

Dietary bioflavonoids induce apoptosis in human leukemia cells

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Received 21 July 2004; accepted 11 November 2004
Available online 19 January 2005

Abstract

Dietary bioflavonoids are secondary metabolites of plants that are known to have a variety of bio-effects, including anti-cancer activity. In this study, we examined the effects of flavonoids on the growth of human leukemia cells and found that certain flavonoids induce apoptosis in a variety of human leukemia cells. The apoptosis induced by bioflavonoids was dose-dependent and was accompanied by a disruption of the mitochondrial transmembrane potential and the activation of caspase. Our data suggests that dietary bioflavonoids may be useful chemotherapeutic reagents for leukemia patients.

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Keywords: Bioflavonoid; Apoptosis; Acute lymphoblastic leukemia; Precursor-B-cell

1. Introduction

Flavonoids are ubiquitously occurring and widely consumed secondary metabolites of plants [1,2]. Flavonoids can be divided into three main groups: Flavones, Flavonones (2,3-dihydroflavones), and isoflavones, which differ in structure and ring substitutions [3]. They have diverse pharmacological properties, including antioxidant, cytoprotective, and anti-inflammatory activities [1,2], and have also been reported to display anti-viral [4] and anti-parasitic [5] activities.

Moreover, some flavonoids are known to act as anti-cancer reagents. For example, Yoshida et al. reported that Quercetin markedly inhibited the growth of human gastric cancer cells [6]. Record et al. also described the inhibition of B16 melanoma cells by Genistein, both in vivo and in vitro [7]. Huang et al. demonstrated that Luteolin and Quercetin

significantly inhibited the proliferation of epidermoid carcinoma A431 cells with an overexpression of epidermal growth factor receptor [8]. Indeed, some bioflavonoids like Quercetin and Genistein have already been used as chemotherapeutic agents in phase trials [9,10].

In an attempt to examine the effects of flavonoids on the growth of human leukemic cells, we challenged cultured human leukemic cell lines with several kinds of flavonoids. In the present study, we demonstrated that certain flavonoids can induce significant apoptosis in a variety of human leukemia cells.

2. Materials and methods

2.1. Cells and reagents

The cell line BV-173 that were established from a patient in an acute relapse who most likely had Ph1-positive chronic myelogenous leukemia [11]; the acute-phase of chronic myelogenous leukemia-derived cell lines K-562 (Japanese Cancer Research Resources Bank, JCRB, Tokyo, Japan)

Abbreviations: ALL, acute lymphoblastic leukemia; CD, cluster of differentiation; FITC, fluorescein isothiocyanate; PE, phycoerythrin; PC-5, PE-Cy-5; PC-7, PE-Cy-7; topo, topoisomerase

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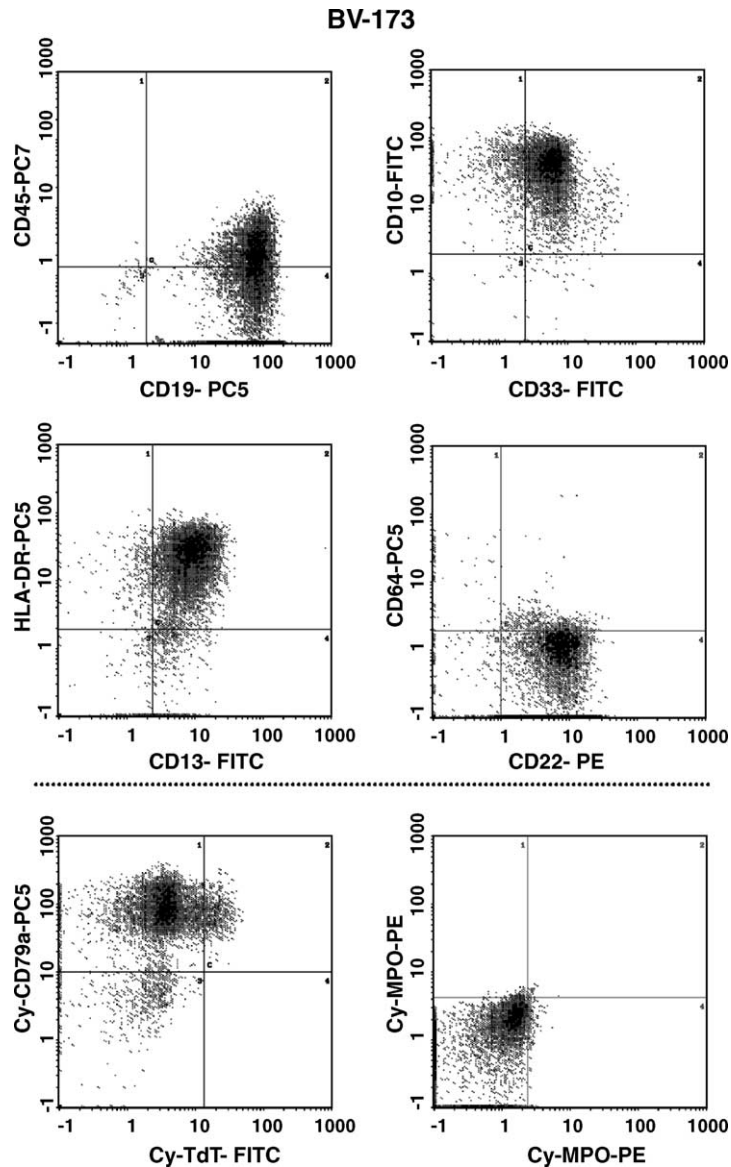


