Pergamon

European Journal of Cancer, Vol. 35, No. 10, pp. 1517–1525, 1999

Printed in Great Britain
0959-8049/99/$ - see front matter

PII: S0959-8049(99)00168-9

Original Paper

Induction of Apoptosis by Apigenin and Related Flavonoids Through Cytochrome c Release and Activation of Caspase-9 and Caspase-3 in Leukaemia HL-60 Cells

I.-K. Wang,[1] S.-Y. Lin-Shiau[2] and J.-K. Lin[1]

[1]Institutes of Biochemistry; and [2]Toxicology, College of Medicine, National Taiwan University, No. 1, Section 1, Jen-Ai Road, Taipei, Taiwan, R.O.C.

The aim of this study was to investigate the mechanism of flavonoid-induced apoptosis in HL-60 leukaemic cells. Thus, the effect of structurally related flavonoids on cell viability, DNA fragmentation and caspase activity was assessed. Loss of membrane potential and reactive oxygen species generation were also monitored by flow cytometry. The structurally related flavonoids, such as apigenin, quercetin, myricetin, and kaempferol were able to induce apoptosis in human leukaemia HL-60 cells. Treatment with flavonoids (60 μM) caused a rapid induction of caspase-3 activity and stimulated proteolytic cleavage of poly-(ADP-ribose) polymerase (PARP). Furthermore, these flavonoids induced loss of mitochondrial transmembrane potential, elevation of reactive oxygen species (ROS) production, release of mitochondrial cytochrome c into the cytosol, and subsequent induction of procaspase-9 processing. The potency of these flavonoids on these features of apoptosis were in the order of: apigenin > quercetin > myricetin > kaempferol in HL-60 cells treated with 60 μM flavonoids. These results suggest that flavonoid-induced apoptosis is stimulated by the release of cytochrome c to the cytosol, by procaspase-9 processing, and through a caspase-3-dependent mechanism. The induction of apoptosis by flavonoids may be attributed to their cancer chemopreventive activity. Furthermore, the potency of flavonoids for inducing apoptosis may be dependent on the numbers of hydroxyl groups in the 2-phenyl group and on the absence of the 3-hydroxyl group. This provides new information on the structure–activity relationship of flavonoids. © 1999 Elsevier Science Ltd. All rights reserved.

Key words: flavonoid, apigenin, apoptosis, mitochondrial transmembrane potential, reactive oxygen species (ROS), cytochrome c, caspase-9, caspase-3, PARP
Eur J Cancer, Vol. 35, No. 10, pp. 1517–1525, 1999

INTRODUCTION

FLAVONOIDS ARE a group of common phenolic plant pigments. They are widely distributed in the plant kingdom and occur naturally in a broad range of fruits and vegetables [1]. Those flavonoids considered to be dietary anticarcinogens [2] and anti-oxidants [3] have been utilised for clinical purposes [4]. In our previous report [5], we showed that apigenin could inhibit 12-O-tetradecanoyl-phorbol-13-acetate (TPA)-induced c-*jun* and c-*fos* expression and TPA-mediated tumour promotion.

Apoptosis is a normal physiological process which occurs during embryonic development as well as during the maintenance of tissue homeostasis [6]; it can be induced by a variety of treatments, such as ultraviolet (UV) irradiation [7], Fas/APO-1 ligand [8, 9] and cytotoxic chemotherapy [10, 11]. Cells which die by apoptosis usually suffer similar morphological changes, including nuclear condensation, cytoplasmic blebbing and DNA fragmentation.

The transduction and execution of apoptotic signals requires the co-ordinated action of a cascade of caspases (aspartate-specific cysteine proteases) [12]. The caspases are present in cells as inactive procaspases, with the active tetramer being formed by removal of the prodomain and cleavage between the large and small subunits [13]. Caspase activity is responsible, either directly or indirectly, for cleavage of cellular proteins which are characteristically proteolysed during apoptosis. For example, caspase-2, -3, -7 and -9 can cleave poly-(ADP-vibose) polymerase (PARP) [14], and caspase-6 can cleave nuclear lamins [15]. The active caspase-4 cleaves

Correspondence to J.-K. Lin, e-mail: jklin@ha.mc.ntu.edu.tw
Received 30 Jun. 1999; accepted 13 Jul. 1999.

the precursor form of caspase-1 to generate the mature form [16], and activated caspase-1 cleaves the zymogen of caspase-3 to produce the active form [17].

In response to certain apoptotic stimuli, cytochrome c is released from mitochondria [18, 19]. Once it is in the cytoplasm, however, cytochrome c has an entirely different function. The released cytochrome c induces the formation of a caspase activation complex (CAC) with Apaf-1, that directly binds the zymogen form of caspase-9 via a homophilic interaction involving CARD (caspase recruitment domain) motifs [20]. The CARD domain is a region of highly conserved residues in the amino terminus of molecules which are recruited for complex signalling during apoptosis. Activated caspase-9 can proteolytically process the zymogen form of downstream caspases, such as caspase-3 [21].

In the present study, we have demonstrated flavonoid-induced apoptosis in HL-60 cells. Flavonoid-induced apoptosis can activate caspase-3 which leads to the cleavage of PARP. We also explored flavonoid-induced loss of mitochondrial transmembrane potential, reactive oxygen species (ROS) production and induction of procaspase-9 processing.

MATERIALS AND METHODS

Cell culture and chemicals

Human promyelocytic leukaemia HL-60 cells were grown in 85% RPMI 1640 and 15% fetal bovine serum (GIBCO BRL, Grand Island, New York, U.S.A.), supplemented with 2 mM glutamine (GIBCO), 1% penicillin/streptomycin (10 000 U penicillin/ml and 10 mg/ml streptomycin). Cells were plated at a density of 2×10^5 cells/ml in 100 mm petri dishes and grown at 37°C in a humidified, 5% CO_2 atmosphere. Flavonoids (apigenin, quercetin, myricetin, and kaempferol) and genistein were purchased from Sigma Chemical Co. (St Louis, Missouri, U.S.A.) and dissolved in dimethyl sulphoxide (DMSO). Epicatechin 3-gallate (ECG), epigallo-catechin (EGC), and epigallocatechin 3-gallate (EGCG) were isolated from Lonjing tea as described [22] and dissolved in DMSO. The inhibitors for caspase-3 (Z-Asp-Glu-Val-Asp-fluoromethylketone; Z-DEVD-FMK) and caspase-1 (acetyl-Tyr-Val-Ala-Asp-methylcoumany 1-7-amide; Ac-YAVD-CMK) were purchased from Calbiochem (La Jolla, California, U.S.A.).

