FEBS 23853 FEBS Letters 476 (2000) 213–217

Flavones and flavonols at dietary levels inhibit a transformation of aryl hydrocarbon receptor induced by dioxin

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Received 20 April 2000

Edited by Pierre Jolles

Abstract Dioxins invade the body mainly through the diet, and produce toxicity through the transformation of aryl hydrocarbon receptor (AhR). An inhibitor of the transformation should therefore protect against the toxicity and ideally be part of the diet. We examined flavonoids ubiquitously expressed in plant foods as one of the best candidates, and found that the subclasses flavones and flavonols suppressed antagonistically the transformation of AhR induced by 1 nM of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, without exhibiting agonistic effects that transform AhR. The antagonistic IC_{50} values ranged from 0.14 to 10 μM, close to the physiological levels in human.

Key words: Flavonoid; Dioxin antagonist; Aryl hydrocarbon receptor; Transformation inhibitor; Cancer prevention; Dietary phytochemical

1. Introduction

Dietary exposure to the environmental pollutants dioxins is a serious health concern. Dioxins transform the cytosolic aryl hydrocarbon receptor (AhR), resulting in its translocation into the nucleus and interaction with a specific DNA element known as the dioxin-responsible element (DRE) [1,2]. Subsequently, this induces the gene expression of several carcinogenesis-related enzymes, and is associated with teratogenesis, tumor promotion, immunotoxicity and epithelial hyperplasia [3–6]. As the transformation of AhR is a primary and key step in the development of dioxin toxicity, an inhibitor of the transformation would protect against the toxicity. The inhibitors should ideally be factors in our daily diet, because dioxins enter the body mainly through the diet. However, the only inhibitors identified to date are synthetic drugs [7–9].

AhR has been found to favor compounds that are hydrophobic and have van der Waals dimensions of 14×12×5 Å [1,2,8]. Flavonoids have these chemical characteristics, and are abundant in plant foods [10,11]. The subclasses flavones and flavonols are usually ingested from vegetables, fruits, teas and red wine, and flavanones, isoflavones and catechins from citrus fruits, beans and teas, respectively [12,13]. The daily intake is estimated to be 23 mg per capita in The Netherlands, 170 mg in the USA and 70 mg in Japan [14]. In addition, recent information suggests that several flavonoids act as inhibitors [15]. Therefore, we considered the flavonoid a good dietary candidate for preventing dioxin toxicity. The purpose of this study was to investigate the inhibitory IC_{50} values of flavonoids for the transformation of AhR induced by 1 nM of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), and thereby to understand which flavonoids are available as inhibitors by comparing their IC_{50} values to the physiological levels.

2. Materials and methods

2.1. Materials

The flavonoids apigenin and hesperetin were obtained from Sigma (St. Louis, MO). Baicalein, daidzein, eriodictyol, galangin, isorhamnetin, kaempferol, luteolin, luteolin-7,3′-*O*-bis-glucoside, tamarixetin, and tangeretin were from Extrasynthèse (Genay, France). Chrysin and myricetin were from Aldrich (Tokyo, Japan). Fisetin, flavanone, genistein, quercetin, and rutin were from Wako Pure Chemical (Tokyo, Japan). Flavone, morin, naringenin, and quercitrin were from Nacalai Tesque (Kyoto, Japan). Flavonol and naringin were from Tokyo Kasei Kogyo (Tokyo, Japan). Puerarin was from Funakoshi (Tokyo, Japan). The catechins (+)-catechin, (−)-gallocatechin, (−)-catechin gallate, (−)-gallocatechin gallate, (−)-epicatechin, (−)-epigallocatechin, (−)-epicatechin gallate, and (−)-epigallocatechin gallate were purchased from Kurita Kogyo (Tokyo, Japan). The purity of each chemical was confirmed by a nuclear magnetic resonance spectral analysis with a Bruker AC-250 (Bruker Analytik GmbH). α-Naphthoflavone, β-naphthoflavone and 4,7-phenanthroline were from Aldrich. Ellipticine was obtained from Sigma. TCDD and 20-methylcholanthrene (MC) were purchased from Accu Standard (New Haven, CT, USA) and Nacalai Tesque, respectively. All other reagents used were of the highest grade available from commercial source.