Fig. 1. Immunophenotypic analysis of BV-173 cells. The BV-173 cells were stained with fluorescence-labeled specific monoclonal antibodies against leukocyte antigens, as indicated, and analyzed by flow cytometry. The resulting histograms are shown. (Cy-) Cytoplasmic antigen stained after cell-permeabilization treatment.

and KU-812 (Institute for Fermentation, Osaka, Japan); precursor-B-acute lymphoblastic leukemia (ALL)-derived cell lines, including NALM-16, NALM-20, HPB-NUL and NALM-17 [12]; Burkitt's lymphoma-derived cell lines, Daudi and Ramos (JCRB); the histiocytic lymphoma-derived cell line U-937 (JCRB) and the acute monocytic leukemia-derived cell line THP-1 (JCRB) were used. Cells were cultured in RPMI1640 supplemented with 10% FCS at 37 °C in a humidified 5% CO₂ atmosphere.

Fluorescence-labeled monoclonal antibodies against leukocyte antigens were obtained from Beckman/Coulter Inc. (Westbrook, MA). Bioflavonoids, including Flavone, Genistein, Genistin, Quercetin, Fisetin, Luteolin, Apigenin and Rutin, and the anti-cancer drug VP-16 (which exhibits topoisomerase (topo) II-inhibitor activity) were purchased from

Sigma-Aldrich (St. Louis, MO). The peptide-inhibitors for the caspases were obtained from Calbiochem-Novabiochem Co. (San Diego, CA). Bioflavonoids, VP-16 and the caspase inhibitors were desorbed in DMSO and then added to the cell cultures. All other chemical reagents were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan), unless otherwise indicated.

2.2. Immunofluorescence study and detection of apoptosis

A multi-color immunofluorescence study was performed using a combination of fluorescein isothiocyanate (FITC), phycoerythrin (PE), PE-Cy-5 (PC-5) and PE-Cy-7 (PC-7). Cells were stained with fluorescence-labeled monoclonal

antibodies and analyzed by flow cytometry (EPICS-XL, Beckman/Coulter), as described previously [13]. Staining of the cytoplasmic antigens was performed using Cytotfix/Cytoperm™ Kits (Becton Dickinson), according to the manufacturer's protocol.

To quantitate the incidence of apoptotic cells, cells were stained with FITC-labeled annexin V using a MEBCYTO®-Apoptosis Kit (Medical & Biological Laboratories (MBL) Co. Ltd., Nagoya, Japan) and then analyzed by flow cytometry according to the manufacturer's protocol. Experiments were performed in triplicate, and the mean \pm S.D. of the cells that bound annexin V are shown. Caspase-3 activity was assessed with a PhiPhiLUX™ G1D2 kit (MBL) and analyzed by flow cytometry according to the manufacturer's protocol. The disruption of the mitochondrial transmembrane potential was detected by the MitoCapture Apoptosis Detection Kit (MBL) and analyzed by flow cytometry according to the manufacturer's protocol.

2.3. Examination of morphological appearance

BV-173 cells were immobilized onto glass slides with Cytospin 2 (Shandon Inc., Pittsburg, PA), Giemsa-stained, and their morphological appearance was examined by light microscopy (BX-61, Olympus, Tokyo, Japan).

3. Results

3.1. Immunophenotypic analysis of BV-173 cells

First, we examined the cell surface and cytoplasmic antigens expressed in BV-173 cells originally derived from a patient with Ph1-positive acute leukemia. As shown in Fig. 1, the BV-173 cells expressed B-cell antigens, such as cluster of differentiation (CD)19, CD22 and cytoplasmic CD79a, as determined by flow cytometry. Together with the expression of CD10 and HLA-DR (Fig. 1) and the absence of surface IgM (data not shown), the cell line was thought to have originated from a precursor-B-cell. However, flow cytometric analysis also revealed that the BV-173 cells simultaneously expressed myeloid antigens, including CD13 and CD33 (Fig. 1). Therefore, BV-173 was thought to exhibit biphenotypic leukemia characteristics with both precursor-B-cell and myeloid lineages. This cell line was mainly used in the following experiments.

