Flavonoids potentiate the efficacy of cytarabine through modulation of drug-induced apoptosis

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Recent studies have provided strong evidence for potential beneficial effects of flavonoids in chemoprevention or in combination with chemotherapeutics in tumor cells treatment. The aim of this work was to compare the antioxidant properties of four flavonoids with emphasis on association of these antioxidant properties with their effects on the therapeutic efficacy of cytarabine (AraC) using L1210 leukemia cells. The results of antiproliferative studies showed that antiproliferative potential of flavonoids tested decreased in the order: isorhamnetin > kaempferol > myricetin > rutin, while their antioxidant properties decreased in the order: rutin > myricetin > kaempferol > isorhamnetin. Combinational treatment of isorhamnetin, kaempferol and myricetin with AraC led to synergism in their antiproliferative activities (CIs < 1). Rutin exhibited antagonism with AraC (CIs > 1). Apoptotic DNA fragmentation and flow cytometry analyses revealed that synergism in antiproliferative activities of compounds tested might be due to potentiation of AraC-induced apoptosis. In conclusion, our results clearly indicate that isorhamnetin, kaempferol and myricetin despite their antioxidant properties might be used to increase the sensitivity of leukemia cells to AraC treatment.

Keywords: chemotherapy, modulator, flavonoids, apoptosis, cisplatin, cytarabine

Many naturally occuring compounds have beneficial effects on human health. It is widely accepted that regular consumption of fruits and vegetables can reduce the risk of development of cancer and some degenerative diseases. Recently it has been shown that among numerous phytochemicals, the "beneficial" compounds can be classified as polyphenols. Several hundreds of different polyphenols have been identified so far. The two main types of polyphenols are flavonoids and phenolic acids. Flavonoids belong to a class of polyphenols with the chemical structure consisting of 15 carbon atoms. Various substitutions on the skeleton are responsible for their typical chemical diversity [1, 2]. Due to chemical structure, they are strong scavengers of oxygen-derived free radicals and metal chelators [3]. They are oxidized by free radicals, resulting in less reactive and more stable radicals. Other mechanisms, by which flavonoids exhibit their effects, involve the cell cycle arrest, inhibition of cell proliferation, induction of apoptosis and differentiation and/or inhibition of carcinogen activation [4]. Previously it has been shown that some flavonoids may positively or negatively affect the efficacy of chemotherapeutics [5–7]. As there is clear evidence on negative effects of some antioxidants on chemotherapy effectivity, the *in vitro* experiments evaluating the association between antioxidant and antiproliferative properties of flavonoids should proceed their applications in cancer therapy [5, 8].

In the presented work, four flavonoids (myricetin, kaempferol, isorhamnetin and rutin) were studied for their antioxidant and antiproliferative activities. Additionally, association between their antioxidant properties and ability to modulate the therapeutic potential of cytarabine (AraC) were investigated using L1210 leukemia cells.

Materials and methods

Drugs. Cytarabine (AraC) and flavonoids were purchased from Sigma. Flavonoids were dissolved in DMSO (0.1 M) and stored at –20°C. The final concentration of DMSO in the medium was < 0.02% and did not affect cell growth.

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Cell line. Murine leukemia L1210 cell line was obtained from the ATCC (Rockville, MD, USA). Cells were maintained in RPMI-1640 medium supplemented with 10% FCS, 100 U/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine in an atmosphere of 5% CO_2 in humidified air at 37°C.

Drug treatment. Exponentially growing cells were harvested by centrifugation and resuspended in fresh medium to achieve culture density of 3 x 10^5 cells/mL. The cells were treated with 0.05 – 0.5 µM AraC, 10 – 40 µM flavonoids, or with their combinations for 24 h. Cell viability was determined by trypan blue staining.

