Mutagenesis vol.12 no.5 pp.383–390, 1997

Involvement of rat cytochrome 1A1 in the biotransformation of kaempferol to quercetin: relevance to the genotoxicity of kaempferol

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Kaempferol is a flavonoid widely distributed in edible plants and has been shown to be genotoxic to V79 cells in the absence of external metabolizing systems. The presence of an external metabolizing system, such as rat liver homogenates (S9 mix), leads to an increase in its genotoxicity, which is attributed to its biotransformation to the more genotoxic flavonoid quercetin, via the cytochrome P450 (CYP) mono-oxygenase system. In the present work we investigated the mechanisms of the genotoxicity of kaempferol further. Special attention has been given to the role of CYP in the genotoxicity of this flavonoid. We studied the induction of mutations in *Salmonella typhimurium* TA98 in the presence and in the absence of S9 mix and the induction of chromosomal aberrations (CAs) and micronuclei (MN) by kaempferol in V79 cells in the presence and in the absence of S9 mix. To evaluate the role of different CYP in the biotransformation of kaempferol we studied the induction of CAs and MN in V79 cells genetically engineered for the expression of rat CYP 1A1, 1A2 and 2B1. In addition we performed CYP inhibition studies using the above-mentioned indicators and high performance liquid chromatography (HPLC) analysis. The results obtained in this work suggest that rat CYP 1A1 is, among the cytochromes studied, the one that plays the major role in the transformation of kaempferol into quercetin. The relevance of these findings to the human situation is discussed.

Introduction

Natural products from plants and micro-organisms have traditionally provided the pharmaceutical industry with one of its most important sources of compounds in the search for new drugs and medicines. Over the last few decades, researchers have turned to traditional medicines in order to identify natural products that have therapeutic properties and find a way of taking advantage of these properties in order to develop drugs with improved efficacy. Plants containing polyphenols, namely flavonoids, have been given particular attention. Indeed, flavonoids have been reported to possess bactericidal action (Havsteen, 1983), to inhibit various enzymes such as glutathione reductase (Elliot *et al.*, 1992), mitochondrial succinoxidase (Hodnick *et al.*, 1986), cyclo-oxygenase and lipoxygenase (Laughton *et al.*, 1989), to inhibit the biotransformation of benzo[*a*]pyrene (B[*a*]P) and 2-amino-3-methyl-imidazo[4,5-*f*]-quinoline (Chae *et al.*, 1991; Lee *et al.*, 1994), and to be able to act as antioxidants due to their ability to chelate iron and to scavenge reactive oxygen species (Ueno *et al.*, 1984; Robak and Gryglewski, 1988; Afanas'Ev *et al.*, 1989; Jovanovic *et al.*, 1994). These properties can be useful in drug design. However the structure–activity relationships have to be clarified since some flavonoids, in certain conditions, can also display toxic effects as it has been shown for quercetin and myricetin, two flavonoids that are very common in edible plants, and that have been shown to be able to act as pro-oxidants (Laughton *et al.*, 1989; Sahu and Gray, 1991, 1993; Gaspar *et al.*, 1994, 1996; Duarte Silva *et al.*, 1996).

Quercetin, kaempferol and myricetin are the most common flavonoids found in edible plants in a glycosylated form (Brown, 1980). Two recent cohort studies on the relationship between the intake of flavonoids and the risk of coronary heart disease estimate a mean baseline flavonoid intake of 25.9 mg/day in The Netherlands (Hertog *et al.*, 1993) and of 20.1 mg/day in the USA (Rimm *et al.*, 1996). Both studies found that the major sources of flavonoids in the diet were tea, onions and apples (Hertog *et al.*, 1993; Rimm *et al.*, 1996) and that the three primary flavonoids ingested were quercetin (16.3 mg/day; 15.4 mg/day), kaempferol (8.2 mg/day; 3.6 mg/day) and myricetin (–; 0.9 mg/day) (Hertog *et al.*, 1993; Rimm *et al.*, 1996). Little is known about the absorption and distribution of these compounds. In man it has been shown that flavonoid glycosides, when taken orally, are hydrolysed to their aglycons by bacterial glycosidases present in the digestive tract (Brown, 1980). The flavonoid aglycons can thereafter be absorbed into the intestine epithelium and then into the blood. The major part of the flavonoids is probably delivered to the liver through the portal vein (Havsteen, 1983; Manach *et al.*, 1995).

There have been numerous studies carried out on the genotoxicity of quercetin and it has been proven to be mutagenic in both prokaryotic and eukaryotic cells (MacGregor and Jurd, 1978; Brown and Dietrich, 1979; MacGregor *et al.*, 1983; Rueff *et al.*, 1986; Gaspar *et al.*, 1994). However, only a few studies report on the mutagenicity of kaempferol. Kaempferol, in the presence of a rat liver metabolizing system (S9 mix), has been shown to induce mutations in *Salmonella typhimurium* (MacGregor and Jurd, 1978; Hardigree and Epler, 1978), in *Drosophila* (Watson, 1982), in the *tk* locus of Chinese hamster ovary (CHO) cells and to induce chromosomal aberrations (CAs) in CHO cells with and without metabolic activation (Carver *et al.*, 1983). In V79 cells it has been shown to induce mutations in the *hprt* locus in the presence of S9 mix and to induce CAs in the presence and in the absence of S9 mix (Maruta *et al.*, 1979; Duarte Silva *et al.*, 1996b). The increased genotoxicity of kaempferol in the presence of S9 mix, as measured by the induction of CAs in V79 cells, has been attributed to its biotransformation to quercetin by cytochrome P450 (CYP) present in Aroclor 1254-induced rat liver homogenates (Duarte Silva *et al.*, 1996b).