2.2. Preparation of rat hepatic cytosol

Rat hepatic cytosol was used as a source of AhR to determine the transformation in a cell-free system. Livers from male Sprague–Dawley rats (6 weeks old, 140–170 g) (Japan SLC; Shizuoka, Japan) were perfused with ice-cold phosphate-buffered saline and homogenized in a double volume of HEDG buffer (25 mM HEPES pH 7.4, 1.5 mM EDTA, 1.0 mM dithiothreitol, 10% glycerol). The homogenate was centrifuged at 105 000×*g* for 70 min, and the supernatant was referred to as cytosol.

2.3. Evaluation of the AhR transformation

The cytosol (4.0 mg protein/ml) was incubated with TCDD or MC in dimethylsulfoxide (DMSO) in HEDG buffer at 20°C for 2 h, and with DMSO (10 μl/ml) alone as a control. In the antagonism test, flavonoid or catechin at various concentrations was added to the mixture 10 min prior to the incubation.

Transformation of AhR in the cytosol was evaluated by a gel mobility shift assay [16], using a prepared oligonucleotide for DRE. The DRE probe corresponded to the 26-bp AhR binding site [3]: 5′-GAT CTG GCT CTT CTC ACG CAA CTC CG-3′ (coding) and 5′-GAT CCG GAG TTG CGT GAG AAG AGC CA-3′ (non-coding). The prepared double-strand oligonucleotide was 5′-end labeled with T4-polynucleotide kinase (Takara Biomedicals, Kusatsu, Japan) and [γ-^{32}P]ATP. The reaction mixture for the binding of AhR/DRE consisted of 20 μg of cytosolic protein, 200 ng of poly[dI-dC] (Boehringer Mannheim GmbH, Germany) and ^{32}P-labeled DRE probes (100 kcpm, 20 fmol) in a final volume of 25 μl of HEDG buffer containing 150 mM KCl. The mixture was loaded on 4% non-stacking native polyacrylamide gels containing TAE buffer (6.7 mM Tris–HCl, pH

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0014-5793/00/$20.00
PII: S0014-5793(00)01730-0

7.5, 3.3 mM sodium acetate, 1 mM EDTA), and electrophoresed in the same buffer at 135 V for 30 min before loading and for 150 min after loading. The AhR/DRE complex was identified by competition assay with a 50-fold excess of non-labeled DRE probe and determined by autoradiography with a Digital Imaging System Is-1000 (Alpha Innotech).

3. Results

3.1. Antagonism of flavonoid on AhR transformation

On incubating AhR with 1 nM TCDD, effects of flavonoid on the complex of AhR/DRE were determined by gel mobility shift assay. Fig. 1A shows that additions of flavonoid prior to TCDD suppressed the formation of the AhR/DRE complex while additions after AhR had been transformed by TCDD were ineffective against the suppression. Thus, flavonoids inhibited the transformation of AhR competing with the agonist TCDD, i.e. antagonistically. Fig. 1B,C shows typical results for the antagonistic action of flavonoid. Quercetin suppressed TCDD-induced transformation dose-dependently (Fig. 1B). Luteolin similarly suppressed the transformation induced by 5 nM of MC (Fig. 1C), which is another strong agonist [17].