DPPH radical scavenging assay. Antioxidant activity of flavonoids was measured in terms of DPPH radical scavenging ability with slight modifications [9]. L-ascorbic acid was used as a reference. Flavonoids (50 µL) at different concentrations were placed in a cuvette and 1 ml of 23.7 g/mL ethanolic solution of DPPH radical was added. The decrease in absorbance at 517 nm was determined after 30 min incubation using Spekol 221. All determinations were performed in three replicates. The percentage of DPPH radical inhibition by the samples was calculated according to the following formula:

$$\% \text{ of scavenging of DPPH} = [(A_{C(0)} - A_{A(t)})/A_{C(0)}] \times 100,$$

where $A_{C(0)}$ is the absorbance of the control at t = 0 min and $A_{A(t)}$ is the absorbance of the antioxidant at t = 30 min. A dose–response curve was plotted for determining the SC_{50} values. SC_{50} was defined as a concentration sufficient to obtain 50% of maximum scavenging capacity.

Hydroxyl radical scavenging assay. The hydroxyl radicals (•OH) were generated in an L-ascorbic acid/$CuSO_4$ system by reduction and were assayed by the oxidation of cytochrome *c* [10]. In this experiment, the •OH were generated in 1 mL of 0.15 mM sodium phosphate buffer (pH 7.4) containing 100 µM L-ascorbic acid, 100 µM $CuSO_4$, 12 µM cytochrome *c*, and the samples to be tested at different concentrations. The mixture was incubated at 25°C for 90 min. Change in transmittance caused by color change of cytochrome *c* was measured at 550 nm using Spekol 221. The inhibition of •OH generation by 500 µg/mL thiourea was taken as 100%. The inhibition ratio was calculated using the following formula:

$$\% \text{ of scavenging of •OH radicals} = [(T - T_2) / (T - T_1)] \times 100,$$

where T was the transmittance of the hydroxyl radical generation system, and T_1 and T_2 were the transmittances of the control (no •OH generation) and test systems, respectively.

Analysis of drug combination. The cell viability data were analyzed using CalcuSyn Version 2.0 program to determine the IC_{50} of each drug alone. The CI-isobologram by Chou and Talalay was used to analyze the drug combinations [11]. Variable ratios of drug concentrations were used in the studies, and mutually exclusive equations were applied to determine the CIs. Each CI was calculated from the mean affected fraction at each drug ratio concentration (triplicate). CI > 1, CI = 1, and CI < 1 indicate antagonism, additive effect, or synergism, respectively.

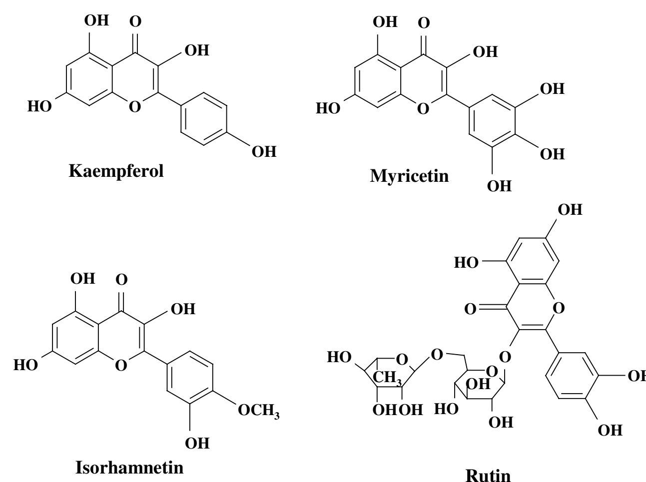

Figure 1. Chemical structure of flavonoids.

Electrophoretic analysis of DNA fragmentation. Untreated and drug-treated cells (1 x 10^6) were harvested, washed in PBS (4°C) and lysed with 100 µL of solution (10 mM Tris, 10 mM EDTA, 0.5% Triton X-100) supplemented with proteinase K (1 mg/mL). Samples were then incubated at 37°C for 1 h and heated at 70°C for 10 min. Following lysis, RNAse (200 µg/mL) was added and repeated incubation at 37°C for 1 h followed. The samples were subjected to electrophoresis at 40 V for 2 h in 1.5% agarose gel complemented with EtBr (1 µg/mL). Separated DNA fragments were visualized using UV transilluminator (254 nm).