3.2. Dietary bioflavonoids induce apoptosis in BV-173 cells

Next, we tested whether the administration of dietary bioflavonoids induced any cytotoxic effects on BV-173 cells. When BV-173 cells were treated with 200 μ M of Flavone for 24 h and then examined morphologically by light microscopy, a portion of the cells exhibited condensation (arrow-head) and cleavage (arrow) of the nuclei, findings that are typical

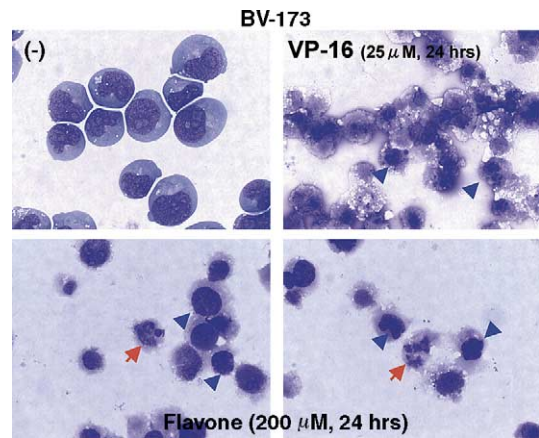


Fig. 2. Morphological examination of Flavone-treated BV-173 cells. BV-173 cells were cultured in the presence or absence of either Flavone or VP-16, as a positive control of apoptosis induction, for 24 h as indicated in the figure, then cytocentrifuged on the slide glasses. After Giemsa-staining, the morphological appearance of the cells was examined using light microscopy. The arrowheads indicate nuclear condensation. Typical apoptotic cells, characterized by cleaved nuclei, are indicated by the arrows. Magnification 400 \times .

of apoptosis (Fig. 2). No such figures were observed in untreated cells (Fig. 2). The following results clearly show that the administration of Flavone indeed induced apoptosis in BV-173 cells. First, DNA prepared from BV-173 cells treated with Flavone for 24 h showed oligonucleosomal ladder fragmentation on agarose gel electrophoresis (Fig. 3). Second, the number of cells binding to annexin V increased significantly after Flavone-treatment (Fig. 4). As shown in Fig. 4, other bioflavonoids, including Luteolin, Genistein, Quercetin, and

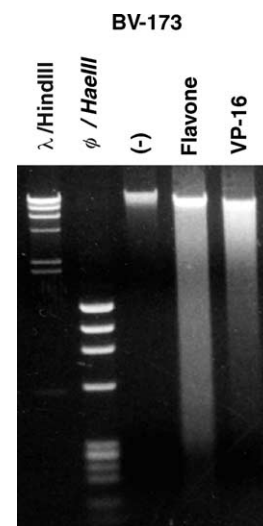


Fig. 3. DNA ladder formation in BV-173 cells after Flavone-treatment. BV-173 cells were treated with (lane 4) or without (-, lane 3) 200 μ M of Flavone for 24 h. The extracted DNA (1.5 μ g per lane) from each sample was separated by 1% agarose gel electrophoresis. The λ /HindIII and ϕ /HaeIII DNA markers were applied to the same gel (left two lanes). As a positive control for DNA ladder formation, DNA extracted from BV-173 cells treated with 25 μ M of VP-16 for 24 h was also examined (VP-16, lane 5).