Cell viability assay

Cells were plated at a density of 2×10^5 cells/well into 96-well plates. After overnight growth at 37°C, cells were treated with different flavonoids for 12 h, and then the cell viability was assayed with CellTiter 96 Non-Radioactive Cell Proliferation Assay Kit (Promega, Madison, Wisconsin, U.S.A.). Briefly, 20 µl of combined solution of a tetrazolium compound MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium: inner salt) and an electron-coupling reagent, phenazine methosulphate, were added to each well. After incubation for 2 h at 37°C in a humidified 5% CO_2 atmosphere, the absorption of $A_{490\,nm}$ was recorded using an enzyme-linked immunosorbent array (ELISA) plate reader.

DNA extraction and electrophoretic analysis

HL-60 cells (4×10^5 cells/ml) were harvested, washed in phosphate-buffered saline (PBS) and then lysed by digestion buffer containing 0.5% sarkosyl, 0.5% mg/ml proteinase K, 50 mM Tris (hydroxy methyl) aminomethane (pH 8.0) and 10 mM EDTA at 55°C for 3 h. RNase A (0.5 µg/ml) was added and incubated at 55°C for 24 h. The DNA was extracted by phenol–chloroform–isoamyl alcohol and analysed by 1.8% agarose gel electrophoresis (containing ethidium bromide) at 50 V for 120 min. Approximately 20 µg of DNA was loaded in each well, visualised under a UV light and photographed.

Western blotting

The nuclear and cytosolic proteins were isolated from human promyelocytic leukaemia HL-60 cells (4×10^5 cells/ml) after treatment with 60 µM flavonoids for 0, 3, 6, 9, and 12 h. Total proteins were extracted by adding 200 µl of cold lysis buffer (50 mM Tris–HCl, pH 7.4; 1 mM sodium fluoride (NaF); 150 mM NaCl; 1 mM EGTA; 1 mM phenylmethylsulphonyl fluoride (PMSF); 1% NP 40; and 10 µg/ml leupeptin) to the cell pellets. After 30 min on ice the cell debris was pelleted by centrifugation at 10 000 ***g*** for 30 min at 4°C. The cytosolic fraction (supernatant) proteins were measured by bicinchoninic acid assay (BCA; Promega, Madison, Wisconsin, U.S.A.). The samples (50 µg of total protein) were mixed with 5× sample buffer, containing 0.3 M Tris–HCl (pH 6.8), 25% 2-mercaptoethanol, 12% sodium-dodecyl sulphate (SDS), 25 mM EDTA, 20% glycerol, and 0.1% bromophenol blue. The mixtures were boiled at 95°C for 5 min, and subjected to SDS-PAGE (polyacrylamide gel electrophoresis) at a constant current of 20 mA. Following electrophoresis, proteins on the gel were electro-transferred onto an immobile membrane (PVDF; Millipore, Bedford, Massachusetts, U.S.A.) in transfer buffer composed of 25 mM Tris–HCl (pH 8.9), 192 mM glycine and 20% methanol. The membranes were blocked with 20 mM Tris–HCl (pH 7.4), 125 mM NaCl, 0.2% Tween 20, 1% bovine serum albumin (BSA), and 0.1% sodium azide. The membranes were then immunoblotted with primary antibodies (rabbit polyclonal antibodies to human PARP at a dilution of 1/1000 (UBI, Lake Placid, New York, U.S.A.), mouse MAbs to cytochrome c (RDI, Flanders, New Jersey, U.S.A.) at a dilution of 1/1000 or mouse MAbs to caspase-9 at a dilution of (Pharmingen, Becton Dickinson, San Diego, California, U.S.A.), at room temperature for 3 h. This was followed by the addition of horseradish peroxidase-labelled second antibody at a dilution of 1/5000 and development using the ECL system (Amersham Life Science, Amersham, Bucks, U.K.).

Activity of caspase

Cells were collected and washed with PBS and resuspended in 25 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 5 mM EDTA, 5mM dithiothreitol (DTT), 2 mM PMSF, 10 µg/ml pepstatin A and 10 µg/ml leupeptin after treatment. Cell lysates were clarified by centrifugation at 12 000 rpm for 5 min and clear lysates containing 50 µg of protein were incubated with 50 µM substrate Ac-DEVD-AMC or Ac-YVAD-AMC at 30°C for 1 h. Levels of released AMC were measured using a spectrofluorometer (Hitachi F-4500) with excitation at 360 nm and emission at 460 nm (Promega's CaspACE™ Assay System, Madison, Wisconsin, U.S.A.).

Cytochrome c release

Mitochondrial and cytosolic fractions were prepared by resuspending cells in ice-cold buffer A (250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 17 µg/ml PMSF, 8 µg/ml aprotinin

and 2 μg/ml leupeptin (pH 7.4)). Cells were passed through a needle ten times. Unlysed cells and nuclei were pelleted by centrifugation for 10 min at 750*g*. The supernatant was then centrifuged at 100 000*g* for 15 min. This pellet was resuspended in buffer A and represents the mitochondrial fraction. The supernatant was again centrifuged at 100 000*g* for 1 h. The supernatant from this final centrifugation step represents the cytosolic fraction.

Analysis of mitochondrial transmembrane potential and generation of reactive oxygen species (ROS)

Loss of mitochondrial transmembrane potential was monitored by flow cytometry. Briefly, HL-60 cells were exposed to flavonoids. Mitochondrial transmembrane potential was measured directly using 40 nM 3,3′-dihexyloxacarbocyanine (DiOC6(3)); (Molecular Probes, Eugene, Oregon, U.S.A.). Fluorescence was measured after staining the cells for 15 min at 37°C. To assess ROS generation by flow cytometry, cells were treated with 20 μM 2′,7′-dichlorofluorescein diacetate (DCFH-DA) (Molecular Probes) for 1 h at 37°C.

RESULTS

Flavonoids cause reduction in cell viability

Previous studies have shown that flavonoids are potent antiproliferation [23–25] and anticancer agents [3]. Here, we investigated four structurally related flavonoids: apigenin, myricetin, quercetin and kaempferol. The structures of these flavonoids are illustrated in Figure 1. We tested their effects on cell viability in human promyelocytic leukaemia HL-60 cells with different concentrations of flavonoids. After 12 h of treatment, the percentage of living cells was determined and is shown in Figure 2, myricetin and quercetin induced a dose-dependent decrease in cell viability after 40 μM treatment, but the affected cell viability was lower than that by apigenin. Apigenin appeared to be more potent, with an IC_{50} of 50 μM and dose-dependent inhibition of cell viability. In contrast, kaempferol only slightly affected cell viability.

