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FLAVONOIDS AS INHIBITORS OF RAT LIVER MONOOXYGENASE ACTIVITIES

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Abstract. Flavanone and six hydroxylated derivatives, and cianidanol and eight ethers and esters thereof, were investigated as inhibitors of cytochrome P-450 mediated reactions in rat liver microsomes. The IC_{50} values towards aminopyrine *N*-demethylation varied over a 20-fold range and were shown to depend on the pattern of hydroxylation (flavanone derivatives) and on lipophilicity (cianidanol derivatives). In the latter case, a bilinear relationship exists, the optimal log P being 2.92. Using selected compounds, IC_{50}, K_m and V_{max} values were determined for aminopyrine *N*-demethylation, biphenyl 4-hydroxylation, and biphenyl 2-hydroxylation. Depending on the inhibitor and on the activity examined, non-competitive, competitive, or mixed inhibition was seen. Interaction with cytochrome P-450 was also studied spectrally and was always found to result in a modified type II difference spectrum (ligand binding). A dual binding mode is postulated, involving electrostatic and lipophilic interactions.

The flavonoids are a group of natural and semi-synthetic compounds displaying to variable extents a wide range of pharmacodynamic properties [1, 2]. Of particular interest for their therapeutic potential are such effects as stabilisation of cellular membranes in mastocytes and hepatocytes [e.g. 3–5], decrease of capillary permeability [e.g. 1], influence on immune regulation [e.g. 2, 6], trapping of free radicals and inhibition of lipoperoxidation [e.g. 3, 7, 8], impairment of neutrophil response [9], decrease of platelet aggregation and antithrombotic action [e.g. 10–12], gastric anti-ulcer activity [13], antiviral activity [2], and antinecrotic hepatoprotective effects [e.g. 14]. A number of references quoted here demonstrate that minor changes in the chemical structure of flavonoids result in important modifications of biological activity.

The molecular mechanisms underlying the pharmacodynamic effects of flavonoids are insufficiently understood in some cases, while in other cases underlying inhibition or activation of enzymes has been demonstrated. Thus, inhibition of histidine decarboxylase [13] and cyclic AMP phosphodiesterase [e.g. 15, 16] has been reported. Similarly, some flavonoids influence key enzymes involved in the metabolism of arachidonic acid, e.g. inhibition or activation of cyclooxygenase [e.g. 12, 17], inhibition of lipoxygenases [e.g. 2, 12, 18].

The effects of flavonoids on monooxygenases are complex and may have pharmacodynamic and pharmacokinetic consequences. In a careful study, Kellis and Vickery [19] have demonstrated that a number of flavones are strong competitive inhibitors of androstenedione and testosterone aromatisation in human placental and ovarian microsomes. Similarly, quercetine (3,5,7,3′,4′-pentahydroxyflavone) inhibits various cytochrome P-450 activities in rat liver microsomes [20]. A large number of hydroxylated flavone derivatives were also shown to inhibit benzo[*a*]pyrene hydroxylation in human liver microsomes, an effect partly due to inhibition of cytochrome P-450 reduction [21]. In contrast, flavone and other non-hydroxylated analogs acted as activators of benzo[*a*]pyrene hydroxylation and aflatoxin B1 metabolism [21, 22], an activation effect later shown to occur only with some cytochrome P-450 isozymes, while others indeed are inhibited [23].

When administered to animals in relatively high doses over longer periods of time, some flavonoids such as quercetin tend to produce an increase in hepatic microsomal monooxygenases [24] while others such as flavanone are inactive [25]. Taken globally, the effects of flavonoids on monooxygenases thus appear rather complex. While some of them can act *in vitro* as activators or *in vivo* as weak inducers, a number of these compounds are relatively strong *in vitro* inhibitors of cytochromes P-450, a behaviour displayed by many phenols [26]. These effects, which may contribute to some of the pharmacological actions of flavonoids, could also have toxicological or pharmacokinetic consequences, for example interference with cytochromes P-450 mediated physiological reactions, and drug–drug interactions. A better understanding of the mechanisms of monooxygenase inhibition and relevant structure–activity relationships is thus indicated for a more rational development of therapeutic flavonoids.