Taking into account the intensive research on the beneficial effects of flavonoids and a tendency to use them as therapeutic agents, it is desirable to study the possible toxic effects of

these compounds and their metabolites, especially if we bear in mind that human exposure through a normal diet is by itself significant.

The present study was undertaken to investigate the mechanisms of the genotoxicity of kaempferol further. Special attention has been given to the role of CYP in the genotoxicity of this flavonoid. We studied the induction of mutations in *S.typhimurium* TA98 in the presence and in the absence of S9 mix and the induction of CAs and micronuclei (MN) by kaempferol in V79 cells in the presence and in the absence of S9 mix. To evaluate the role of different CYPs in the biotransformation of kaempferol we studied the induction of CAs and MN in V79 cells genetically engineered for the expression of rat CYP 1A1, 1A2 and 2B1. In addition we performed CYP inhibition studies using the above mentioned indicators and HPLC analysis.

Materials and methods

Chemicals and culture media

Kaempferol, mitomycin C (MMC), B[*a*]P, cyclophosphamide (CP), 2-aminoanthracene (2-AA) cytochalasin B, newborn calf serum, Ham's F-10 medium, phosphate-buffered saline (PBS), quercetin and antibiotic G418 were obtained from Sigma (St Louis, MO, USA). Dimethylsulphoxide (DMSO), acetonitrile, acetic acid and Giemsa dye were from Merck (Darmstadt, Germany). Colchicine was from Fluka (Buchs, Switzerland). Penicillin, streptomycin and amphotericin B were from Irvine Scientific (Santa Ana, CA, USA).

Salmonella typhimurium reversion assay

The tester strain, *S.typhimurium* TA98, has been described by Maron and Ames (1983). The influence of the test compounds on the reversion of the tester strain was determined using the plate incorporation assay (Maron and Ames, 1983). The test compound dissolved in DMSO was added to the top agar and then plated (DMSO did not exceed 0.28 mmol/plate). When metabolic activation was used, 500 µl of S9 mix together with the test compound were added to the top agar and then plated. Aroclor 1254-induced S9 from male Wistar rats and the respective S9 mixes were prepared as described in detail elsewhere (Laires *et al.*, 1982; Maron and Ames, 1983). In inhibition studies 0.1 µmol of α-naphthoflavone, an inhibitor of the CYP 1A subfamily (Tassaneeyakul *et al.*, 1992; Newton *et al.*, 1995), was also added to the top agar. Each assay was carried out in triplicate. Reversion specificity and activity of S9 mix were confirmed with B[*a*]P (5 µg/plate). Each experiment included a determination of the genotoxicity of quercetin (10 µg/plate) as a concurrent reference standard.

V79 Chinese hamster ovary cells

Cells were cultured in 5 ml Ham's F-10 medium supplemented with 10% newborn calf serum, penicillin (50 U/ml), streptomycin (50 µg/ml) and amphotericin B (25 µg/ml) and incubated at 37°C under an atmosphere of 5% CO_2.

Genetically-engineered V79 Chinese hamster ovary cells

V79 Chinese Hamster cells genetically engineered for the expression of rat CYP 1A1 (V79-MZ-r1A1), 1A2 (V79-MZ-r1A2) and 2B1 (V79-MZ-r2B1) were kindly provided by Professor H.R.Glatt (Mainz and Potsdam). These cell lines have been described elsewhere (Doehmer *et al.*, 1988; Dogra *et al.*, 1990; Wolfel *et al.*, 1991). The validation of these cell lines in regard to the expression of the different CYP has been done using different endpoints and test compounds (Ellard *et al.*, 1991; Rueff *et al.*, 1996). In our laboratory this validation has been done using the CA assay, in the same conditions described in this paper and the sister chromatid exchange assay, and the positive control compounds tested were CP, 2-AA and B[*a*]P for the cell lines V79-MZ-r2B1, V79-MZ-r1A2 and V79-MZ-r1A1, respectively (Rodrigues *et al.*, 1993, 1994).

Cells were cultured in 5 ml Ham's F-10 medium supplemented with 10% newborn calf serum, penicillin (50 U/ml), streptomycin (50 µg/ml) and amphotericin B (25 µg/ml) and incubated at 37°C under an atmosphere of 5% CO_2. The selection of the genetically-engineered cells was maintained by addition of the antibiotic G418 (400 µg/ml).

