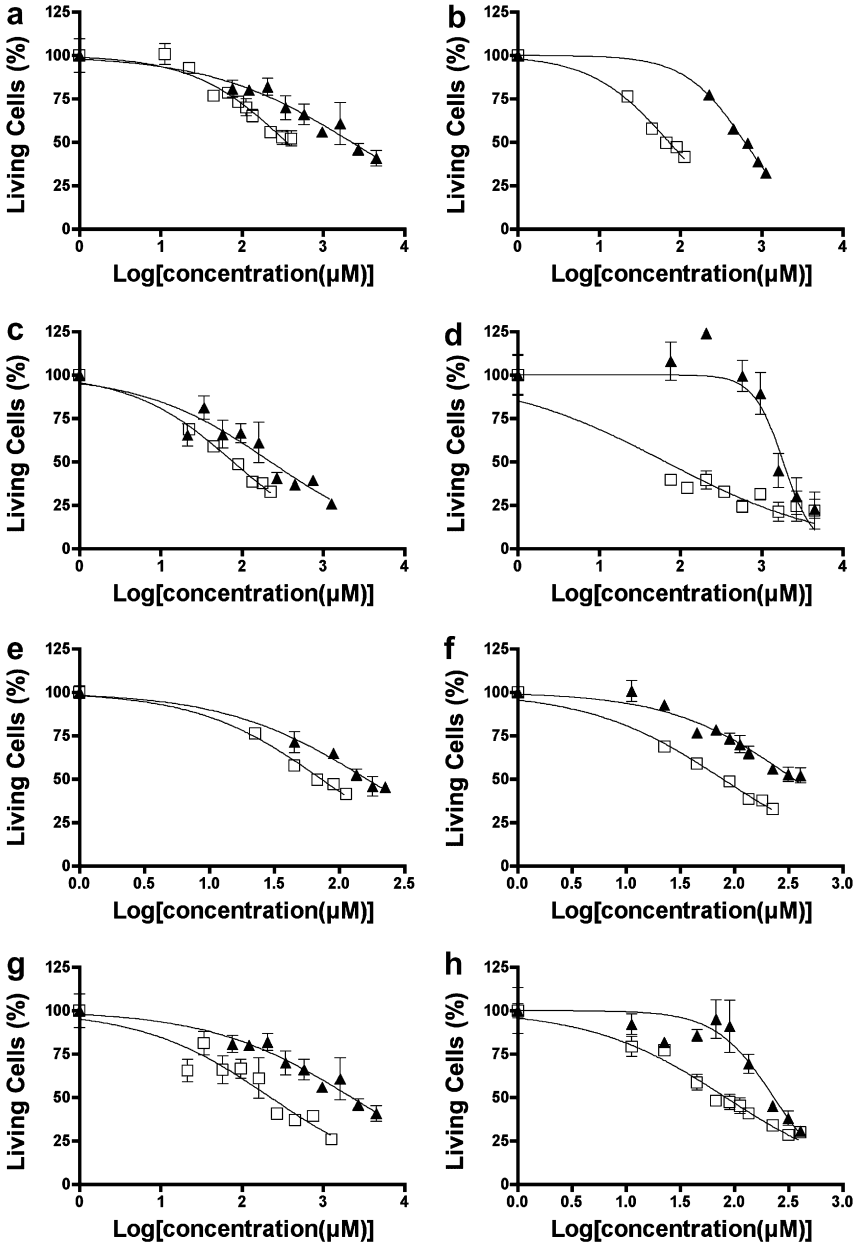

Fig. 3. Structural components associated with cytotoxicity. The presence of a 2–3 double bond (a–c), the presence of a 4-carbonyl group (d), the absence of a 3-hydroxyl group (e–g) and *ortho*- versus *meta*-hydroxylation in the B ring (h) are associated with enhanced cytotoxicity. Data sets from 24 h-cytotoxicity experiments (d from 48 h-experiments) were compared by an *F*-test for statistical significant differences between EC_{50} values (*, statistically significant defined as $P < 0.05$). (a) Quercetin (□) versus Taxifolin (▲), $^{*}P < 0.0001$. (b) Apigenin (□) versus Naringenin (▲), $^{*}P < 0.0001$. (c) Luteolin (□) versus Eriodictyol (▲), $^{*}P < 0.0001$. (d) Taxifolin (□) versus Catechin (▲), $^{*}P = 0.0022$. (e) Apigenin (□) versus Kaempferol (▲), $^{*}P < 0.0001$. (f) Luteolin (□) versus Quercetin (▲), $^{*}P < 0.0001$. (g) Eriodictyol (□) versus Taxifolin (▲), $^{*}P < 0.0001$. (h) Quercetin (□) versus Morin (▲), $^{*}P < 0.0001$.

positively correlates with the maximum solubility of the substances (Fig. 5b). The inverse correlation between S_{max} and cytotoxicity is statistically significant ($P = 0.0031$), indicating that hydrophobicity is one key parameter for flavonoid-triggered cytotoxicity. In line with this, *O*-methoxylation is associated with a decrease in solubility and an increase in toxicity (Table 1 and Fig. 4a–d). In contrast to these findings, glucuronidation, which increases solubility of baicalein and scutellarein by a factor of 7–8 (Table 1), induced significantly more cell death than unglucuronidated compounds (Fig. 4e–f). Consequently, glucuronidated flavonoids do not fit within the correlation between EC_{50} and S_{max} described in Fig. 5c, indicating that hydrophobicity may rather represent a surrogate marker for flavonoid-cytotoxicity than a causative trigger.

The presence of the B ring is associated with enhanced cytotoxicity

In order to investigate the contribution of the B ring to flavonoid-induced cytotoxicity, we compared the chalcone phloretin with phloroacetophenon, a compound that lacks