In a previous study [27], we have examined the effects of cianidanol [(+)-catechin, (+)-cyanidan-3-ol, compound 11 in Table 1] on rat hepatic monooxygenase activities. In rat liver microsomes, the drug non-competitively inhibited a number of cytochrome P-450 mediated reactions, displaying weak IC_{50} values close to or above 1 mM. Its binding to ferricytochrome P-450 elicited a modified type II

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spectrum as do a number of oxygenated compounds, suggesting that it acts as a Fe(III) ligand by stabilising the low-spin form of the enzyme and preventing its reduction [26]. When tested *in vivo*, a weak but statistically significant inhibition of [^{14}C]aminopyrine demethylation was detected after oral dosing during 5 days. In the present study, the inhibition of rat liver monooxygenase activities is examined using a number of flavanones and of 3-*O*-alkylated and 3-*O*-acylated cianidanol derivatives.

MATERIALS AND METHODS

Chemicals. The origin of the various chemicals and reagents has been described [27]. Cianidanol and all other flavonoids used here (see Table 1) were supplied by the Chemical Research Department, Zyma SA, and were of pharmaceutical grade. The log *P* values of the flavonoids (*P* = octanol/water partition coefficient) have been reported [5] (see Table 1). Briefly, experimental log *P* values were measured for a number of basic flavonoid structures with various hydroxylation patterns, hydrophobic fragmental constants being then used to calculate the lipophilicity of derivatives.

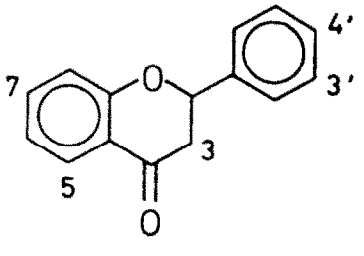
Animals and preparation of microsomes. Adult male RAI rats. (*ca.* 200 g) were obtained from Ciba-Geigy AG (Sisseln, Switzerland). The pretreatment of the animals and the preparation of microsomes have been described [27].

In vitro assays. The aminopyrine *N*-demethylation assay (Nash reaction) and the biphenyl 2- and 4-hydroxylation assays (GLC) have been described [27]. We note again that the presence of semicarbazide (45 μmol) in the aminopyrine *N*-demethylation assay is necessary to trap the nascent formaldehyde and prevent what appears to be a flavonoid-catalysed transfer of HCHO to protein amino groups. Semicarbazide at the concentration used did not detectably inhibit aminopyrine *N*-demethylation.

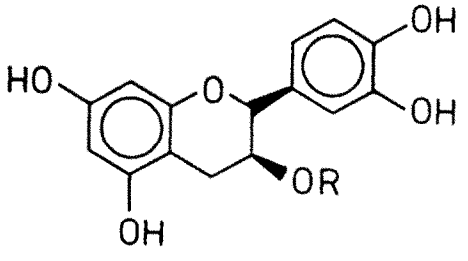
The soluble flavonoids were dissolved in water (pH 7.4, phosphate buffer). For the poorly water-soluble compounds (see Table 1) a 200 mM acetonic solution was prepared and then diluted with water (pH 7.4, phosphate buffer) under sonication (3 min, Cell Disfractor B12, Branson Sonic Power Co., Danbury, U.S.A.) to obtain a highly dispersed 2.4 mM suspension. The latter was added to the incubation flask to obtain the test concentration (0.01–1 mM). When dissolved under such conditions, 1 mM 3-palmitoyl-cianidanol inhibited aminopyrine *N*-demethylation by 49.7 ± 0.9% (see later, Table 3). In contrast, incomplete dissolution and variable inhibition ranging from 17 to 29% were obtained when mechanical agitation (Vortex-Genie, Bender-Hobein, Zurich, Switzerland) replaced sonication.