CA assay

Cultures (20 h; ~1×10^6 cells) were washed with Ham's F-10 medium reconstituted in 0.01 M phosphate buffer (pH 7.4) and grown in 5 ml of this medium for 2 h in the presence of kaempferol dissolved in DMSO (maximum concentration of DMSO was 84 mM). For positive controls, 3 µM MMC was used. When metabolic activation was required, 500 µl of S9 mix and 4.5 ml of medium were used. CP (21.5 µM) was used as a positive control. In the inhibition studies 0.1 µmol of α-naphthoflavone (10 µl of a 10 mM solution of α-naphthoflavone in DMSO) were added during the 2 h treatment period. After the treatment cells were washed with culture medium and grown for another 15 h. Colchicine was added at a final concentration of 6 µg/ml, and cells were grown for a further 3 h. Cells were then harvested by trypsinization. After a 3 min hypotonic treatment with KCl [0.56% (w/v), 37°C] cells were fixed with methanol:acetic acid (3:1) and slides were prepared and stained with Giemsa (4% in 0.01 M phosphate buffer, pH 6.8) for 10 min. Two independent experiments were carried out with each test compound and 100 metaphases were scored for each dose level treatment group in each experiment. Scoring of the different types of aberrations followed the criteria described by Rueff *et al.* (1993). The cytotoxicity of kaempferol was assessed by counting the mitotic indexes (MI; i.e. percentage of metaphases in 2000 cells) and when the dose tested induced a decay in the MI >50%, when compared with the control it was considered to be cytotoxic.

MN assay

Cultures (20 h; ~3×10^5 cells), grown in microwell plates with a glass slide on the bottom, were washed with Ham's F-10 medium reconstituted in 0.01 M phosphate buffer (pH 7.4) and grown in 3 ml of this medium for 2 h in the presence of kaempferol (dissolved in DMSO). When metabolic activation was required 300 µl of S9 mix, and 2.7 ml of medium were used. After the treatment cells were washed with culture medium and grown for another 17 h in the presence of cytochalasin B (6 µg/ml). Cells were then washed five times with PBS (4°C) fixed with cold methanol for 5 min, washed and fixed again for 20 min and then stained with Giemsa (4% in 0.01 M phosphate buffer, pH 6.8) for 15 min. Two independent experiments were carried out with each test compound and 1000 binucleated cells were scored for each dose.

Incubations for HPLC

Kaempferol (524 nmol) was incubated with 500 µl of rat liver S9 mix, at 37°C for different times. In the inhibition studies 0.1 µmol α-naphthoflavone in DMSO was added to the mixture. These conditions are equivalent to those used in the CA assay in regard to the relative amounts of kaempferol, inhibitor and S9 mix. The reaction was stopped by addition of 0.1 M H_3PO_4 in methanol. The mixture was centrifuged and the supernatant was evaporated to dryness. The residue was reconstituted in 70 µl DMSO and then diluted 1:14 in DMSO. This mixture (60 µl) was diluted 1:3 in 10% acetic acid, filtered, and analysed in a Merck–Hitachi L-6200 HPLC apparatus with a diode array Merck–Hitachi L-3000 and a RP-18 column. The solvents used were 10% acetic acid (solvent A) and acetonitrile (solvent B) and the gradient used was 0–20 min: 15–90% solvent B with a flow of 0.7 ml/min. The detection was followed at 370 nm. The identification of the peaks was carried out by comparison of retention times and UV spectra between the samples and external and also internal standards.

Results

The results on the induction of CAs by kaempferol in V79 cells with and without S9 mix and in V79 cells genetically-engineered for the expression of rat CYP 1A1, 1A2 and 2B1 are shown in Table I. There is a dose-dependent induction of CAs in V79 cells which increases in the presence of S9 mix. There is no significant difference in the induction of CAs in the cell lines that express rat CYP 1A2 and 2B1 when compared with parental V79 cells. However the results obtained for the cell line that expresses rat CYP 1A1 are significantly higher when compared with parental V79 cells and not significantly different from the results obtained in V79 cells in the presence of S9 mix (values of significance according to χ^2 test are shown in Table I). It must be noted that the highest concentration tested (139.2 µM) was cytotoxic to all cell lines as measured by the decay in the mitotic index when compared with negative controls. The concentration of 104.4 µM was not cytotoxic and was chosen for further tests. In the presence of α-naphthoflavone the results obtained on the induction of CAs by kaempferol (104.4 µM) in parental V79 cells with S9 mix (Table II) are significantly lower when compared with its absence but are not significantly different from the results obtained in parental V79 cells (values of significance according

Table I. Induction of chromosomal aberrations by kaempferol in V79-MZ cells, V79-MZ cells expressing rat CYP 1A1, 1A2 and 2B1 and V79-MZ cells in the presence of S9 mix