Cell cycle analysis. Untreated and drug-treated cells (0.5 × 10^6) were harvested, washed in PBS (4°C) and fixed using 70% ethanol. Samples were exposed to 0.05% Triton X-100 in PBS supplemented with RNAse (50 µg/mL) for 25 min at 37°C. Afterwards, DNA was stained by PI (50 µg/mL) for 15 min at 4°C. Samples were analyzed by FACStar (Beckman-Dickinson, USA) with the use of software provided by the manufacturer. A minimum of 20,000 cells per sample were analyzed at flow rate of 200 cells/s.

Results and discussion

In the recent years, polyphenols are considered as promising compounds for clinical applications as modulators of the therapeutic efficacy of chemotherapeutics [12, 13]. However many reports showed, that some polyphenols with strong antioxidant properties might have negative effects on efficacy of clinically used chemotherapeutics [5, 8, 13]. Due to these results, the combinational experiments assessing the positive vs. negative effects of polyphenols on efficacy of chemotherapy is highly desirable.

In this work, four flavonoids (myricetin, kaempferol, isorhamnetin and rutin; Fig. 1) were studied for their antioxidant and antiproliferative activities. Additionally, association

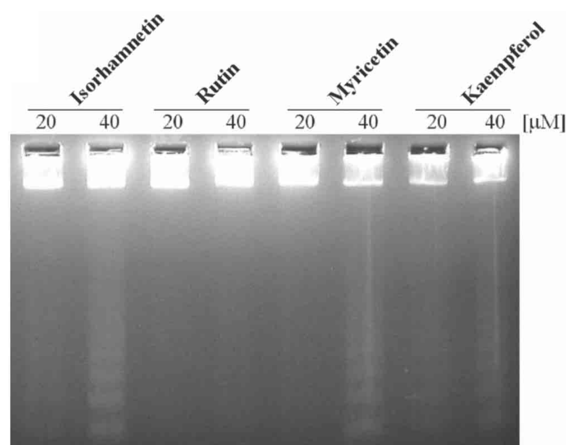

Figure 2. Apoptotic DNA fragmentation in L1210 leukemia cells treated with flavonoids for 24 h. The figure is representative of three independent experiments.

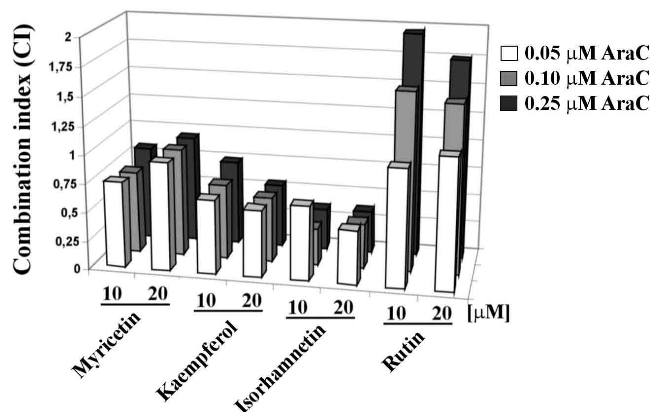

Figure 3. Effects of flavonoids in combination with cytarabine (AraC) on growth of L1210 leukemia cells after 24 h treatment. CI values were calculated as described in "Materials and Methods". ***Note:*** **CI > 1.0 antagonism, CI = 1.0 additive effect, CI < 1.0 synergism.**