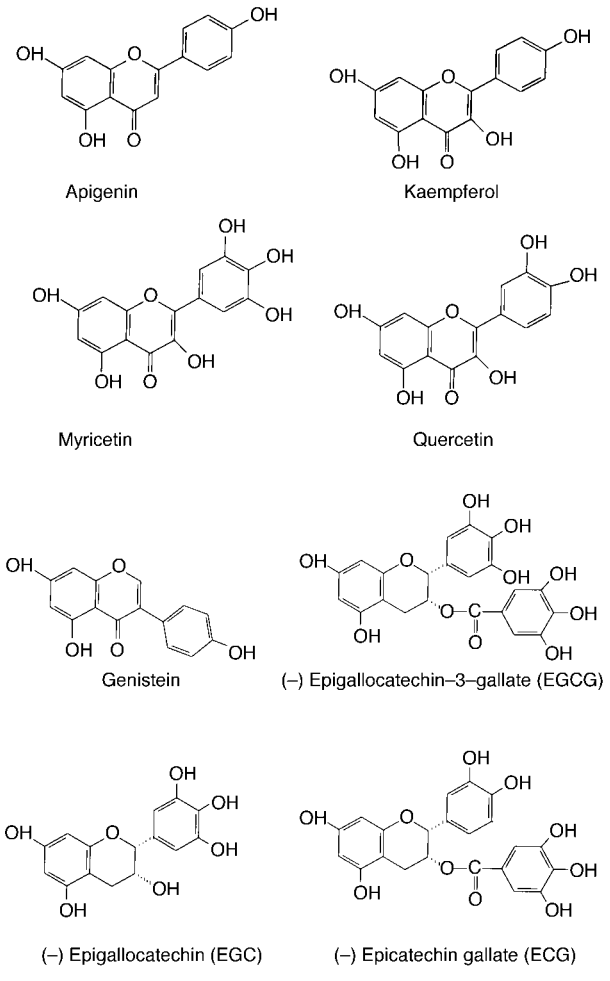

Figure 1. Chemical structure of the flavonoids, apigenin, kaempferol, quercetin and myricetin; an isoflavonoid, genistein; and the flavanoids, ECG, EGC, and EGCG.

Effect of flavonoids on DNA fragmentation of HL-60 cells

Flavonoid induction of DNA fragmentation was demonstrated by incubating HL-60 cells with different concentrations of flavonoids for 12 h (Figure 3a). DNA fragmentation became apparent with between 20 and 40 μM apigenin treatment, and this DNA fragmentation response was dose-dependent (Figure 3a). When cells were treated with 60 μM apigenin, DNA ladders were just visible 6 h after treatment, and increasing DNA fragmentation was observed from 6 to 12 h (Figure 3b). Myricetin and quercetin exhibited very similar DNA ladders in a dose-dependent fashion (Figure 3A). No DNA fragmentation was obtained in HL-60 cells treated with kaempferol (Figure 3). Within 12 h of treatment with 60 μM flavonoids, the potency of these flavonoids on DNA fragmentation was: apigenin > quercetin > myricetin > kaempferol (Figure 3b).

Flavonoids stimulated caspase-3 activity in a time- and dose-dependent manner

Caspases are believed to play a central role in mediating various apoptotic responses. To monitor the enzymatic activity of caspases during flavonoid-induced apoptosis, we used two fluorogenic peptide substrates: Ac-YVAD-AMC is a specific substrate for caspase-1, whereas Ac-DEVD-AMC is a specific substrate for caspase-3. Caspase activities were measured following treatment of HL-60 cells with different

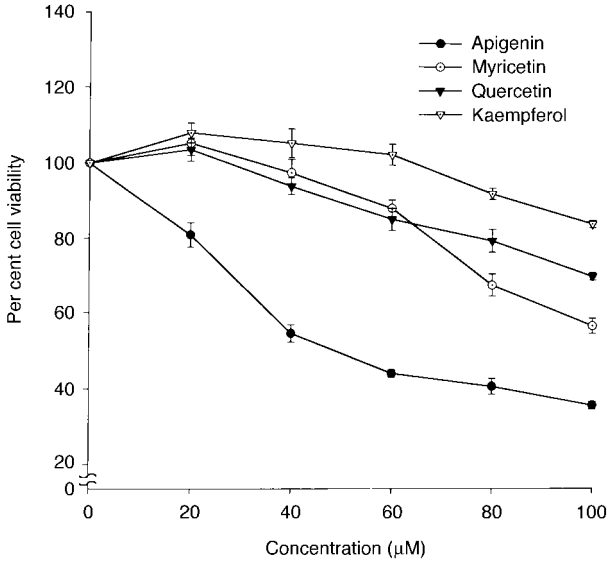

Figure 2. Effects of different flavonoids on cell viability. The percentage of cell viability was calculated as the ratio, A_{490}, of treated cells to control cells. Data points represent means for three independent experiments; bars = standard error.

concentrations of these flavonoids. As shown in Figure 4a, the induction of caspase-3 activity by apigenin paralleled the dose-dependent pattern of apoptosis. Activities of myricetin and quercetin also followed a dose-dependent pattern. At lower concentrations, myricetin induced lower caspase-3 activity than did quercetin, but the order was reversed at high concentrations. In contrast, kaempferol only slightly induced caspase-3 activity. Again, a very low level of caspase-1 activity was induced by these flavonoids (Figure 4b).

We further studied the time course of caspase-3 activation of flavonoids in HL-60 cells. Caspase-3 activity was determined by treatment with different flavonoids (60 μM). Myricetin, quercetin, and kaempferol induced a slow and slightly time-dependent caspase-3 activation (Figure 4c). Apigenin induced a rapid rise in caspase-3 activity to approximately a 16-fold increase after the addition of apigenin for 12 h (Figure 4c).

Caspase-3 inhibitor, Z-DEVD-FMK, decreases apoptosis induced by flavonoids

The above results clearly indicate that caspase-3 protease is activated in response to apoptosis induced by flavonoids. To determine whether the activation of caspase-3 is required for the induction of cell death by flavonoids, we pretreated HL-60 cells with caspase inhibitors. As shown in Figure 5, an inhibitor of caspase-3 protease, Z-DEVD-FMK, was able to inhibit flavonoid-stimulated DNA fragmentation (Figure 5a) and caspase-3 activity (Figure 5b). In contrast, Ac-YVAD-CMK, an inhibitor of caspase-1 activity, had little effect at similar concentrations (Figure 5a), consistent with the high substrate specificity of different caspases. Similar results were also obtained when apoptosis was induced by other