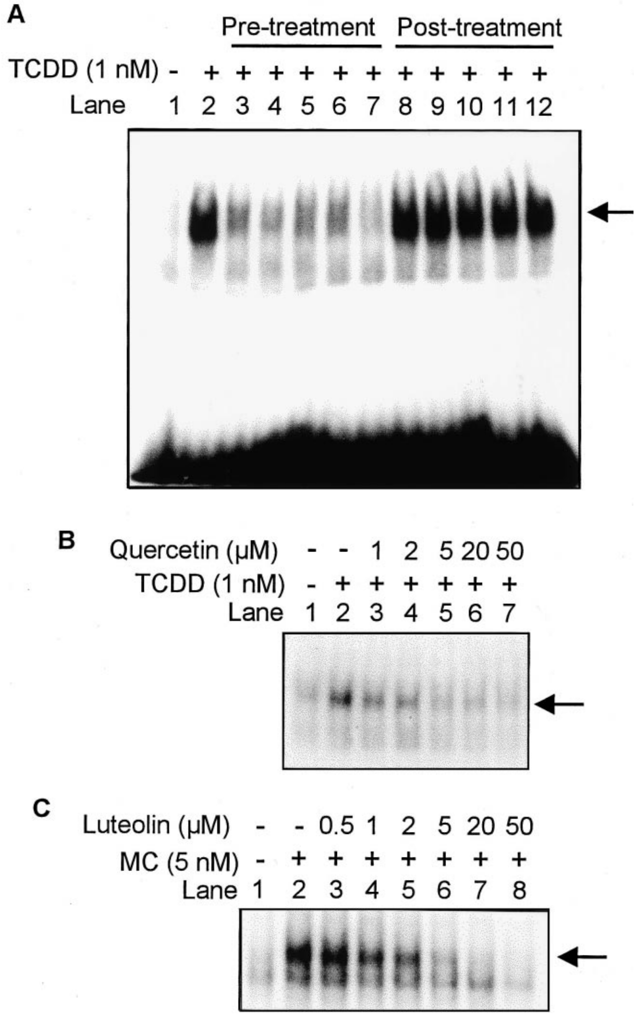


Fig. 1. Inhibitory effect of flavonoids on TCDD- or MC-induced transformation of AhR. A: A typical gel retardation assay for the effect of flavones and flavonols. Arrows indicate AhR/DRE complex. Rat hepatic cytosol was incubated with 5 μM flavonoids before (pre-treatment, lanes 3–6) or after (post-treatment, lanes 7–10) the transformation of AhR by 1 nM TCDD. Lane 1, the vehicle DMSO alone; 2, 1 nM TCDD alone; 3 and 8, flavone; 4 and 9, chrysin; 5 and 10, flavonol; 6 and 11, galangin; 7 and 12, flavanone. B: Dose-dependent effect of flavonoids on transformation. Quercetin was added to the cytosol at a concentration of 0–20 μM before the treatment with 1 nM TCDD. C: Inhibitory effect of flavonoids on the MC-induced system. Luteolin was added to the cytosol at 0–50 μM before treatment with 5 nM MC.