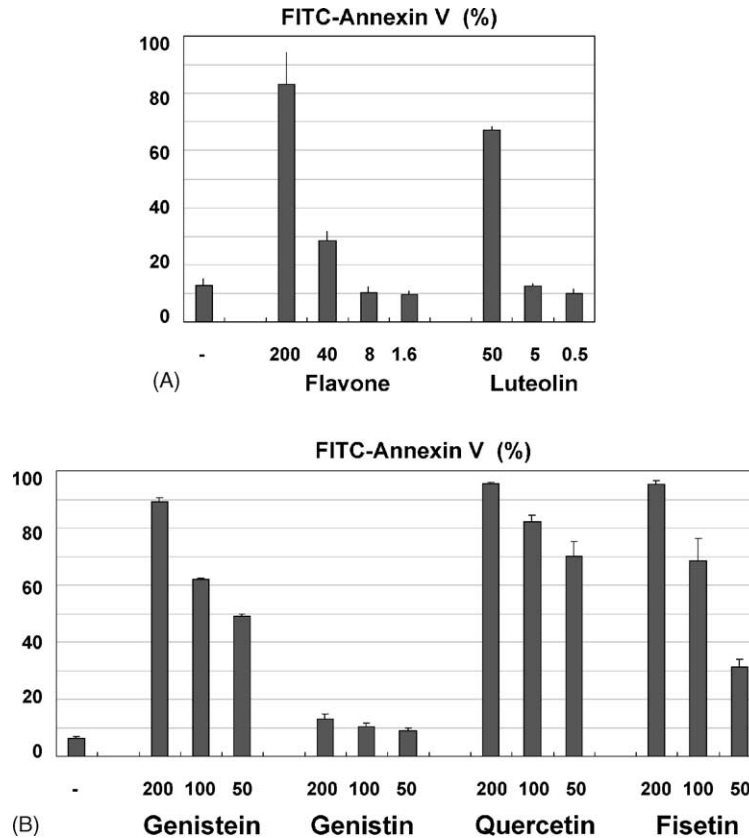


Fig. 4. Detection of annexin V binding cells after flavonoid-treatment. After culturing for 24 h in the presence of the indicated concentrations of each flavonoid, BV-173 cells were incubated with FITC-conjugated annexin V and then analyzed by flow cytometry. Experiments were performed in triplicate and the means + S.D. of the percentages of annexin V bound cells are indicated.

Fisetin, but not Genistein, also induced an increase in the number of cells binding to annexin V.

3.3. Characterization of bioflavonoid-induced apoptosis

The apoptosis induced by bioflavonoids in BV-173 cells was further characterized. In healthy cells, MitoCapture, a cationic dye, accumulates and aggregates in the mitochondria, giving off a bright red fluorescence. When the mitochondrial transmembrane potential is disrupted, however, this dye remains in the cytoplasm in its monomeric form, fluorescing green. As shown in Fig. 5, flow cytometric analysis revealed that treatment with Flavone significantly increased the number of cells with green fluorescence indicating that the mitochondrial transmembrane potential was disrupted after the induction of Flavone-induced apoptosis. In addition, Luteolin and Apigenin, but not Rutin, also disrupted the mitochondrial transmembrane potential (Fig. 5). Next, we examined the activation of caspase-3 in the process of bioflavonoid-induced apoptosis. Flow-cytometric analysis with PhiPhiLux™ G1D2 indicated that treatment with Flavone significantly increased the number of cells in which caspase-3 was activated (Fig. 6). The incidence of caspase-3-activated cells induced by Flavone-treatment was much higher than that induced by VP-16-treatment (Fig. 6).

Luteolin and Apigenin also increased the number of cells in which caspase-3 was activated (Fig. 6).

We further examined whether the activation of caspase was indeed involved in the bioflavonoid-induced apoptosis. As shown in Fig. 7, when BV-173 cells pretreated with either z-DEVD-fmk (a tetrapeptide inhibitor of caspase-3), z-IETD-fmk (a tetrapeptide inhibitor of caspase-8), or z-VAD-fmk (a tripeptide inhibitor of a broad range of caspases), a reduction in annexin V-positive cells after Flavone treatment was observed (Fig. 7). Of these inhibitors, z-VAD-fmk produced the most significant inhibition of Flavone-induced apoptosis in BV-173 cells.

3.4. Bioflavonoids induce apoptosis in other leukemia cell lineages

Next, we tested the effect of dietary bioflavonoids on other leukemia cell lines. As shown in Fig. 8, all of the leukemia cell lines tested in this study, including the pro-B cell lines NALM-16 and NALM-20, the pre-B cell lines HPB-NUL and NALM-17, the Burkitt's lymphoma cell lines Ramos and Daudi, the erythroleukemia cell line K-562, the basophilic leukemia cell line KU-812, the histiocytic lymphoma cell line U-937, and the acute monocytic leukemia cell line THP-1, were found to be sensitive to the apoptosis-inducing effect