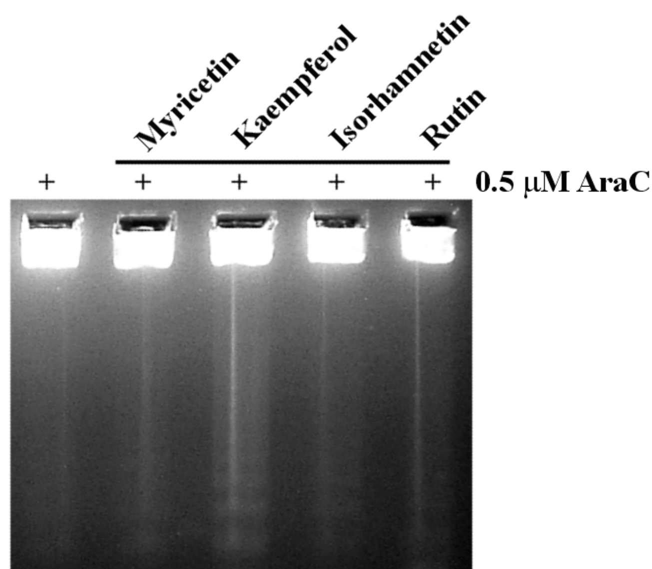

Figure 4. Effect of flavonoids (10 μM) on apoptotic potential of cytarabine (AraC) toward L1210 leukemia cells after 24 h treatment. The figure is representative of three independent experiments.

between antioxidant properties and ability of flavonoids to modulate the therapeutic potential of cytarabine (AraC) were investigated using L1210 leukemia cells.

The antioxidant activity of flavonoids was evaluated by DPPH and OH radicals scavenging assays. As shown in Table 1, the antioxidant activities of flavonoids increased in the order isorhamnetin < kaempferol < myricetin < rutin. Rutin exhibited the strongest antioxidant activity with SC_{50} values of 85.8 ± 2.3 μM (DPPH radical assay) and 45.3 ± 6.7 μM (OH radicals assay), respectively. Isorhamnetin showed to be the weakest antioxidant with SC_{50} values of 471.2 ± 3.1 μM (DPPH radical assay) and 97.2 ± 4.2 μM (OH radicals assay), respectively. Recent study showed that rutin, myricetin and kaempferol can protect erythrocytes from free radical-induced oxidative hemolysis [14]. Additionally, it has been shown that isorhamnetin might increase the activity of SOD, the enzyme that participates in antioxidant defence of the cell. Moreover, isorhamnetin supressed ROS overproduction and increased survival of cells by inhibiting the oxidized LDL's incidence [15]. Presumption that the antioxidant activity of flavonoids is related to the presence of a $C_2 = C_3$ bond, a 3-OH substituent, a C_4 keto group and a 3',4'-dihydroxy moiety [16], is explaining the fact, why isorhamnetin exerted the weakest antioxidant activity in our experiments. On the other hand,

Table 1. Antioxidant activity of flavonoids determined by DPPH and OH radicals scavenging assays.

Drugs	DPPH	•OH
Rutin	85.8 ± 2.3	45.3 ± 6.7
Myricetin	117.9 ± 1.9	69.7 ± 3.6
Kaempferol	217.9 ± 3.6	86.3 ± 5.8
Isorhamnetin	471.2 ± 3.1	97.2 ± 4.2
Ascorbic acid	23.4 ± 1.5	14.8 ± 1.7

– data represent SC_{50} values ± s.d. [μM] of three independent experiments.
– 500 μg/mL of thiourea = 100% of scavenging activity.

Table 2. Antiproliferative activity of flavonoids toward L1210 leukemia cells after 24 h treatment.

Drugs	IC_{50} [μM]
Isorhamnetin	11.1 ± 1.4
Kaempferol	15.9 ± 2.1
Myricetin	24.6 ± 2.5
Rutin	45.8 ± 3.6

– data represent mean values ± s.d. of three independent experiments.

Table 3. Cell cycle profile of L1210 leukemia cells treated with cytarabine (AraC), flavonoids or with their combinations for 24 h.