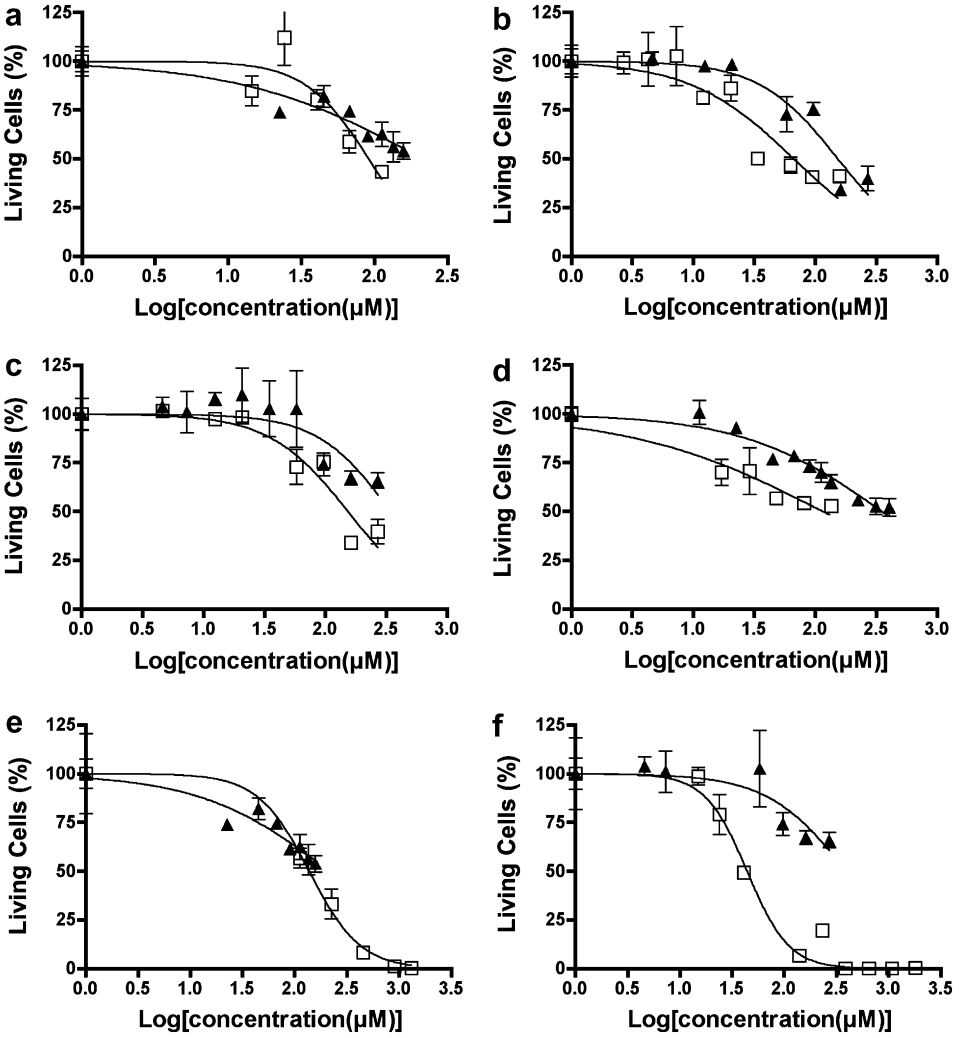

Fig. 4. Chemical modifications found in metabolites increase flavonoid cytotoxicity. *O*-methylation (a–d) and glucuronidation (e–f) are associated with enhanced cytotoxicity. Data sets derived from 24 h-cytotoxicity experiments. (a) MeO-Baicalein (□) versus Baicalein (▲), $^{*}P = 0.0250$. (b) Cirsimaritin (□) versus Hispidulin (▲), $^{*}P = 0.0103$. (c) Hispidulin (□) versus Scutellarein (▲), $^{*}P = 0.0111$. (d) Isorhamnetin (□) versus Quercetin (▲),$^{*}P = 0.0074$. (e) Baicalein-7-glucuronide (□) versus Baicalein (▲), $^{*}P = 0.0327$. (f) Scutellarein-7-glucuronide (□) versus Scutellarein (▲), $^{*}P < 0.0001$.

the hydroxybenzyl moiety present in phloretin. Phloroacetophenon was about 50 times less cytotoxic than phloretin ($P < 0.0001$), demonstrating the importance of the B ring for flavonoid-triggered cytotoxicity (graph not shown, for comparison of EC_{50}s see Table 1). A further reduction of the backbone (phloroacetophenon versus phloroglucin) additionally decreased the cytotoxicity by a factor of 10 ($P = 0.0050$) (graph not shown, for comparison of EC_{50}s see Table 1). Due to the marked reduction of toxicity, we used the 48 h time point for this analysis, in order to be able to reliably determine EC_{50}.

Discussion

In this study we identified several structural characteristics associated with flavonoid-triggered cytotoxicity. One identified structure element is the presence of a 4-carbonyl group which has been reported to correlate with inhibition of fatty acid synthesis, an event discussed to be a causative factor in flavonoid-induced cytotoxicity [8]. Another important structural characteristic for enhanced cytotoxic activity is the presence of the 2–3 double bond, leading to a planar structure of the A and C ring of the flavonoid backbone. The presence of this double bond has been linked to efficient binding and inhibition of the P-glycoprotein (P-gp) [15], a plasma membrane transporter responsible for the export of chemotherapeutic agents. Inhibition of this important cellular detoxification system may thus contribute to flavonoid-induced cytotoxicity. In line with our results, the presence of the 2,3 double bond has been correlated with mitochondrial damage and cell death [16].