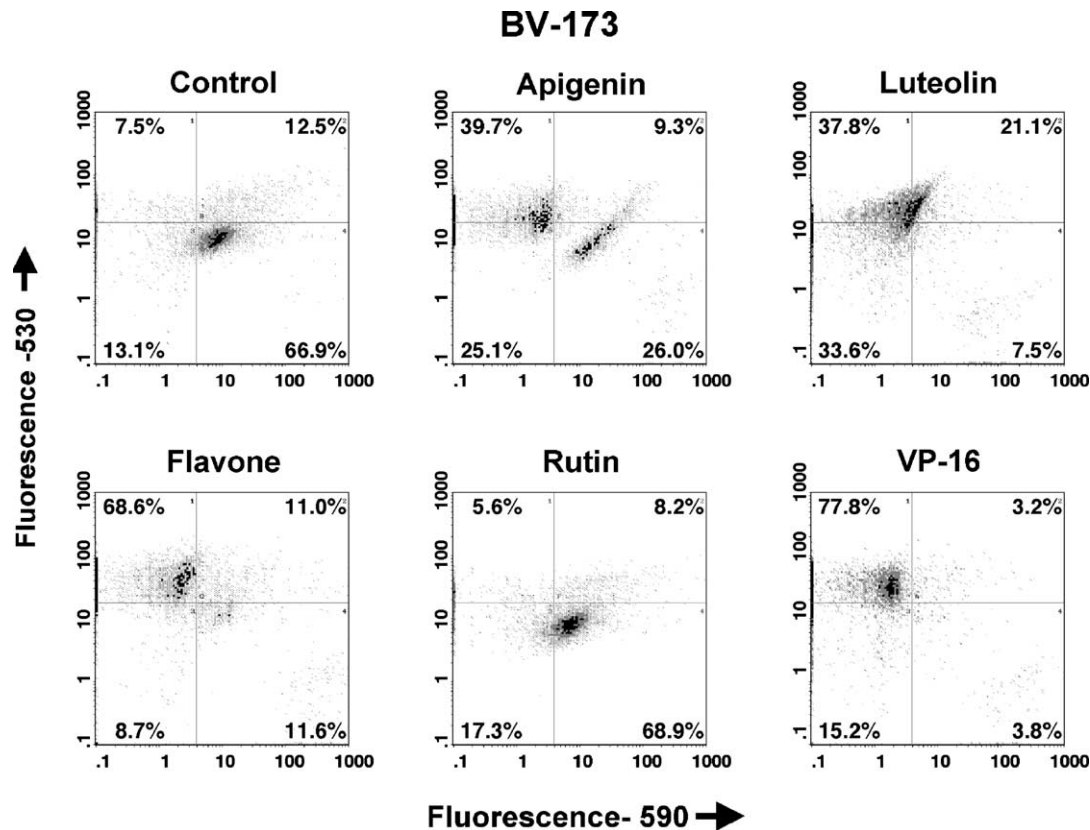


Fig. 5. Disruption of the mitochondrial transmembrane potential in BV-173 cells after bioflavonoid-treatment. BV-173 cells treated with or without flavonoids (Apigenin, 200 μ M; Luteolin, 50 μ M; Flavone, 200 μ M; Rutin, 200 μ M) or VP-16 (25 μ M) for 24 h were examined using a MitoCapture Apoptosis Detection Kit and analyzed by flow cytometry. The resulting histograms are shown. X-axis, intensity of fluorescence -590 (red); Y-axis, intensity of fluorescence -530 (green). A shift in the fluorescence from red to green indicates the disruption of the mitochondrial transmembrane potential.

of dietary bioflavonoids. Among these cell lines, however, NALM-6 and K-652 exhibited a relatively lower sensitivity. Although the Burkitt's cell lines showed a limited sensitivity to VP-16 cytotoxicity, they showed a very high sensitivity to Flavone-mediated apoptosis induction.

4. Discussion

Our data clearly indicates that certain, but not all, bioflavonoids induce apoptosis in a variety of human leukemia cell types. As shown in the present study, Flavone, Luteolin, Genistein, Quercetin, and Fisetin induced significant apoptosis in BV-173 cells, while Genistein and Rutin did not. The apoptosis-inducing effect of Apigenin was intermediate. As demonstrated in the present study, all of the human leukemia cells that were tested were effectively induced to undergo apoptosis after bioflavonoid treatment. The bioflavonoid-induced apoptosis occurred in a dose-dependent manner and was accompanied by the disruption of the mitochondrial transmembrane potential and the activation of caspase-3 and perhaps caspase-8. Indeed, the apoptosis was diminished by pretreatment of the cells with anti-caspase inhibitors.

A number of studies have reported the potential ability of bioflavonoids to act as anti-cancer drugs. The precise mechanism of this phenomenon, however, remains unclear, although several effects of bioflavonoids on cell growth and cell death have been reported. For example, bioflavonoids are reported to have topo inhibitor activity. Luteolin is reported to inhibit both topo I and II and induces apoptosis in *Leishmania* cells [5,14]. Strick et al. [15] reported that certain bioflavonoids induce MLL gene cleavage through the inhibition of topo II.