Drugs	[μM]	sub-G_0	G_0/G_1	S	G_2/M
Control	–	1.5 ± 0.5	28.5 ± 2.6	58.3 ± 1.6	13.2 ± 2.6
Myricetin	10	2.2 ± 1.1	32.3 ± 1.9	49.2 ± 3.4	18.5 ± 3.1
Kaempferol	10	1.6 ± 0.8	27.3 ± 3.4	53.6 ± 3.3	19.1 ± 2.9
Isorhamnetin	10	1.9 ± 0.6	29.8 ± 2.8	55.3 ± 4.8	14.9 ± 4.8
Rutin	10	2.6 ± 1.3	32.6 ± 3.9	54.9 ± 2.9	12.5 ± 2.1
AraC	0.5	2.1 ± 0.2	26.5 ± 4.5	65.4 ± 5.7	8.1 ± 4.5
AraC + Myricetin	0.5 + 10	12.9 ± 2.3	25.4 ± 1.7	53.9 ± 1.6	20.7 ± 3.6
AraC + Kaempferol	0.5 + 10	19.6 ± 2.4	39.3 ± 5.1	38.8 ± 2.5	21.9 ± 2.7
AraC + Isorhamnetin	0.5 + 10	8.3 ± 1.9	22.1 ± 3.3	56.4 ± 3.6	21.5 ± 3.2
AraC + Rutin	0.5 + 10	3.6 ± 1.1	24.8 ± 2.1	57.7 ± 2.8	17.5 ± 2.8

– data represent mean values ± s.d. of three independent experiments.

the strongest antioxidant activity of rutin is probably due to its sugar part.

To examine antiproliferative properties of flavonoids, L1210 cells were treated with flavonoids for 24 h. As it is shown in Table 2, isorhamnetin exhibited the strongest antiproliferative activity (IC_{50} = 11.1 ± 1.4 μM), while rutin exhibited the weakest activity (IC_{50} = 45.8 ± 3.6 μM). The antiproliferative activities of flavonoids correlated with induction of apoptosis (Fig. 2) and with changes in cell cycle profiles (Table 3).

To assess the association between antioxidant properties of flavonoids and their abilities to modulate the therapeutic potential of cytarabine (AraC), the combinational treatment was performed, with emphasis on the combination indexes, cell cycle profile changes and apoptotic DNA fragmentation analyses. As shown in Fig. 3, isorhamnetin, kaempferol and myricetin combined with AraC synergistically enhanced the antiproliferative activity of AraC (CIs < 1). On the contrary, rutin exhibited antagonism with AraC (CIs > 1). Cell cycle analysis revealed that synergism of flavonoids (10 μM) with AraC (0.5 μM) is due to significant changes in cell cycle profile of L1210 cells (Table 3). Treatment of cells with combination of flavonoids and AraC induced a block of cells in G_2/M phase. Additionally, appearance of sub-G_0 cell fraction (Table 3) and potentiation of apoptotic DNA fragmentation was observed (Fig. 4), pointing out the fact that synergism of flavonoids with AraC is due to potentiation of AraC-induced apoptosis. Previuosly it has been shown that some flavonoids potentiate the efficacy of chemotherapeutics through modulation of apoptotic cell response [5-7]. The synergism of flavonoids used in our study with chemotherapeutics is probably more general, as we observed strong synergism between flavonoids and chemotherapeutics that act even through different mechanisms (e.g. cisplatin, temozolomide, VP-16 and doxorubicin; unpublished results). Similar resuts were observed when naturally occuring compound, resveratrol, was used as sensitizer of leukemia cells to different chemotherapeutics [17].

In conclusion, our results clearly demonstrate that isorhamnetin, kaempferol and myricetin are promising drugs and might be used to positively modulate the efficacy of chemotherapy with AraC. On the contrary to rutin, or other flavonoids tested previously [5,6], the antioxidant activity of isorhamnetin, kaempferol and myricetin does not interfere with chemotherapeutic efficacy of AraC. To better understand the precise molecular mechanism of synergistic activities of these flavonoids, further experiments elucidating the changes in cell cycle regulations and apoptotic pathways signalling are in progress.

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