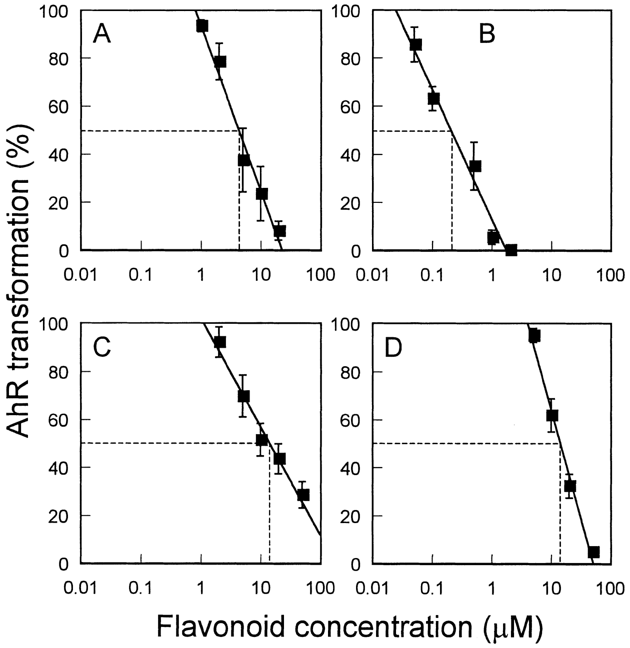


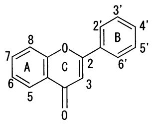
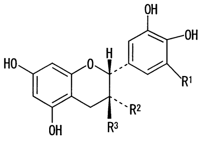
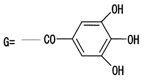
Fig. 2. Determination of IC_{50} values of flavonoids for suppressing the AhR transformation. Rat hepatic cytosol was incubated with various concentrations of flavonoids: tangeretin (A), galangin (B), eriodictyol (C), and rutin (D), before being treated with 1 nM TCDD, and the transformation of AhR was determined as mentioned in Fig. 1. The ratio of transformation was compared to those of both a positive control (1 nM TCDD) and a negative one (vehicle alone) and plotted as % of transformation against a log of the dose concentration. The determination was carried out three times independently, and then the amount requiring 50% suppression was calculated.

3.2. Antagonistic IC_{50} and agonistic ED_{50} values of flavonoids

We determined the IC_{50} values by plotting a log of the dose concentration against the ratio of transformation. As shown in Fig. 2, using both a positive control (1 nM TCDD, 100%) and a negative one (vehicle alone, 0%) at each time, the suppressing activity of flavonoid was plotted as % of transformation from independent triplicate assays. On the other hand, some flavonoids have been reported to exhibit agonistic action and produce toxicity [15,18]. Therefore, we also determined the agonistic ED_{50} values.

Table 1 lists the IC_{50} values for 34 natural flavonoids and four known antagonistic drugs, along with the agonistic effects for comparison. Almost all of the natural flavonoids showed antagonism without agonism. Flavone and tamarixetin had agonistic effects, but the ED_{50} values were higher by one or two orders of magnitude than the corresponding antagonistic IC_{50} values. Chrysin, flavonol, quercetin, flavanone, and daidzein at 100 μM had a weak agonistic effect. This means that natural flavonoids at low concentrations inhibit the dioxin action and are not in themselves toxic.

Table 1
Effects of flavonoids on the transformation of AhR[a]

Chemical (position of substituent)	Antagonistic IC_{50} (μM) against		Agonistic ED_{50} (μM or maximum % when added at 100 μM)[b]
	1 nM TCDD	5 nM MC	
			
Flavones			
Flavone (none)	0.14	0.62	23
Chrysin (5,7-OH)	4.2	3.8	weak (32 ± 6)
Baicalein (5,6,7-OH)	4.3	5.3	no effect
Apigenin (5,7,4′-OH)	3.2	7.1	no effect
Luteolin (5,7,3′,4′-OH)	6.5	8.4	no effect
Tangeretin (5,6,7,8,3′-OCH_3)	9.0	8.2	no effect
Luteolin-7,3′-*O*-bis-glucoside	28	–	no effect
Flavonols			
Flavonol (3-OH)	0.42	1.2	weak (33 ± 3)
Galangin (3,5,7-OH)	0.22	1.3	no effect
Kaempferol (3,5,7,4′-OH)	2.1	9.0	no effect
Fisetin (3,7,3′4′-OH)	5.5	–	no effect
Morin (3,5,7,2′,4′-OH)	7.4	> 50	no effect
Quercetin (3,5,7,3′,4′-OH)	1.5	10	weak (15 ± 2)
Myricetin (3,5,7,3′,4′,5′-OH)	7.6	–	no effect
Tamarixetin (3,5,7,3′-OH, 4′-OCH_3)	2.4	–	89
Isorhamnetin (3,5,7,4′-OH, 3′- OCH_3)	10	–	no effect
Quercetin-3-*O*-rhamnoside (quercitrin)	3.7	35	no effect
Quercetin-3-*O*-rutinoside (rutin)	13	> 50	no effect
Flavanones (2–3 is saturated)			
Flavanone (none)	0.65	–	weak (12 ± 2)
Naringenin (5,7,4′-OH)	6.7	–	no effect
Eriodictyol (5,7,3′,4′-OH)	11	–	no effect
Hesperetin (5,7,3′-OH, 4′-OCH_3)	14	–	no effect
Naringenin-7-*O*-rutinoside (naringin)	25	–	no effect
Isoflavones (B-ring binds to 3-position)			
Daidzein (7,4′-OH)	> 50	> 50	weak (34 ± 1)
Genistein (5,7,4′-OH)	> 50	> 50	no effect
Daidzein-8-glucoside (puerarin)	> 50	–	no effect
			