Some topo II-inhibitors, such as VP-16 and doxorubicin, are widely used as anti-cancer reagents and have been linked with therapy-related leukemia induction due to topo II-inhibition. The effect of topo II-inhibiting substances on cells is thought to consist of two stages [16,17]. During the first stage, topo II-inhibitors stabilize topo II-cleavable complexes by forming drug:topo II:DNA ternary complexes on chromosomal DNA. This stage is reversible by DNA religation or by DNA repair. However, cellular processing of the accumulating ternary complexes triggers the initiation of apoptotic DNA cleavage, an irreversible process (secondary stage of the pathway). At this stage, it is reported that caspase-8 is activated through FADD/TRADD-dependent mechanism and plays a critical role in caspase-3 activation and apoptotic

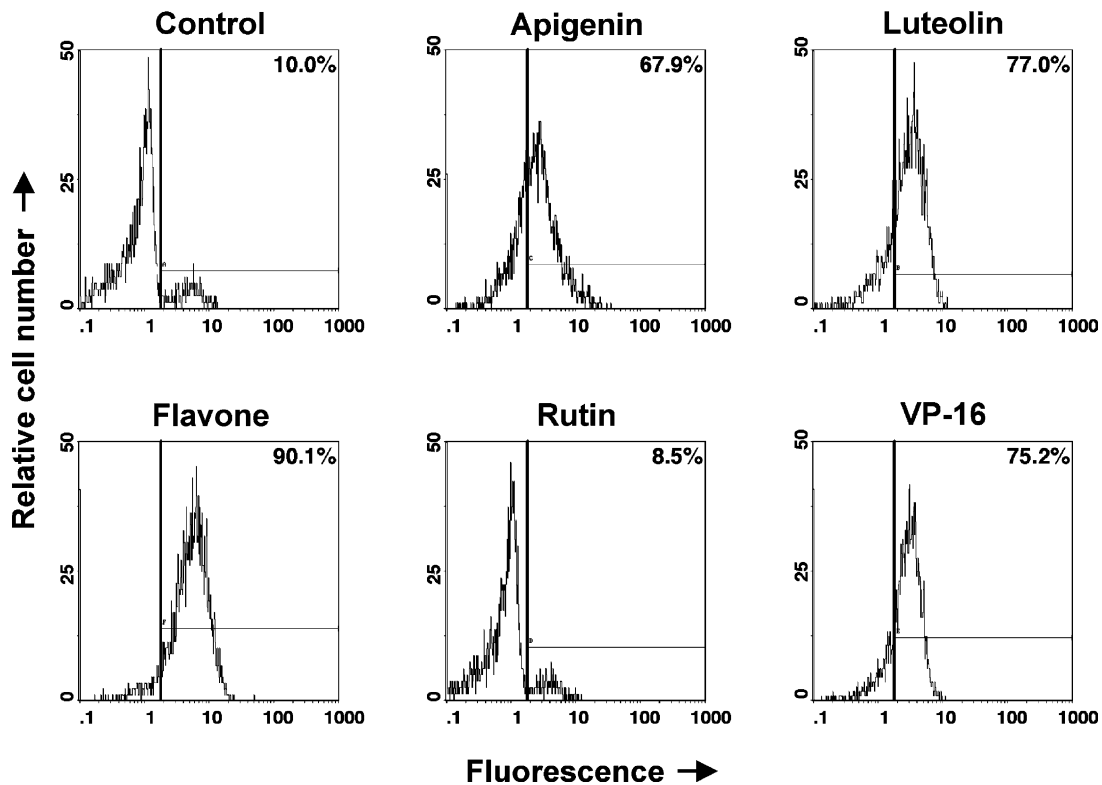


Fig. 6. Caspase-3 activity in BV-173 cells after bioflavonoid-treatment. To measure caspase-3 activity in BV-173 cells after bioflavonoid-treatment, cells prepared as in Fig. 5 were examined using PhiPhiLux™ GiD2 and analyzed by flow cytometry. The resulting histograms are shown. X-axis, fluorescence intensity; Y-axis, relative cell number.

cell death [18]. In parallel, caspase-9 is also activated by apoptosome-mediated mechanism as a result of mitochondrial dysfunction. However, latter pathway seems to play a much less role in caspase-3 activation [18]. Considering the above evidence, including similar activation pattern of caspase pathway (Figs. 6 and 7), it seems reasonable to assume that the anti-cancer effect of the bioflavonoids originated in their topo II-inhibitor activity.

However, as we presented in Fig. 8, Ramos Burkitt's cells, which were resistant to VP-16, were sensitive to Flavone-

induced apoptosis. In addition, Strick et al. have reported that Luteolin exhibits more strong topo II-inhibitory effect than that of Flavone [15], whereas apoptosis-inducing effect of Flavone is not lower than that of Luteolin (Fig. 8). Therefore, the induction of apoptosis by flavonoids cannot be explained solely by their topo II-inhibitory effect, and additional possible anti-cancer effects may be involved.