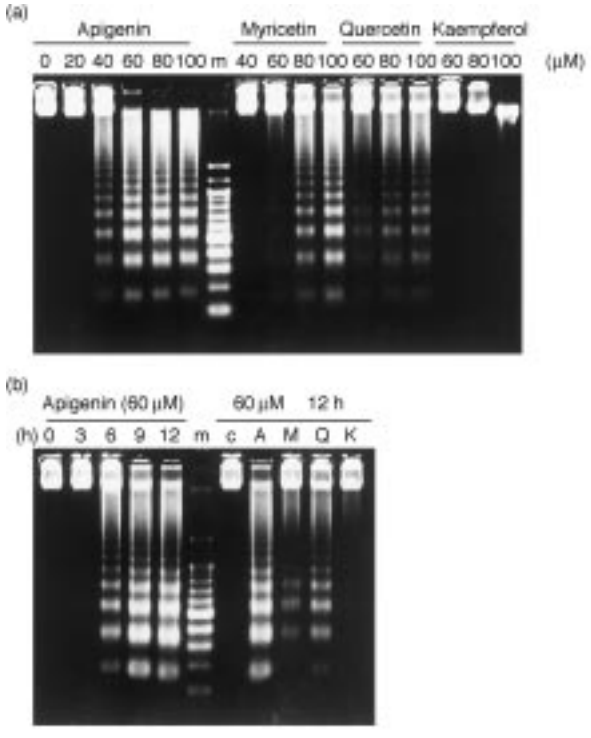

Figure 3. Induction of DNA fragmentation in HL-60 cells by flavonoids. (a) HL-60 cells treated with increasing doses of flavonoids for 12 h. (b) Time-dependent increase of DNA fragmentation ladders induced by 60 μM apigenin, and HL-60 cells treated with different flavonoids (60 μM) for 12 h. Agarose gel analysis of DNA fragmentation was performed. m, DNA ladder marker; C, control; A, apigenin; M, myricetin; Q, quercetin; K, kaempferol.

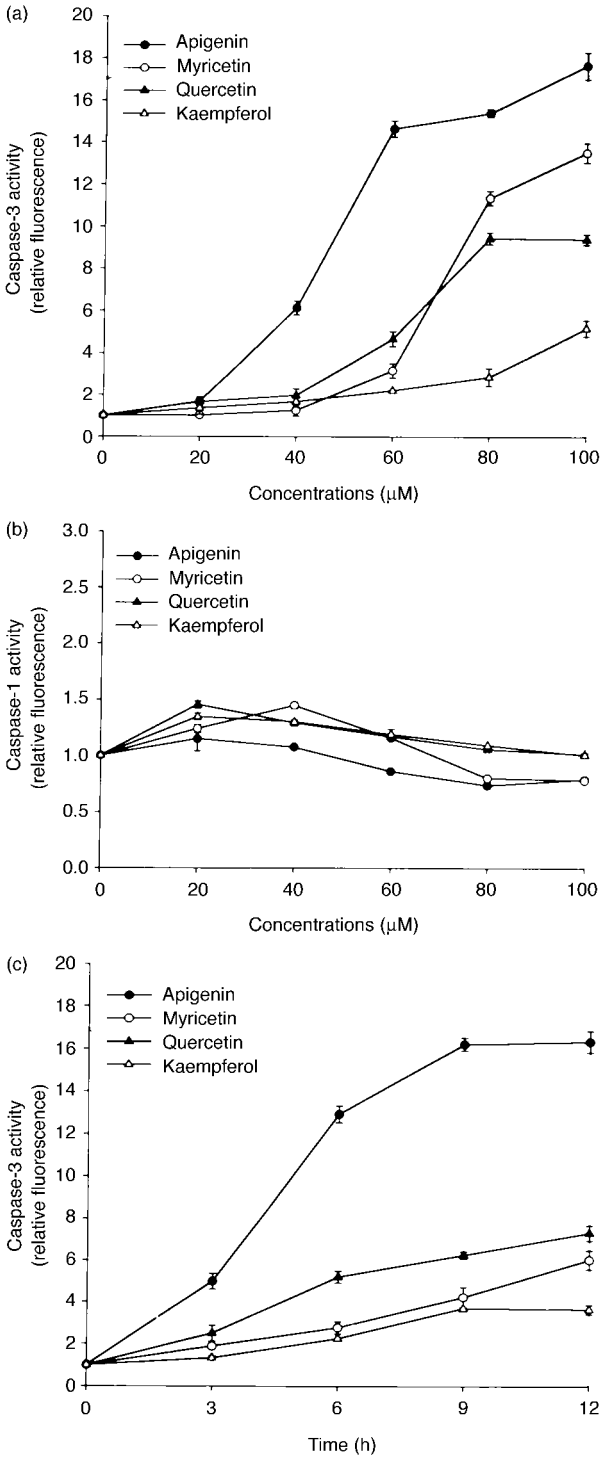

Figure 4. Induction of caspase activities. (a,b) Dose-dependent activation of caspases by flavonoids. Following treatment with different concentrations of flavonoids for 12 h, caspase activities were analysed. (c) Kinetics of caspase-3 activation by flavonoids. HL-60 cells were treated for indicated time periods, and caspase-3 activity was analysed as described in Materials and Methods. Data points represent means for three independent experiments and are expressed relative to 0 h; bars = standard error.

flavonoids (data not shown). Thus, these data suggest that the induction of caspase-3 activity is a specific biochemical event brought about by apoptosis-inducing flavonoids.

Flavonoid induced cleavage of PARP

Activation of caspase-3 leads to the cleavage of poly-(ADP-ribose) polymerase (PARP). The cleavage of PARP is the hallmark of apoptosis. PARP (116 kDa) is cleaved to produce an 85-kDa fragment during apoptosis [26]. We examined the cleavage of PARP in flavonoid-induced apoptosis. Treatment of HL-60 cells with 60 μM apigenin for more than 6 h caused a time-dependent proteolytic cleavage of PARP, with accumulation of the 85-kDa fragments and a concomitant disappearance of the full-length 116-kDa protein (Figure 6). In addition, we determined the cleavage of PARP with myricetin, quercetin, and kaempferol (60 μM) for 12 h. The potency of quercetin was greater than that of myricetin and kaempferol, but they were all lower than that of apigenin (Figure 6). These data are consistent with DNA fragmentation and caspase-3 activity.