We observed a statistically significant correlation between EC_{50} and S_{max}. One possible explanation could be an enhanced membrane permeability of the more lipophilic flavonoids, leading to higher intracellular concentrations [17]. In line with this, xanthohumol—the most toxic flavonoid in our study—has a prenyl group in C6 and these

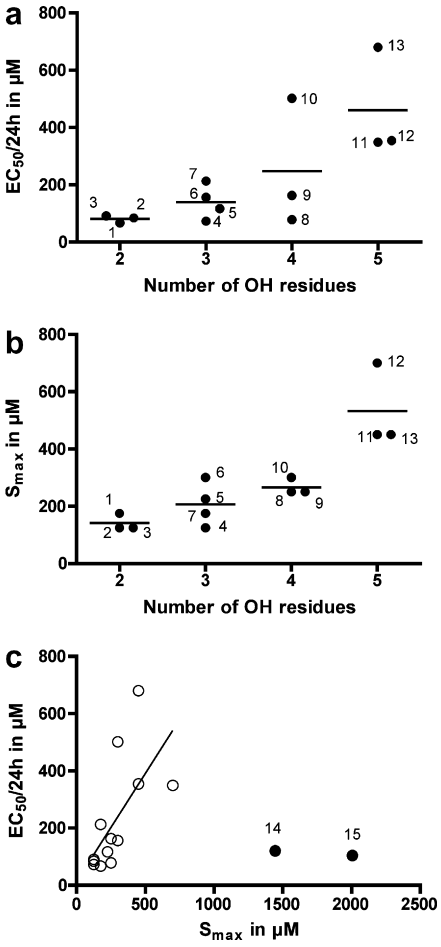

Fig. 5. Flavonoid-induced cytotoxicity negatively correlates with solubility. Flavones and flavonols used in this study were grouped according to the number of hydroxyl residues and correlated with EC_{50}/24 h (a) or S_{max} (b). Cirsimaritin (1), Chrysin (2), 7-MeO-Baicalein (3), Apigenin (4), Isorhamnetin (5), Hispidulin (6), Baicalein (7), Luteolin (8), Kaempferol (9), Scutellarein (10), Quercetin (11), Myricetin (12), Morin (13). (c) Correlation of EC_{50}/24 h with S_{max} and linear regression (considering compounds 1--13 depicted in open circles (○) showing significant ($P = 0.0031$) correlation between the two parameters in a nonparametric test (two-tailed P with a 95% confidence interval). Black dots (●) represent baicalein-glucuronide (14) and scutellarein-glucuronide (15) that do not fit into linear regression.

groups are known to interact with cell membranes [18]. Apparently, high lipophility seems to be associated with enhanced cytotoxicity for other polyphenols as well [19], suggesting that a low S_{max}, although a prerequisite for efficient intracellular accumulation, may not directly be involved in the cytotoxic mechanism. This is supported by our observation that glucuronidation, a structural characteristic associated with increased solubility (Table 1), further enhances the cytotoxicity of flavonoids (Fig. 4e–f).

The exact molecular mechanism by which flavonoids induce cell death is still to be elucidated. Several mechanisms have been proposed, including the production of cytotoxic hydrogen peroxide in the culture medium during autoxidation of the polyphenols, a reaction that is further enhanced by the presence of iron ions in the growth medium [20,21]. In our hands, however, neither treatment with a dilution series of 10–1000 U/ml of catalase nor iron chelation with a dilution series of 0.1–3.0 mM EDTA inhibited quercetin- or xanthohumol cytotoxicity (data not shown), suggesting that the full spectrum of flavonoid-triggered cytotoxicity requires additional mechanisms. Indeed, there is evidence that the nature of flavonoid-triggered cell death may be multifactoral, involving the inhibition of topoisomerases [9], inhibition of the proteasome [6,7], inhibition of fatty-acid synthesis [8], inhibition of phosphatidyl-inositol 3-kinase [10], induction of cell cycle arrest [11], accumulation of p53 [12] and enhanced expression of c-fos and c-myc [13]. Moreover, other characteristics influence the cytotoxic potential of flavonoids as well, such as their ability to support ROS-formation [21], the membrane permeability [17] and cellular absorption [22], affinity to p-glycoprotein-mediated export [15] and flavonoid metabolism [22]. These different activities may be associated with individual structure–activity relationships that merge and interfere with each other when analysing a combined end-point such as cytotoxicity. This may account for the mixed results when analyzing the influence of hydroxylation of the B ring: whereas kaempferol (4′-OH in B ring) is more cytotoxic than quercetin (3′-OH, 4′-OH in B ring) ($P = 0.0007$), naringenin (4′-OH in B ring) is less cytotoxic than eriodictyol (3′-OH, 4′-OH in B ring) ($P = 0.0007$) (graphs not shown, see EC_{50}s in Table 1). Similarly, the analysis of the influence of the chalcone versus chromane backbone on cytotoxicity produces contradicting results: apigenin (closed C ring) is more cytotoxic than naringeninchalcon (open C ring) ($P = 0.0301$) but naringenin (closed C ring) is less cytotoxic than phloretin (open C ring) ($P = 0.0301$) (graphs not shown, see EC_{50}s in Table 1).