Of note, K-562 cells have been reported to be topo II-resistant [19], whereas our data indicated that K-562 cells are sensitive to VP-16-induced apoptosis and show over than 70% annexin V-positive cells, similar to the flavonoids used. The precise reason for the discrepancy between the previous reports and our data is presently unclear. However, it is also reported that K-562 cells show delay in the VP-16-induced caspase activation in compared with HL-60 cells, leading to a long latent period before initiation of apoptosis, and once the active phase of apoptosis is initiated, a similar proportion of cells are ultimately killed in both cell lines [20]. Therefore, K-562 cells are not completely resistant to topo II and the sensitivity to VP-16-mediated apoptosis may vary among the stocks of K-562 cells in different laboratories.

On the other hand, some bioflavonoids, such as Genistein and Quercetin inhibited tyrosine kinase activity both in vitro and in vivo [21]. Since the overactivation of tyrosine kinases is thought to be involved in oncogenesis in many types of cancer, it seems reasonable that bioflavonoids with anti-tyrosine kinase activity would exhibit an anti-cancer effect.

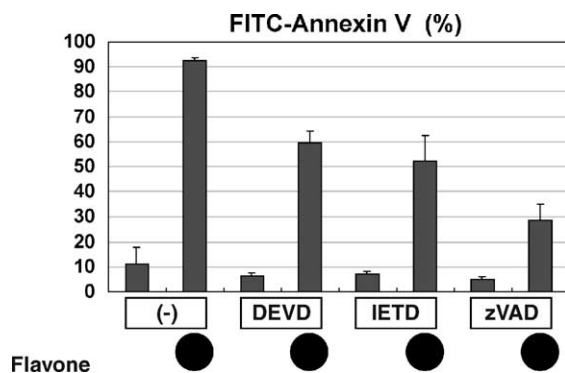


Fig. 7. Effect of caspase-inhibitors on Flavone-induced apoptosis. BV-173 cells pretreated with 50 μ M of caspase-inhibitor, as indicated, were treated with 200 μ M of Flavone for 24 h, as in Fig. 4. Subsequent apoptotic cells were detected by annexin V binding and analyzed by flow cytometry and are indicated as shown in Fig. 4.