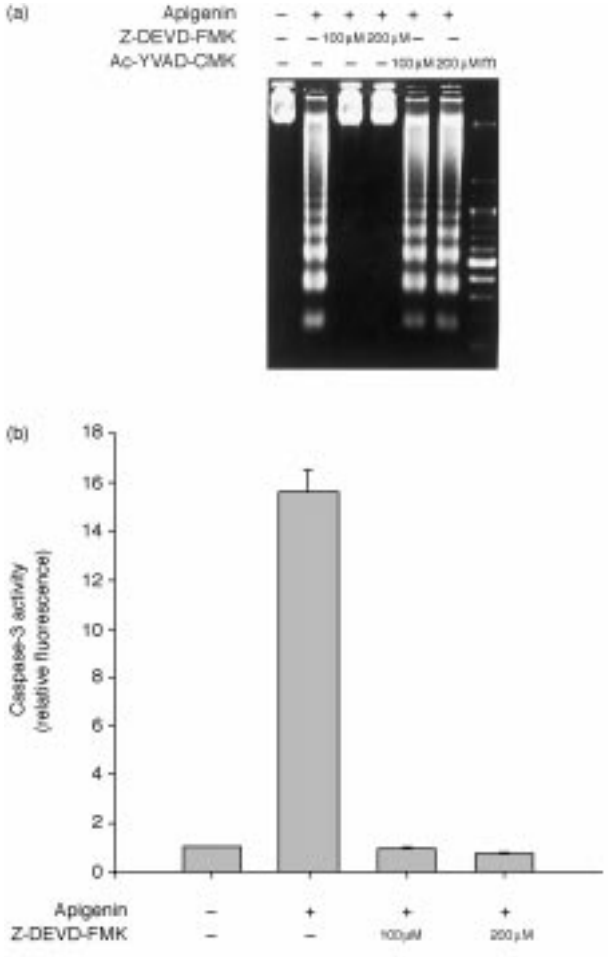

Figure 5. Effects of caspase-3 inhibitors on DNA fragmentation and caspase-3 activity induced by flavonoids. (a) Suppression of flavonoid-induced DNA fragmentation by caspase-3 inhibitors. m = DNA ladder marker. (b) Inhibition of flavonoid-induced caspase-3 activity by caspase-3 inhibitors. HL-60 cells were pretreated for 1 h with caspase inhibitors (Z-DEVD-FMK or Ac-YAVD-CMK) prior to the addition of 60 μM apigenin for 12 h. The caspase-3 activity was detected as described in Materials and Methods. Columns represent means for three independent experiments and are expressed relative to 0 h; bars = standard error.

Effect of flavonoids on mitochondrial transmembrane potential (ψ_m) and ROS generation

There is increasing evidence that altered mitochondrial function is linked to apoptosis and a decreasing mitochondrial transmembrane potential is associated with mitochondrial dysfunction. Thus, we next evaluated the effect of flavonoids on the mitochondrial transmembrane potential (ψ_m). We measured $\Delta\psi_m$ using the fluorescent probe DiOC6(3) and monitored it using flow cytometry. As shown in Figure 7a, HL-60 cells exposed to 60 μM apigenin for only 1 h showed a sharp decline in DiOC6(3) fluorescence (mean = 66.8; Mc). In contrast, the mean of DiOC6(3) fluorescence intensity of untreated control cells was 101.12 (Ma) (Figure 7a). Treatment with other flavonoids for 6 h at 60 μM also induced loss of mitochondrial transmembrane potential; mean DiOC6(3) fluorescence declined from Ma = 101.12 (control) to Mb = 56.71 (myricetin treated) (Figure 7b); 49.65 (quercetin treated) (Figure 7c), and 79.35 (kaempferol treated) (Figure 7d).

Further, we studied the loss of mitochondrial transmembrane potential resulting in the generation of ROS [27] by assessing reactive oxygen species generation using the fluorescent probe, DCFH-DA, and monitoring by flow cytometry. The potency of ROS generation was: apigenin > quercetin > myricetin > kaempferol in HL-60 cells treated with 60 μM flavonoids. The mean of DCFH-DA fluorescence increased from Ma = 111.45 (control) to Mb = 427.32 (apigenin treated for 1 h) (Figure 7e), 158.32 (myricetin treated for 6 h) (Figure 7f), 175.76 (quercetin treated for 6 h) Figure 7g), and 127.46 (kaempferol treated for 6 h) (Figure 7h). These findings point to an effect of flavonoids on mitochondrial function and accumulation of ROS. These features are cues for the induction of apoptosis.

Flavonoid induction of cytochrome c release and caspase-9 activation

The process of cell death may involve the release of cytochrome c from the mitochondria, which subsequently causes apoptosis by activation of the caspases. Together, these data suggest a linear and specific activation cascade between caspase-9 and caspase-3 in response to cytochrome c released from the mitochondria [21]. Treatment of HL-60 cells with 60 μM flavonoids for 12 h caused cytochrome c to be released into the cytosol (Figure 8a). Furthermore, we examined the cleavage of caspase-9 during treatment of cells with flavonoids (60 μM) by immunoblotting. A time-dependent proteolytic cleavage of procaspase-9, with an increase of the 10-kDa fragment, was obtained by 60-μM apigenin treatment (Figure 8b). Procaspase-9 processing was also observed with other

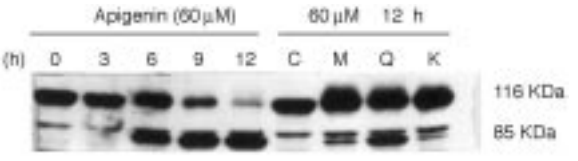

Figure 6. Cleavage of poly-(ADP-ribose) polymerase (PARP) during flavonoid-induced apoptosis. C, control; M, myricetin; Q, quercetin; K, kaempferol.

flavonoids (Figure 8b). These data are consistent with DNA fragmentation, caspase-3 activity, cleavage of PARP and loss of mitochondrial transmembrane potential. Therefore, these results suggest that mitochondrial dysfunction caused cytochrome c to be released into the cytosol, and then the activation of the cascade between caspase-9 and caspase-3.

Effect of apigenin and related flavonoids on DNA fragmentation of HL-60 cells

We further examined the effects of genistein, ECG, EGC and EGCG on DNA fragmentation in HL-60 cells. These compounds are illustrated in Figure 1. HL-60 cells were incubated with different chemical compounds (60 μM) for 12 h. The photograph shown as Figure 9 was overexposed (2/30 s) as compared with that in Figure 3 (1/30 s). DNA fragmentation was obtained with apigenin, myricetin, quercetin, kaempferol and EGC treatment, but not with genistein, ECG or EGCG (Figure 9). The structures of apigenin and genistein are similar (Figure 1), but genistein cannot induce DNA fragmentation (Figure 9). In addition, the structures of ECG, EGCG, and EGC are similar (Figure 1). However, EGC was the most effective inducer of DNA fragmentation.