Catechins			
(+)-Catechin ($R^1 = H$, $R^2 = H$, $R^3 = OH$)	> 200	–	no effect
(−)-Gallocatechin ($R^1 = OH$, $R^2 = H$, $R^3 = OH$)	> 200	–	no effect
(−)-Catechin gallate ($R^1 = H$, $R^2 = H$, $R^3 = OG$)	150	–	no effect
(−)-Gallocatechin gallate ($R^1 = OH$, $R^2 = H$, $R^3 = OG$)	39	–	no effect
(−)-Epicatechin ($R^1 = H$, $R^2 = OH$, $R^3 = H$)	> 200	–	no effect
(−)-Epigallocatechin ($R^1 = OH$ $R^2 = OH$, $R^3 = H$)	> 200	–	no effect
(−)-Epicatechin gallate ($R^1 = H$, $R^2 = OG$, $R^3 = H$)	81	–	no effect
(−)-Epigallocatechin gallate ($R^1 = OH$, $R^2 = OG$, $R^3 = H$)	35	–	no effect
Known antagonistic and agonistic drugs			
7,8-Benzoflavone (α-naphthoflavone)	0.39	–	31
5,6-Benzoflavone (β-naphthoflavone)	no effect	–	2.7
4,7-Phenanthroline	6.8	–	no effect
5,11-Dimethyl-6*H*-pyrido[3,4-*b*]carbazole (ellipticine)	0.013	–	no effect

[a] The amounts of AhR/DRE complex were determined as a percentage of control incubation without flavonoids, and plotted vs. flavonoid concentrations to calculate the IC_{50} values.
[b] 'No effect' is up to 100 μM.

3.3. Relation between the structure and the antagonistic activity

In antagonistic action, flavones, flavonols and flavanones showed low IC_{50} values, and non-polar or less polar molecules were stronger. Subsequently, flavone, flavonol, galangin, and flavanone gave values similar to that of a known antagonistic drug, α-naphthoflavone. The results indicated that hydrophobicity and molecular size were important for AhR. AhR also favors a stereo coplanar structure like the strongest agonist

Table 2
Changes in the IC_{50} values of flavonoid with TCDD concentration

Flavonoid	IC_{50} value against TCDD	
	0.2 nM	5 nM
Apigenin	2.4	6.3
Luteolin	0.83	16
Kaempferol	0.54	20
Quercetin	0.50	18
Rutin	11	> 100

TCDD [1,2,18]. Flavones and flavonols have a plane benzopyranone skeleton, and flavanones a less plane dihydrobenzopyranone skeleton, but catechins are not plane benzodihydropyrans. Subsequently, in antagonistic action the flavonoids ranked in the order of flavones and flavonols > flavanones > catechins. Isoflavones having almost no effect differed markedly in stereo structure from the above flavonoids in that the B-ring was bound to the 3-position.

Luteolin-7, 3′-*O*-bis-glucoside, naringenin-7-*O*-rutinoside and isorhamnetin were weaker than the corresponding aglycones (skeleton without sugar), though a 3-substitute such as quercetin-3-*O*-rhamnoside was not. These results reveal a region of the flavonoid with affinity for the hydrophobic pocket of AhR comprising 7- and 8-carbons, 1-pyran and a B-ring, and this was consistent with a finding for a cytochrome P450 (CYP) 1A1 [19]. CYP 1A1 is one of the enzymes that are expressed through AhR and metabolize hydrophobic xenobiotics [3–5]. The similarity between the hydrophobic pockets of AhR and CYP is purposive.