Dose (μM)		Ctg[a]	Ctb[b]	Int[c]	Chg[d]	Chb[e]	Dic[f]	Other[g]	>10[h]	%Aber. cells includ. gaps	%Aber cells exclud. gaps	Mitotic index (%)
V79-MZ												
0	exp 1	3	1	0	1	0	0	0	0	5	1	6.4
	exp 2	3	1	0	0	0	0	0	0	4	1	12.3
17.4	exp 1	2	0	0	0	0	0	0	0	2	0	
	exp 2	2	1	0	0	0	0	0	0	3	1	
52.2	exp 1	15	3	1	1	0	1	0	0	18	5[i]	
	exp 2	18	4	1	1	0	1	0	0	21	4	
104.4	exp 1	15	8	9	2	0	0	0	0	25	11[j]	6.7
	exp 2	19	7	3	1	0	1	1	0	22	10	6.3
139.2	exp 1	22	13	12	1	0	0	0	0	30	15[j]	1.9
	exp 2	13	7	5	1	0	0	1	0	13	13	3.3
MMC	exp 1	5	7	8	1	0	2	0	2	20	15	
3 μM	exp 2											
V79-MZ-r1A2												
0	exp 1	5	2	0	0	0	0	0	0	7	2	8.4
	exp 2	2	0	0	0	0	0	0	0	2	0	11.3
17.4	exp 1	4	0	0	0	0	0	0	0	4	0	
	exp 2	4	0	0	0	0	0	1	0	5	1	
52.2	exp 1	0	0	2	10	0	0	0	0	2	1[k]	
	exp 2	11	3	1	0	0	0	0	0	14	4	
104.4	exp 1	6	4	4	1	0	1	0	0	16	9[j]	5.2
	exp 2	14	10	6	3	0	2	1	0	27	14	7.2
139.2	exp 1	7	11	10	1	0	0	2	0	25	19[j]	2.5
	exp 2	16	9	14	0	0	1	0	2	27	21	2.2
MMC	exp 1	15	8	9	4	0	1	0	2	27	18	
3 μM	exp 2	7	10	5	3	3	0	0	1	21	15	
V79 MZ-r2B1												
0	exp 1	2	0	1	0	0	0	0	0	3	1	7.8
	exp 2	0	0	0	0	0	0	0	0	0	0	9.2
17.4	exp 1	4	0	1	0	0	0	0	0	5	1	
	exp 2	3	0	0	0	0	0	0	0	3	0	
52.2	exp 1	0	0	0	1	0	0	0	1	1	0[l]	
	exp 2	5	1	2	0	0	0	0	0	8	3	
104.4	exp 1	11	0	6	1	0	1	1	0	17	8[j,m]	6.9
	exp 2	9	7	5	0	0	1	0	0	18	10	7.5
139.2	exp 1	21	6	10	2	0	0	1	0	32	16[j]	3.6
	exp 2	19	8	14	1	0	0	0	0	32	16	4.3
MMC	exp 1	3	4	4	0	0	0	2	1	13	11	
3 μM	exp 2	5	16	13	1	0	1	1	1	28	24	
V79-MZ-r1A1												
0	exp 1	3	2	1	0	0	0	0	0	6	3	10.1
	exp 2	0	0	0	0	0	0	0	0	0	0	9.3
17.4	exp 1	3	0	0	0	0	0	0	0	3	0	
	exp 2	1	0	0	1	0	1	0	0	3	1	
52.2	exp 1	8	6	3	0	0	0	1	0	18	9[j,n]	
	exp 2	15	3	4	0	0	3	2	0	27	11	
104.4	exp 1	18	12	12	0	0	1	0	0	43	19[j,n]	7
	exp 2	15	5	7	7	1	7	3	0	45	21	7.5
139.2	exp 1	16	17	21	0	2	1	2	0	59	33[j,m,o]	2.7
	exp 2	14	12	12	0	2	9	3	0	52	29	1.9
MMC	exp 1	20	12	1	7	3	3	1	0	47	19	
3 μM	exp 2	5	2	3	2	0	7	1	3	20	16	
V79-MZ with S9 mix												
0	exp 1	6	0	0	0	0	0	0	0	5	0	10.6
	exp 2	5	0	0	0	0	0	0	0	5	0	6.6
17.4	exp 1	5	1	0	0	0	0	0	0	6	1	
	exp 2	3	0	0	0	0	1	0	0	4	1	
52.2	exp 1	9	13	0	2	0	0	0	0	16	10	
	exp 2	5	9	0	1	0	0	0	0	15	9[j]	
104.4	exp 1	21	8	3	2	2	7	0	0	34	16[j]	8.1
	exp 2	5	6	3	0	1	8	0	0	20	17	6.7
139.2	exp 1	21	13	4	1	2	2	1	0	36	21[j]	4
	exp2	19	15	4	1	1	3	0	0	34	20	3.8
CP	exp 1	13	16	5	2	0	4	0	0	27	19	
21.5	exp 2	6	8	18	0	1	4	1	1	22	20	

[a]Chromatid gap, [b]chromatid break, [c]interchange, [d]chromosome gap, [e]chromosome break, [f]dicentrics, [g]others, [h]multi-aberrant cells, [i]$P < 0.05$, [j]$P < 0.001$when compared with the control (χ^2 test); [k]$P < 0.01$, [l]$P < 0.001$, [m]$P < 0.05$ when compared with the same dose in V79-MZ cell line in the presence of S9 mix (χ^2 test); [n]$P < 0.05$, [o]$P < 0.001$ when compared with the same dose in V79-MZ cell line (χ^2 test).