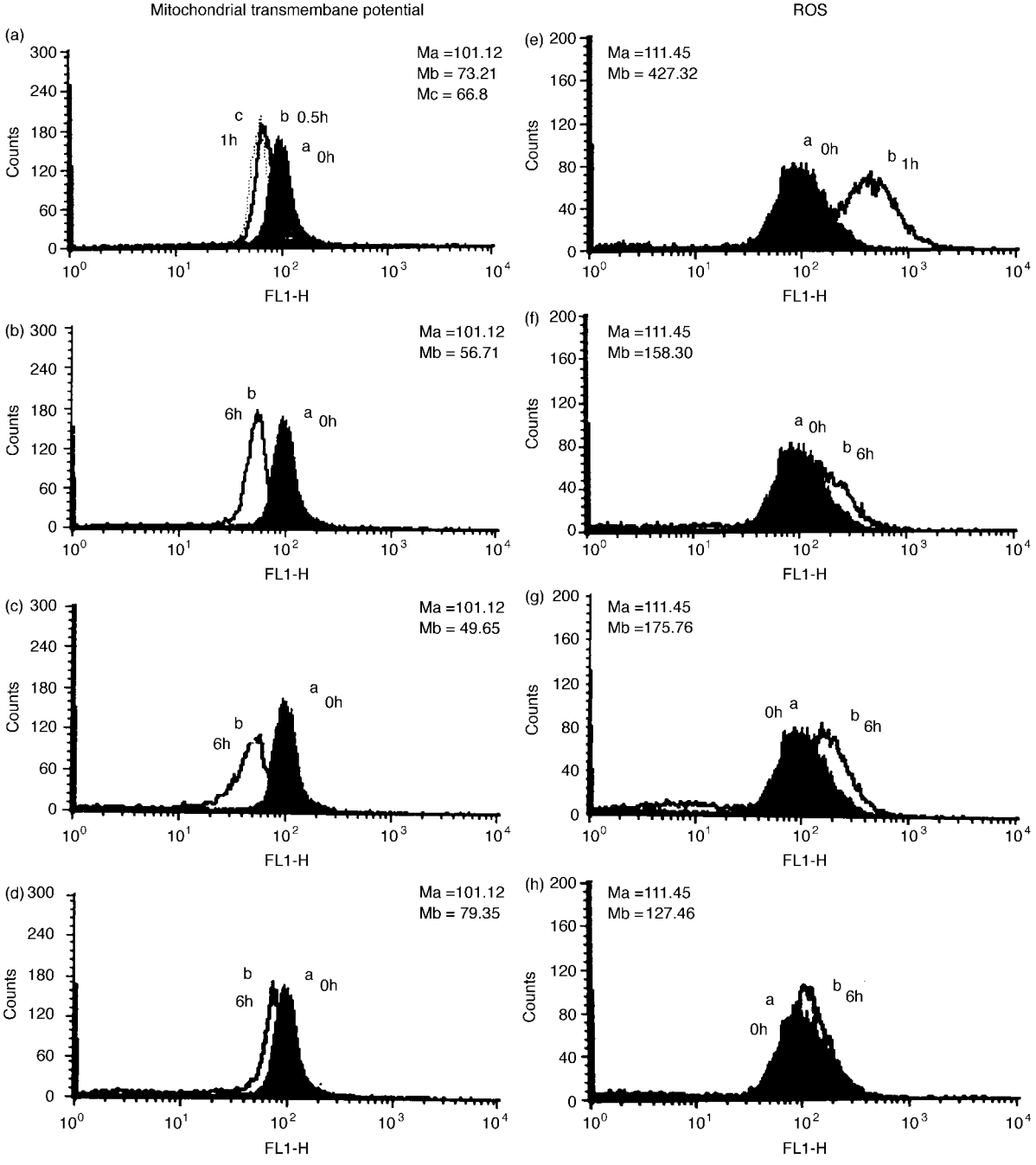

Figure 7. Induction of mitochondrial dysfunction and reactive oxygen species (ROS) generation in HL-60 cells by flavonoids. Apigenin (a); myricetin (b); quercetin (c); or kaempferol (d) induced reduction of mitochondrial transmembrane potential ($\Delta\psi_m$) in HL-60 cells. The percentages reflect the reduction of $\Delta\psi_m$ [DiOC6(3)]. Apigenin (e); myricetin (f); quercetin (g); or kaempferol (h) induced ROS generation in HL-60 cells. The percentages reflect ROS generation.

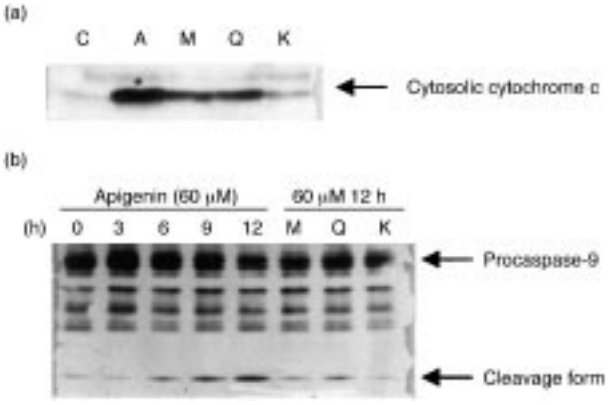

Figure 8. Induction of cytochrome c release and caspase-9 processing in HL-60 cells by flavonoids. (a) Cytochrome c was released from mitochondria into the cytosol. (b) Caspase-9 processing was induced by 60 μM apigenin, and HL-60 cells were treated with different flavonoids (60 μM) for 12 h, then analysed by Western blotting. M, myricetin; Q, quercetin; K, kaempferol.

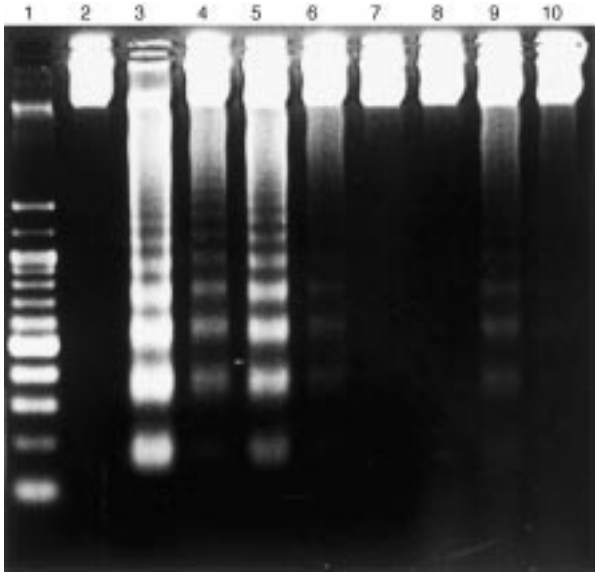

Figure 9. Induction of DNA fragmentation in HL-60 cells by genistein, ECG, EGC, and EGCG. DNA fragmentation was induced by genistein, ECG, EGC and EGCG. Lane 1, DNA ladder marker; 2, control; 3, apigenin; 4, myricetin; 5, quercetin; 6, kaempferol; 7, genistein; 8, ECG; 9, EGC; 10, EGCG.

The data suggest that the potency of induction of apoptosis is attenuated by the substitution of a 3-hydroxyl group.