The multifactoral nature of flavonoid-triggered cytotoxicity also implies that different structure activity relationships may be deduced for different cell types. We have performed some of the experiments presented here (i.e. for quercetin and xanthohumol) also with neuroblastoma cells, showing comparable effects to what we have found with Jurkat cells (data not shown). However, in a study directly comparing the EC_{50}s of different flavonoids on rat hepatocytes and HeLa cells, cell-type specific differences in the order of cytotoxicities were observed [23].

Flavonoids undergo rapid metabolization *in vivo*, forming glucuronides, sulphates and *O*-methylated compounds [24]. We have shown here that *O*-methylation as well as glucuronidation results in an increased cytotoxicity. The pharmacokinetic profile of flavonoids—at least concerning *O*-methylation and glucuronidation—may therefore enhance the cytostatic activity of these compounds. In line with this, we observed a significant increase of toxicity of some compounds over time in our experiments. The most dramatic increase (or delay) of cytotoxicity was observed with taxifolin that displayed a 34 times higher toxicity after 48 h compared to 24 h (see ratio of EC_{50}/24 h and EC_{50}/48 h in Table 1). In contrast, cirsimaritin exhibited only a moderate increase in EC_{50}/48 h compared to EC_{50}/24 h (factor 1.5). We were not able to clearly correlate these differences with structural characteristics. However, the three

flavonoids with the most pronounced delay in toxicity (taxifolin, eriodictyol, naringenin) possess a saturated 2–3 bond. The molecular reasons for this delayed toxicity may range from metabolic activation to different mechanisms of cell death induction associated with different kinetics.

Flavonoids are not only being discussed as candidates for cancer therapy but also to play a role in prevention of cancer. There is accumulating evidence that a diet rich in flavonoids is associated with a reduced cancer risk. Many mechanisms of action have been proposed for flavonoid-mediated prevention of cancer, including estrogenic/antiestrogenic activity, antiproliferative effects, antioxidative effects, induction of detoxification enzymes, regulation of the host immune system, changes in cellular signaling and induction of apoptosis [25]. Plasma levels of flavonoids after a flavonoid-rich diet are in the range of 10–500 nM (about 50 nM for quercetin and 325 nM for hesperetin) [26], which is about 1000–2000 times lower than the concentrations associated with acute cytotoxicity (see S_{max} in Table 1). Moreover, the acute cytotoxic activity of flavonoids observed at relatively high concentrations contrasts with the chemopreventive effects of these substances at lower concentrations. For example, epicatechin has been reported to protect cells against oxidized LDL damage [27].

Taken together, at high concentrations flavonoids exert cytotoxic activity on human leukemia cells and we have identified structure elements associated with enhanced toxicity in this cell type. These data may help to identify novel flavonoid derivatives with optimized cytotoxic activity to be tested for anti-cancer treatment.

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