Table II. Induction of chromosomal aberrations by 104.4 μM kaempferol in V79-MZ cells in the presence and in the absence of S9 mix and in the presence of S9 mix and α-naphthoflavone (ANF)

Treatment		Ctg[a]	Ctb[b]	Int[c]	Chg[d]	Chb[e]	Dic[f]	Other[g]	>10[h]	%Aberrant cells includ. gaps	%Aberrant cells exclud. gaps
Control[i]	exp 1	5	1	0	0	0	0	0	0	3	0
	exp 2	6	0	1	0	0	0	0	0	2	1
Kaempferol	exp 1	14	5	4	0	0	0	1	0	18	9
	exp 2	23	9	5	0	1	1	1	0	28	9
Kaempferol	exp 1	28	14	4	2	0	1	0	0	37	17
+ S9 mix	exp 2	14	14	3	0	0	6	0	0	28	16[j]
Kaempferol + S9	exp 1	16	10	0	0	1	0	0	0	22	8
mix + ANF	exp 2	19	6	0	2	1	0	1	1	22	8[k]

[a]Chromatid gap, [b]chromatid break, [c]interchange, [d]chromosome gap, [e]chromosome break, [f]dicentrics, [g]others, [h]multi aberrant cells, [i]control contains DMSO, S9 mix and α-naphthoflavone, [j]$P < 0.05$ when compared with the absence of S9 mix (χ^2 test), [k]$P < 0.005$ when compared with the absence of α-naphthoflavone (χ^2 test).

Table III. Induction of chromosomal aberrations by 104.4 μM kaempferol in V79-MZ-r1A1 cells in the presence and in the absence of α-naphthoflavone (ANF)

Treatment		Ctg[a]	Ctb[b]	Int[c]	Chg[d]	Chb[e]	Dic[f]	Other[g]	> 10[h]	%Aberrant cells includ. gaps	%Aberrant cells exclud. gaps
Control[i]	exp 1	2	0	0	0	0	0	0	0	2	0
	exp 2	3	4	0	2	0	0	0	0	7	2
Kaempferol	exp 1	13	13	6	1	0	0	7	1	30	22
	exp 2	22	24	5	2	2	1	5	2	36	21
Kaempferol + ANF	exp 1	12	7	2	1	0	2	0	0	22	10
	exp 2	9	2	8	2	0	1	0	0	17	10[j]

[a]Chromatid gap, [b]chromatid break, [c]interchange, [d]chromosome gap, [e]chromosome break, [f]dicentrics, [g]others, [h]multi-aberrant cells, [i]control contains DMSO and α-naphthoflavone, [j]$P < 0.01$ when compared with the absence of α-naphthoflavone (χ^2 test).

χ^2 test are shown in the tables). In the cell line that expresses CYP 1A1 (Table III) the results obtained for the induction of CAs by kaempferol in the presence of α-naphthoflavone are significantly lower when compared with its absence and are not significantly different from the results obtained for V79-MZ cell line.

The results on the induction of MN by kaempferol in V79 cells with and without S9 mix and in V79 cells genetically-engineered for the expression of rat CYP 1A1, 1A2 and 2B1 are shown in Figure 1. There is a dose-dependent induction of MN in V79 cells and this induction significantly increases in the presence of S9 mix. There is no significant difference in the results obtained for the induction of MN in the cell line that express rat CYP 1A2 when compared with parental V79 cells. However, the results obtained for the cell line that expresses rat CYP 1A1 are significantly higher than in parental V79 cells and not significantly different from the values obtained in V79 cells in the presence of S9 mix. In the cell line that expresses CYP 2B1 there is a significant increase in the induction of MN when compared with parental V79 cells; that increase, however, is smaller when compared with the cell line that expresses CYP 1A1 or the parental V79 cell line in the presence of S9 mix (values of significance according to χ^2 test are shown in Figure 1).

The results on the *S.typhimurium* TA 98 reversion assay are shown in Figure 2. Kaempferol is not mutagenic in this tester strain. In the presence of S9 mix, however, there is a clear dose-dependent induction of revertants by kaempferol which is almost completely inhibited in the presence of α-naphthoflavone.

The results obtained using HPLC for CYP inhibition studies are shown in Figure 3a and b. After a 120 min incubation kaempferol is partially transformed into quercetin; however, this transformation does not occur when kaempferol is incubated with S9 mix and α-naphthoflavone.

Discussion

The biotransformation of kaempferol via CYP enhances its genotoxicity, as assessed by the induction of CAs in V79 cells (Duarte Silva *et al.*, 1996b). In this test system the mutagenic potency of quercetin is about four times higher than that of kaempferol (Gaspar *et al.*, 1994; Duarte Silva *et al.*, 1996b). Since CYPs are able to transform kaempferol into quercetin it is conceivable that the increased genotoxicity of kaempferol, in conditions where it can be biotransformed is due to the formation of quercetin (Duarte Silva *et al.*, 1996b).