DISCUSSION

Flavonoids are substantial components of the human diet. They have been suggested to inhibit growth of tumour cells [23-25] and to be anticarcinogens [3]. Recent studies have shown that quercetin induces apoptosis in various tumour cells including K562, Molt-4, Raji and MCAS. The effect was also observed with other tumour cell lines from gastric, colon and lung carcinomas [26]. In addition, apigenin also inhibited tumour growth through cell cycle arrest and induced apoptosis through a p53-dependent mechanism [27]. In the present study, we also show that structurally related flavonoids can induce apoptosis in HL-60 cells. However, to date, the mechanism of apoptosis is unclear. Thus, we investigated the mechanism of apoptosis and the role of the caspase cascade in flavonoid-induced HL-60 cell apoptosis.

This induction of apoptosis occurred within several hours, consistent with the view that flavonoids induce apoptosis by activating the pre-existing apoptosis machinery. Indeed, treatment with flavonoids caused an induction of caspase-3 activity and degradation of PARP, which precedes the onset of apoptosis. Pretreating cells with the caspase-3 inhibitor, Z-DEVD-FMK, inhibited flavonoid-induced caspase-3 activation and DNA fragmentation. However, we were unable to detect any significant changes in the activity of caspase-1 during flavonoid-induced apoptosis, and a specific caspase-1 inhibitor, AC-YVAD-CMK, had little effect on flavonoid-stimulated caspase-3 activation and DNA fragmentation. These results suggest that apoptosis induced by flavonoids involves a caspase-3-mediated mechanism. Questions remain as to how caspase-3 is activated by flavonoids. This raises the possibility that factors or proteases other than caspase-1 are involved in the activation of caspase-3. In fact, *in vitro* studies have previously identified Apaf1, cytochrome c, and caspase-9 as participants in a complex important for caspase-3 activation. *In vitro* depletion of caspase-9 from cytosolic fractions resulted in the failure of caspase-3 activation [21]. Together, these data suggest a linear and specific activation cascade between caspase-9 and caspase-3 in response to cytochrome c release from mitochondria. Release of cytochrome c from mitochondria has been shown to be an almost universal phenomenon during apoptosis, while it is unclear whether the cytochrome c-mediated caspase cascade is triggered only by a few apoptotic stimuli or serves as a general amplification mechanism to accelerate cell death [28]. It has been reported that cytochrome c released from mitochondria can precede dissipation of the voltage gradient (mitochondrial transmembrane potential; $\Delta\psi_m$) across the inner membrane arguing in favour of the specific channel hypothesis, suggesting that the escape of cytochrome c from mitochondria occurs prior to permeability transition pore opening (loss of mitochondrial transmembrane potential) [18, 19]. In this study, we found that flavonoids induce cytochrome c release, cleavage of procaspase-9, and promotion of ROS generation. The increase in ROS was probably due to the affected mitochondria cycling di-oxygen through the electron transport assembly and generating ROS by one electron transfer.

Flavonoids may be considered to be potential compounds for selectively blocking signal transduction pathways. Most of these effects are directed towards the ATP-binding site of the kinase, although other as yet unknown mechanisms may exist [29]. Several recent studies have demonstrated that, depending on their structure, flavonoids may be potent inhibitors of several kinases involved in signal transduction, mainly protein kinase c [30] and tyrosine kinases [31–33]. These studies have shown that the position and number of the hydroxyl group on the 2-phenyl ring strongly influences the conformation of the molecule and modulates their inhibitory effect. As previously shown [33], the introduction of hydroxyl groups on the flavone ring increases the inhibitory potency of the PI3-kinase of flavonoids, the polyhydroxylated flavonol, myricetin, being by far the most effective. The replacement of hydroxyl groups with methoxyl substituents yielded much

weaker inhibitors (diosmetin), suggesting the importance of hydrogen bonds between flavonoids and the kinase. Genistein, daidzein and genistin induced differentiation of mouse erythroleukaemia cells (MEL). Biochanin A and apigenin had no differentiation-inducing effect. These results suggest that the flavonoid structure and the 4′-hydroxyl group on the 3-phenyl ring is essential for the differentiation induction effect [34]. Quercetin has been shown to have good radioprotective effects in γ-ray-irradiated mice, but quercetin tetramethyl-ether, which has methoxyl groups at the 3,7,3′,4′-position, showed poor activity. These also suggest that hydroxyl number is important in flavonoid radioprotective effects [35].

In our study, we demonstrated that flavonoids induce apoptosis in cultured human promyelocytic leukaemia HL-60 cells. The induction of apoptosis may involve the loss of mitochondrial transmembrane potential, cytochrome c release, and caspase-9 and caspase-3 activation. The potency of these flavonoids towards these apoptotic features was: apigenin>quercetin>myricetin>kaempferol in HL-60 cells treated with 60 μM flavonoids. Interestingly, the ability of these flavonoids to activate caspase-3 activity was: apigenin>myricetin>quercetin>kaempferol in HL-60 cells treated with higher flavonoid concentrations (Figure 4a). This finding offers a possibility that flavonoid-induced caspase-3 activation may be dependent on the number of hydroxyl groups in the 2-phenyl group and the existence of the 3-hydroxyl group. Briefly, the 3-hydroxyl group may inhibit the capacity of inducing apoptosis because the potency was: apigenin>kaempferol. The greater the number of hydroxyl groups in the 2-phenyl group, the greater the potency in recovering the ability to induce apoptosis that is inhibited by the existing 3-hydroxyl group. In addition, comparing the analogous compounds, genistein, ECG, EGC and EGCG in Figure 9, we found that only the compound EGC which has a 3-hydroxyl group can induce apoptosis, but with a potency lower than that of apigenin. Other compounds which have functional groups replacing the 3-hydroxyl group will have their ability to induce apoptosis abolished. This confirms the importance of the 3-hydroxyl group in the inhibition of apoptosis again, which agrees with our hypothesis proposed above. A previous study [34] showed that the potency of these flavonoids toward cell growth inhibition was: apigenin>genistein>genistin>biochanin A>daidzein. These structural and functional results are similar to our data related to apoptosis. These provide new information for the design of cancer chemopreventive agents and the study of these functional groups in the future.