3.4. Changes in the IC_{50} values with TCDD level

The dioxin level in the body is usually much less that the 1 nM in Table 1 [1,2]. Therefore, we determined the changes in the IC_{50} values with TCDD concentration in several flavonoids (Table 2). This, together with the results at 1 nM TCDD in Table 1, indicated that the antagonistic action of flavones and flavonols depended on the concentration of agonistic TCDD. Thus, flavonoid was available at lower levels against a smaller amount of TCDD.

4. Discussion

The present study demonstrates that flavonoids, especially flavones and flavonols in aglycones, inhibit the AhR transformation antagonistically, indicating that they protect against dioxin toxicity. Therefore, an important question is whether flavonoids are present in the body at antagonistic levels and reach agonistic levels. The physiological concentrations of dietary flavonoids have been established by several excellent studies.

In absorption experiments in volunteers, onion quercetin, pure rutin and pure aglycone quercetin have been reported to be absorbed into the body at 52, 17 and 24% of the intake amount, respectively [20,21]. The levels in healthy individuals are 0.72–0.76 μM for rutin and 0.5–1.6 μM for other quercetin glycosides in the blood plasma [22]. Hollman and coworkers have examined the changes in physiological levels with a single dose of flavonoid after washing out the internal flavonoid [20,21]. Fried onion equivalent to 68 mg (225 μmol) quercetin gives 0.74 μM in plasma as a peak at 0.7–7 h after the ingestion, and pure rutin 0.3 μM. The disposition has an elimination half-life of about 25 h. The authors concluded that the levels reflect the amount ingested and that repeated intake of quercetin throughout the day would lead to a build-up in the plasma. Indeed, in another experiment by Conquer et al. [23], a capsule containing free quercetin raised the concentration to 1.5 μM after 28 days at a dose of 1 g/day. Quercetin and its glycosides bind to albumin and are carried in the blood stream to the liver, where they are partly metabolized to isorhamnetin and glucurono-sulfated conjugates before being excreted in the urine [24,25]. The other flavonoids have a similar fate. Kaempferol is absorbed better or metabolized to a lesser extent than quercetin [21]. A daily supplement of 500 g of broccoli (providing 12.5 mg of kaempferol and 9 mg of quercetin) for 12 days resulted in small amounts in the urine as conjugates of the non-metabolized aglycone (52–78 ng kaempferol/ml urine) [26]. An oral dose of 500 mg of pure naringin gives naringin, naringenin and naringenin glucuronides in the urine [27]. A capsule of 525 mg of epigallocatechin gallate (roughly equivalent to six cups of green tea) increases the plasma levels to 4.41 μM after 90 min as the free form but not the conjugates [28].

Thus, aglycones and glycosides from the diet are excreted into urine as free aglycones and conjugates thereof. Since the conjugations occur in the intestines and liver [24,25], the dietary flavonoids exist as free aglycones at least temporarily in these tissues, which are major routes for dietary dioxins [1,2]. The physiological levels indicate that the dietary flavones and flavonols can build up to near antagonistic levels and on repeated ingestion reach close to the IC_{50} value, i.e. 1.5 μM for quercetin (Table 1), but do not approach agonistic levels such as 100 μM for quercetin. In addition, the dioxin level is usually pM in the body [1,2], much less that the 1 nM in Table 1. Towards the lower concentration of TCDD, flavones and flavonols acted as antagonists at lower concentrations (Table 2). Probably, the intake from a plant-based diet is enough to inhibit AhR transformation by TCDD. The present results obtained from experiments in a cell-free system suggest strongly that the dietary flavones and flavonols protect against the toxicity of the environmental contaminant dioxin, because the transformation of AhR has been recognized to be a primary and key step in the development of dioxin toxicity [3–6].

References

[1] Poland, A. and Knutson, J.C. (1982) Annu. Rev. Pharmacol. Toxicol. 22, 517–554.

[2] Safe, S.H. (1986) Annu. Rev. Pharmacol. Toxicol. 26, 371–399.

[3] Denison, M.S., Fisher, J.M. and Whitlock Jr., J.P. (1989) J. Biol. Chem. 264, 16478–16482.

[4] Landers, J.P. and Bunce, N.J. (1991) Biochem. J. 276, 273–287.