In the present work we attempted to study the biotransformation of kaempferol and to identify the CYPs that are involved in its biotransformation to the more potent genotoxicant quercetin. The results on the induction of CAs (Table I) and MN (Figure 1) in V79 cell lines genetically engineered for the expression of different rat CYP suggest that CYP 1A1, among the cytochromes studied, is the one that plays the major role in the biotransformation of kaempferol to the more potent genotoxicant. Indeed the induction of CAs and MN in the cell line that expresses rat CYP 1A1 is significantly higher than in parental V79 cell lines and not significantly different from the induction in V79 cells in the presence of Aroclor 1254 induced rat liver S9 mix. To test this hypothesis further, we assessed the induction of CAs in V79 cells with S9 mix (Table II) and in the cell line that expresses rat CYP 1A1 (Table III) in the

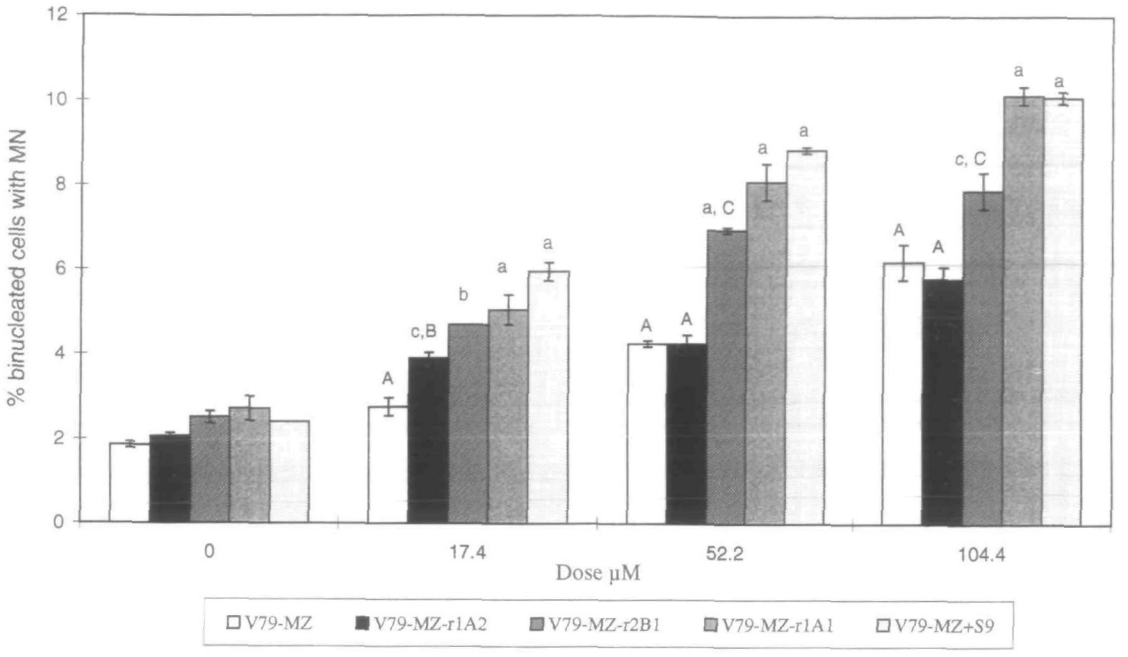

Fig. 1. Induction of micronuclei by kaempferol in V79-MZ cells, V79-MZ cells expressing rat CYP 1A1, 1A2 and 2B1 and V79-MZ cells in the presence of S9 mix. (**a**) $P < 0.001$; (**b**) $P < 0.005$; (**c**) $P < 0.05$ when compared with the same dose in V79-MZ cell line (χ^2 test). (**A**) $P < 0.001$; (**B**) $P < 0.005$; (**C**) $P < 0.05$ when compared with the same dose in V79-MZ cell line in the presence of S9 mix (χ^2 test). Results are the average of two independent experiments; bars show SD.

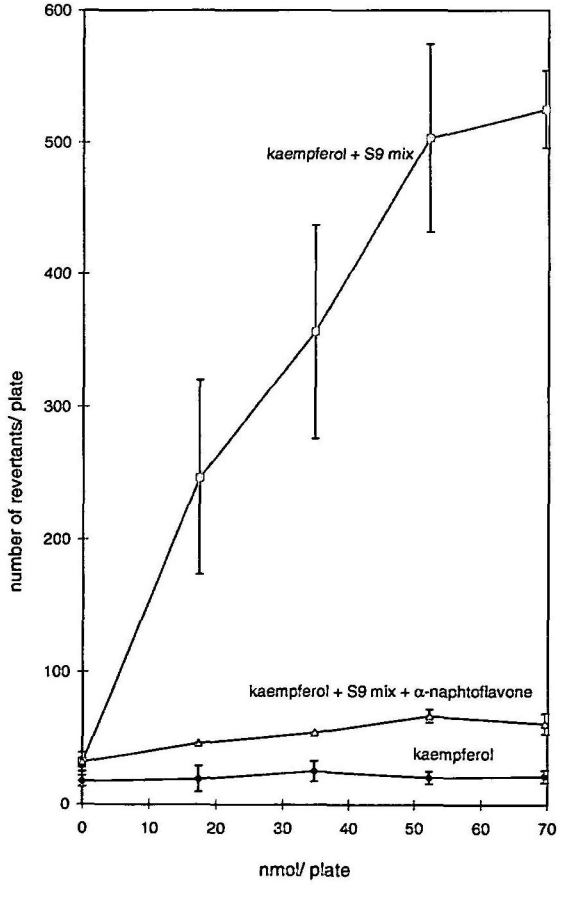

Fig. 2. Induction of *S.typhimurium* TA98 revertants by kaempferol in the presence and in the absence of S9 mix and in the presence of S9 mix and α-naphthoflavone. Bars show SD of three independent experiments.