1. Dunnick JK, Hailey JR. Toxicity and carcinogenicity studies of quercetin, a natural component of foods. *Fund Appl Toxicol* 1992, **19**, 423–431.
2. Fiala ES, Reddy BS, Weisburger JH. Naturally occurring anti-carcinogenic substances in foodstuffs. *Ann Rev Nutrit* 1985, **5**, 295–321.
3. Hollman PC, Van Trijp JM, Buysman MN, *et al.* Relative bioavailability of the antioxidant flavonoid quercetin from various foods in man. *FEBS Lett* 1997, **418**, 152–156.
4. Pratt CB, Relling MV, Meyer WH, Douglass EC, Kellie SJ, Avery L, Phase I. study of flavone acetic acid (NSC 347512, LM975) in patients with pediatric malignant solid tumors. *Am J Clin Oncol* 1991, **14**, 483–486.
5. Huang YT, Kuo ML, Liu JY, Huang SY, Lin JK. Inhibitions of protein kinase c and proto-oncogene expressions in NIH 3T3 cells by apigenin. *Eur J Cancer* 1996, **32A**, 146–151.
6. Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* 1972, **26**, 239–257.
7. McGowan AJ, Fernandes RS, Verhaegen S, Cotter TG. Zinc inhibits UV radiation-induced apoptosis but fails to prevent subsequent cell death. *Int J Radiat Biol* 1994, **66**, 343–349.
8. Strand S, Hofmann WJ, Hug H, *et al.* Lymphocyte apoptosis induced by CD95 (APO-1/Fas) ligand-expressing tumour cells—a mechanism of immune evasion? *Nature Med* 1996, **2**, 1361–1366.
9. Enari M, Hase A, Nagata S. Apoptosis by a cytosolic extract from Fas-activated cells. *EMBO J* 1995, **14**, 5201–5208.
10. Dive C, Hickman JA. Drug–target interactions: only the first step in the commitment to a programmed cell death? *Br J Cancer* 1991, **64**, 192–196.
11. Gunji H, Kharbanda S, Kufe D. Induction of internucleosomal DNA fragmentation in human myeloid leukemia cells by 1-beta-D-arabinofuranosylcytosine. *Cancer Res* 1991, **51**, 741–743.
12. Cotter TG, Glynn JM, Echeverri F, Green DR. The induction of apoptosis by chemotherapeutic agents occurs in all phases of the cell cycle. *Anticancer Res* 1992, **12**, 773–779.
13. Martin SJ, Green DR. Protease activation during apoptosis: death by a thousand cuts? *Cell* 1995, **82**, 349–352.
14. Nagata S. Apoptosis by death factor. *Cell* 1997, **88**, 355–365.
15. Takahashi A, Alnemri ES, Lazebnik YA, *et al.* Cleavage of lamin A by Mch2 alpha but not CPP32: multiple interleukin 1 beta-converting enzyme-related proteases with distinct substrate recognition properties are active in apoptosis. *Proc Natl Acad Sci USA* 1996, **93**, 8395–8400.
16. Faucheu C, Diu A, Chan AW, *et al.* A novel human protease similar to the interleukin-1 beta converting enzyme induces apoptosis in transfected cells. *EMBO J* 1995, **14**, 1914–1922.
17. Tewari M, Quan LT, O'Rourke K, *et al.* Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. *Cell* 1995, **81**, 801–809.
18. Yang J, Liu X, Bhalla K, *et al.* Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. *Science* 1997, **275**, 1129–1132.
19. Kluck RM, Bossy-Wetzel E, Green DR, Newmeyer DD. The release of cytochrome c from mitochondria: a primary site for bcl-2 regulation of apoptosis. *Science* 1997, **275**, 1132–1136.
20. Hofmann K, Bucher P, Tschopp J, The CARD. domain: a new apoptotic signaling motif. *Trends Biochem Sci* 1997, **22**, 155–156.
21. Li P, Nijhawan D, Budihardjo I, *et al.* Cytochrome c and dATP-dependent formation of Apaf-1/Caspase-9 complex initiates an apoptotic protease cascade. *Cell* 1997, **91**, 479–489.
22. Lin YL, Juan IM, Chen YL, Liang YC, Lin JK. Composition of polyphenols in fresh tea leaves and associations of their oxygen-radical-absorbing capacity with antiproliferative actions in fibroblast cells. *J Agric Food Chem* 1996, **44**, 1387–1394.
23. Csokay B, Prajda N, Weber G, Olah E. Molecular mechanisms in the antiproliferative action of quercetin. *Life Sci* 1997, **60**, 2157–2163.
24. Lepley DM, Pelling JC. Induction of p21/WAF1 and G1 cell-cycle arrest by the chemopreventive agent apigenin. *Molec Carcinogen* 1997, **19**, 74–82.
25. Kang TB, Liang NC. Studies on the inhibitory effects of quercetin on the growth of HL-60 leukemia cells. *Biochem Pharmacol* 1997, **54**, 1013–1018.
26. Wei YQ, Zhao X, Kariya Y, Fukata H, Teshigawara K, Uchida A. Induction of apoptosis by quercetin: involvement of heat shock protein. *Cancer Res* 1994, **54**, 4952–4957.
27. Plaumann B, Fritsche M, Rimpler H, Brandner G, Hess RD. Flavonoids activate wild-type p53. *Oncogene* 1996, **13**, 1605–1614.
28. Reed JC. Cytochrome c: can't live with it—can't without it. *Cell* 1997, **91**, 559–562.
29. Middleton E Jr, Kandaswami C. The impact of plant flavonoids on mammalian biology: implications for immunity, inflammation and cancer. In Harborne TH, Liss AR, eds. *Flavonoids: Advances in Research Since 1986*. New York, Chapman and Hall 1994, 619–625.
30. Ferriola PC, Cody V, Middleton E. Protein kinase C inhibition by plant flavonoids. Kinetic mechanisms and structure-activity relationships. *Biochem Pharmacol* 1989, **38**, 1617–1624.

31. Geahlen RL, Koonchanok NM, McLaughlin JL, Pratt DE. Inhibition of protein-tyrosine kinase activity by flavanoids and related compounds. *J Natl Products* 1989, **52**, 982–986.
32. Cushman M, Nagarathnam D, Burg DL, Geahlen RL. Synthesis and protein-tyrosine kinase inhibitory activities of flavonoid analogues. *J Med Chem* 1991, **34**, 798–806.
33. Agullo G, Gamet-Payrastre L, Manenti S, Viala C, Remesy C, Chap H, Payrastre B. Relationship between flavonoid structure and inhibition of phosphatidylinositol 3-kinase: a comparison with tyrosine kinase and protein kinase C inhibition. *Biochem Pharmacol* 1997, **53**, 1649–1657.
34. Jing Y, Waxman S. Structural requirements for differentiation-induction and growth-inhibition of mouse erythroleukemia cells by isoflavones. *Anticancer Res* 1995, **15**, 1147–1152.
35. Shimoi K, Masuda S, Furugori M, Esaki S, Kinae N. Radioprotective effect of antioxidative flavonoids in gamma-ray irradiated mice. *Carcinogenesis* 1994, **15**, 2669–2672.

Acknowledgements—This study was supported by grants from the National Science Council (NSC 88-2316-B-002-015 and NSC 88-EPA-Z-002-021) and from the National Health Research Institute (DOH-HR-403).