[5] Quattrochi, L.C., Vu, T. and Tukey, R.H. (1994) J. Biol. Chem. 269, 6949–6954.

[6] Fernandez-Salguero, P., Pineau, T., Hilbert, D.M., McPhail, T., Lee, S.S.T., Kimura, S., Nebert, D.W., Rudikoff, S., Ward, J.M. and Gonzalez, F.J. (1995) Science 268, 722–726.

[7] Lu, Y.-F., Santostefano, M., Cunningham, B.D.M., Threadgill, M.D. and Safe, S. (1996) Biochem. Pharmacol. 51, 1077–1087.

[8] Henry, E.C., Kende, A.S., Rucci, G., Totleben, M.J., Willey, J.J., Dertinger, S.D., Pollenz, R.S., Jones, J.P. and Gasiewicz, T.A. (1999) Mol. Pharmacol. 55, 716–725.

[9] Casper, R.F., Quesne, M., Rogers, I.M., Shirota, T., Jolivet, A., Milgrom, E. and Savouret, J.-F. (1999) Mol. Pharmacol. 56, 784–790.

[10] Herrmann, K. (1976) J. Food Technol. 11, 433–448.

[11] Wollenweber, E. and Dietz, V.H. (1981) Phytochemistry 20, 869–932.

[12] Hertog, M.G.L., Hollman, P.C.H. and Katan, M.B. (1992) J. Agric. Food Chem. 40, 2379–2383.

[13] Hertog, M.G.L., Hollman, P.C.H. and van de Putte, B. (1993) J. Agric. Food Chem. 41, 1242–1246.

[14] Cook, N.C. and Samman, S. (1996) J. Nutr. Biochem. 7, 66–76.

[15] Ciolino, H.P., Daschner, P.J. and Yeh, G.C. (1999) Biochem. J. 340, 715–722.

[16] Singh, H., Sen, R., Baltimore, D. and Sharp, P.A. (1986) Nature 319, 154–158.

[17] Riddick, D.S., Huang, Y., Harper, P.A. and Okey, A.B. (1994) J. Biol. Chem. 269, 12118–12128.

[18] Gasiewicz, T.A., Kende, A.S., Rucci, G., Whitney, B. and Willey, J.J. (1996) Biochem. Pharmacol. 52, 1787–1803.

[19] Kanazawa, K., Ashida, H. and Danno, G. (1998) in: Functional Foods for Disease Prevention II: Medicinal Plants and Other Foods (Osawa, T., Shibamoto, T. and Terao, J., Eds.), ACS Symposium Series 702, pp. 67–82, Am. Chem. Soc., Washington, DC.

[20] Hollman, P.C.H. and Katan, M.B. (1997) Biomed. Pharmacother. 51, 305–310.

[21] de Vries, J.H.M., Hollman, P.C.H., Meyboom, S., Buysman, M.N.C.P., Zock, P.L., van Staveren, W.A. and Katan, M.B. (1998) Am. J. Clin. Nutr. 68, 60–65.

[22] Paganga, G. and Rice-Evans, C.A. (1997) FEBS Lett. 401, 78–82.

[23] Conquer, J.A., Maiani, G., Azzini, E., Raguzzini, A. and Holub, B.J. (1998) J. Nutr. 128, 593–597.

[24] Zhu, B.T., Ezell, E.L. and Liehr, J.G. (1994) J. Biol. Chem. 269, 292–299.

[25] Manach, C., Morand, C., Crespy, V., Demigné, C., Texier, O., Régérat, F. and Rémésy, C. (1998) FEBS Lett. 426, 331–336.

[26] Nielsen, S.E., Kall, M., Justesen, U., Schou, A. and Dragsted, L.O. (1997) Cancer Lett. 114, 173–174.

[27] Ishii, K., Furuta, T. and Kasuya, Y. (1997) J. Chromatogr. 704, 299–305.

[28] Nakagawa, K., Okuda, S. and Miyazawa, T. (1997) Biosci. Biotechnol. Biochem. 61, 1981–1985.