presence of α-naphthoflavone, (Tassaneeyakul *et al.*, 1992; Newton *et al.*, 1995). The results obtained confirmed our hypothesis since, in both test systems and in the presence of this inhibitor, the genotoxicity of kaempferol was not significantly different from its genotoxicity in parental V79 cells implying that it has not been metabolized to quercetin. Although α-naphthoflavone also inhibits CYP 1A2 we can rule out the involvement of this CYP in the biotransformation of kaempferol to quercetin since the results on the induction of CAs and MN in the cell line that expresses this CYP are not significantly different from the results obtained in V79 parental cells.

The results obtained with HPLC after incubation of kaempferol with S9 mix in the presence and in the absence of α-naphthoflavone further corroborate the involvement of rat CYP 1A1 in the biotransformation of kaempferol to quercetin. Indeed in the absence of the inhibitor we see that kaempferol has been partially transformed into quercetin but that transformation did not occur when the incubation was performed in the presence of α-naphthoflavone (Figure 3). It is noteworthy that the relative amounts of S9 mix, kaempferol and inhibitor used in these incubations were exactly the same that were used in the CA assay therefore we can interpret the results on the induction of CAs in light of the results obtained with HPLC. Taken as a whole these results suggest that CYP 1A1 is responsible for the biotransformation of kaempferol into the more potent genotoxicant quercetin.

Given the present results we considered that it would be interesting to verify whether the mechanisms of mutagenicity in kaempferol are comparable in bacteria and in eukaryotic cells. To do so we performed the *Salmonella* reversion assay in strain TA98 in the presence and in the absence of S9 mix and of α-naphthoflavone. When the biotransformation of kaempferol is inhibited by α-naphthoflavone there is no induction of revertants in strain TA98 (Figure 2). Therefore, as in