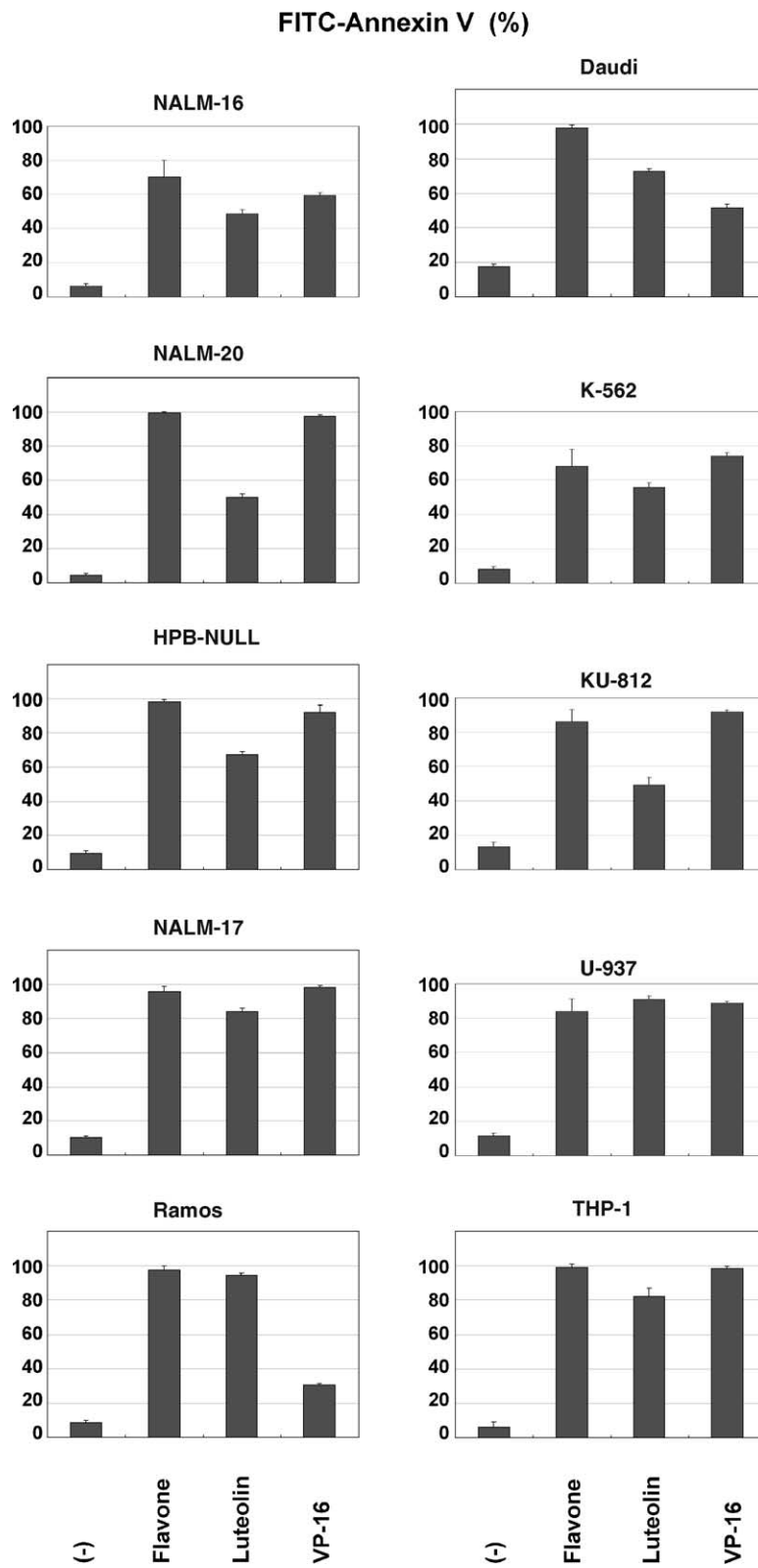


Fig. 8. Apoptosis-inducing effect of bioflavonoids in other leukemia cell lines. A variety of leukemia cell lines, as indicated in the figure, were treated with 200 μ M of Flavone, 50 μ M of Luteolin, or 25 μ M of VP-16 (as a positive control for apoptosis induction), as in Fig. 4. Subsequent apoptotic cells were detected by annexin V binding and analyzed by flow cytometry and are indicated as shown in Fig. 4.

Bioflavonoids have also been reported to be involved in cell cycle regulation. For example, Quercetin was found to downregulate the expression of mutant p53 protein in human breast cancer cell lines, leading to an arrest of the cells in the G2–M phase of the cell cycle [21]. In the case of human leukemic T-cells, Quercetin was found to arrest the cells in late G1 phase. In addition, Luteolin has been reported to arrest the cell cycle in the G1 phase of human melanoma cells [22], and Genistein induces cell cycle arrest at the G2–M stage and the inhibition of cdc2 kinase activity [23]. The arrest of the cell cycle in turn reduces cell growth and results in apoptosis induction.

Another explanation for the anti-cancer activity of bioflavonoids is suggested by their ability to interact with hormone receptors [21]. Certain bioflavonoids have been reported to bind to estrogen binding sites in estrogen receptors, there by interrupting estrogen binding. Indeed, flavonoids, such as Daidzein, Genistein, Quercetin, and Luteolin, were found to suppress the induction of the proliferation-stimulating activity of environmental estrogens in human breast cancer cell lines [24]. However, whether estrogen binding induces the proliferation of leukemic cells has not been reported. Alternatively, flavonoids may interact with the binding sites of growth factors other than estrogen, thereby inhibiting the growth of leukemia cells.

In conclusion, dietary bioflavonoids exhibited an apoptosis-inducing effect in various human leukemia cells. Although further studies must be performed to elucidate the mechanism by which bioflavonoids induce apoptosis in leukemia cells, the present data indicates that dietary bioflavonoids might be useful chemotherapeutic reagents for leukemia patients.

Acknowledgements

We thank S. Yamauchi for her excellent secretarial work. We also thank Dr. Y. Matsuo (Hayashibara Biochemical Laboratories Inc.) for gifting the cell lines. This work was supported in part by Health and Labour Sciences Research Grants from the Ministry of Health, Labour and Welfare of Japan, MEXT. KAKENHI 15019129, JSPS. KAKENHI 15390133 and 15590361, grant from the Japan Health Sciences Foundation for Research on Health Sciences Focusing on Drug Innovation, a grant from Sankyo Foundation of Life Science.

Contributions: J. Matsui contributed to the concept and design, interpreted and analyzed the data, provided drafting of the article, and gave final approval. N. Kiyokawa contributed to the concept and design, interpreted and analyzed the data, provided drafting of the article, and gave final approval, and obtained a funding source. H. Takenouchi interpreted and analyzed the data, provided critical revisions and important intellectual content. T. Taguchi and K. Suzuki interpreted and analyzed the data. Y. Shiozawa provided administrative support, provided critical revisions and important intellectual content. M. Saito and W.-R. Tang provided administrative

support. H. Okita provided critical revisions and important intellectual content. J. Fujimoto provided critical revisions and important intellectual content, and obtained a funding source.

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