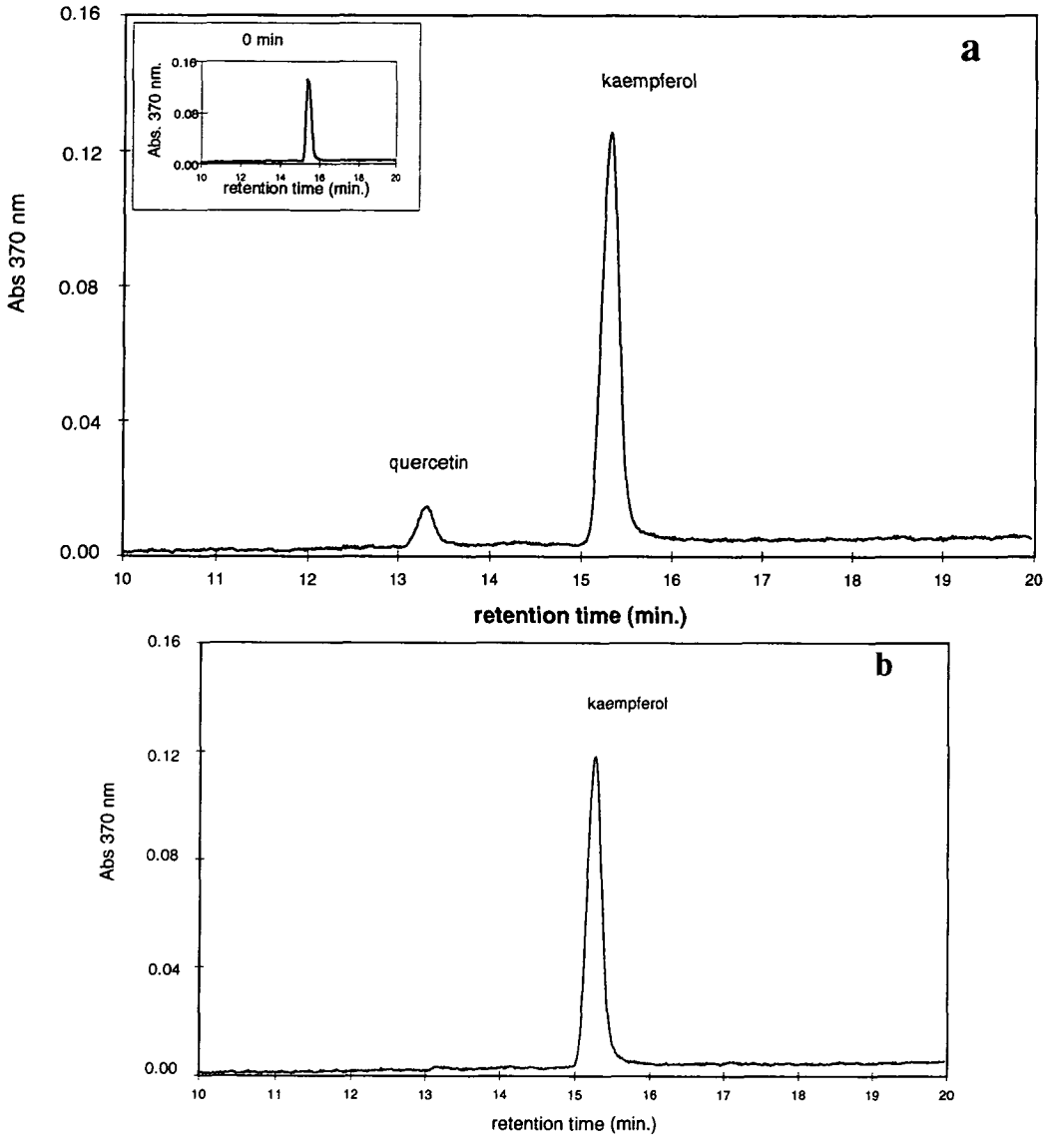

Fig. 3. HPLC analysis of biotransformation of kaempferol to quercetin after a 120 min incubation with S9 mix in (**a**) the absence and (**b**) the presence of α-naphthoflavone. Inset shows the histogram of the sample that contains kaempferol and S9 mix in the absence of α-naphthoflavone at time 0 min.

eukaryotic cells, the biotransformation of kaempferol into quercetin seems to be important to its mutagenicity.

It is important to note that the major forms of CYP expressed in Aroclor 1254 induced rat liver S9 mix are 2B1 (37%), 1A1 (27%) and 1A2 (22%) (Ryan and Levin, 1990). In conditions where CYP 2B1 is expressed, but CYP 1A1 and 1A2 are not, such as in the presence of S9 mix and α-naphthoflavone, there is no transformation of kaempferol to quercetin, as followed by HPLC and the *Salmonella* reversion assay. Therefore we can assume that CYP 2B1 does not metabolize kaempferol to quercetin. The results obtained in the MN assay with the cell line that expresses CYP 2B1 (Figure 1) suggest, however, that this CYP is able to transform kaempferol into a more potent mutagenic product, which, on the basis of our results, cannot be demonstrated to be quercetin.

Flavonoids have been shown to inhibit the biotransformation of the procarcinogen B[*a*]P as measured by the decrease in aryl hydrocarbon hydroxylase activity (Chae *et al.*, 1991; Kansanen *et al.*, 1996) and the suppression of B[*a*]P-induced MN in mice bone marrow polychromatic erythrocytes (Heo *et al.*, 1992). The mechanisms by which flavonoids inhibit the CYP-dependent mono-oxygenase system appear to be multifactorial. For example, it has been suggested that quercetin inhibits CYP 1A1-mediated ethoxyresorufin *O*-deethylase in hepatic microsomes from β-naphthoflavone-treated rats by competing with the substrate and/or by altering the substrate binding site (Sousa and Marletta, 1985; Chae *et al.*, 1991).

In this work we show that kaempferol is a substrate of rat CYP 1A1 and that it is transformed to quercetin by this CYP. These results suggest that kaempferol can be a competitive inhibitor of CYP 1A1 in the activation of procarcinogens such as B[*a*]P, but in doing so kaempferol itself is transformed to the more potent genotoxicant quercetin. Flavonoids have often been referred to as potential chemopreventive agents (Chae *et al.*, 1991; Lee *et al.*, 1994; Elangovan *et al.*, 1994) since they inhibit the biotransformation of some procarcinogens; however, the relative toxicity of the flavonoids themselves has been disregarded. The results obtained in this work indicate that special attention should be paid to the toxicity of flavonoids and hence care should be taken when using them as therapeutic agents.

It has been suggested that CYP 1A1 plays a substantial role in the activation of carcinogens in extrahepatic tissues (Yamazoe *et al.*, 1992). This CYP is not expressed in human liver but it is present in other human tissues such as oesophagus, colon and rectum (Mercurio *et al.*, 1995; Nakajima *et al.*, 1996), placenta, lymphocytes (Fujino *et al.*, 1982), lung and skin (Yamazoe *et al.*, 1992). Man is exposed to kaempferol through the diet; the expression of CYP 1A1 in the digestive tract is, therefore, of special relevance with regard to the mutagenicity of kaempferol.

Considerable evidence has been accumulating that orthologous rat and human CYP forms oxidize numerous chemicals in a very similar manner. Nevertheless, certain specific substrates of rat CYP are not as well oxidized by the orthologous human forms and vice versa. In the case of CYP 1A1, it is believed that there is a similarity in substrate specificity between human CYP 1A1 and those of other mammalian species (Yamazoe *et al.*, 1992). Therefore, although this study was performed with rat CYP 1A1, it is highly indicative of the effects of the genotoxicity of kaempferol in man.

Acknowledgements

Our current research is supported by the European Commission and JNICT. We also acknowledge the collaboration of the Centre for Malaria and other Tropical Diseases-UNL. I.Duarte Silva is supported by a doctoral fellowship from Junta Nacional de Investigação Científica e Tecnológica. We are grateful to Mr Romão from the Gulbenkian Institute of Sciences for his excellent technical assistance. The tester strain, *S.typhimurium* TA98, was kindly provided by Professor B.Ames (CA, USA) and the V79 Chinese hamster cells were kindly provided by Professor H.R.Glatt (Mainz and Potsdam, Germany).

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Received on March 5, 1997; accepted on May 6, 1997