The method of dissolution was validated by replacing the sonicated 2.4 mM dispersion with a

Table 1. Flavonoids investigated



Flavanone



(+)-Cianidanol derivatives

No.	Compounds‡	Substituents	log P†	Water solubility‡
1	Flavanone (F)	–	3.14	–
2	5-Hydroxy-F	5-OH	2.80	–
3	7-Hydroxy-F	7-OH	2.93	–
4	5,7-Dihydroxy-F	5,7-$(OH)_2$	3.02	–
5	Naringenin	5,7,4'-$(OH)_3$	2.52	–
6	Eriodictyol	5,7,3',4'-$(OH)_4$	2.02	–
7	(+)-Taxifolin	3,5,7,3',4'-$(OH)_5$	0.95	+
11	(+)-Cianidanol (C)	R = H	0.51	+
12	3-Methyl-C	R = CH_3	1.00	+
13	3-*n*-Propyl-C	R = $(CH_2)_2CH_3$	2.12	+
14	3-*n*-Butyl-C	R = $(CH_2)_3CH_3$	2.64	+
15	3-*n*-Heptyl-C	R = $(CH_2)_6CH_3$	4.20	–
16	3-*n*-Dodecyl-C	R = $(CH_2)_{11}CH_3$	6.79	–
17	3-Palmitoyl-C	R = $CO\text{-}(CH_2)_{14}CH_3$	8.70	–
18	3-Benzoyl-C	R = $CO\text{-}C_6H_5$	3.44	–
19	3-(3,4-Dihydroxybenzoyl)-C	R = $CO\text{-}C_6H_3$-m,p-$(OH)_2$	2.45	+

* Taxifolin, cianidanol and all cianidanol derivatives were used in pure stereoisomeric form. All other compounds were racemates.

† Log of octanol/water partition coefficient, determined as explained under Methods.

‡ Good solubility: +; poor solubility: –.

40 mM solution of 3-palmitoyl-cianidanol in dimethylsulfoxide (DMSO). Under these conditions, DMSO was present in the incubation flasks at a 2.5% concentration which totally dissolved the flavonoid and which alone inhibited aminopyrine *N*-demethylation by 50.7 ± 1.8%. In contrast, the reaction was inhibited by 77.2 ± 2.6% in the presence of DMSO 2.5% plus 1 mM 3-palmitoyl-cianidanol. In other words, the flavonoid inhibited *ca.* 50% of the remaining activity, confirming the sonication results.

The IC_{50} values (concentrations inhibiting the reaction by 50%) were calculated by probit analysis from 6 concentrations in the range 0.01–1.0 mM. For the determination of K_m and V_{max} values, 4–6 (usually 5) concentrations of the substrate were used, while the inhibitors were assayed at a concentration close to their IC_{50}.

Spectral measurements. Cytochrome P-450 was determined by the method of Omura and Sato [28]. To examine the influence of flavonoids on cytochrome P-450 concentrations, the microsomal suspension containing the flavonoid was distributed in both cells, and the method of Raj and Estabrook (as described by Levin *et al.* [29]) was used to quantitate cytochrome P-450 concentrations in the presence of CO generated during incubation.

The binding spectra were determined, and the spectral dissociation constants were calculated, as described by Schenkman *et al.* [30]. Liver microsomes from untreated rats were used at a concentration of 2 mg protein/ml, while the concentrations of the ligands were in the range 0.025–1.00 mM. For further details, see Ref. 27.

RESULTS

Inhibition of aminopyrine N-*demethylation*

For each of the 16 compounds in Table 1, we have measured their inhibitory effect (IC_{50} values) on aminopyrine *N*-demethylase in liver microsomes from untreated rats. The detailed results for taxifolin are presented in Table 2 as a representative example. Table 3 shows the IC_{50} values and 95% confidence limits determined for the 16 compounds. In the regressions, the r^2 values ranged from 0.963 to 0.995. Compound No. 2 (5-hydroxyflavanone) with r^2 = 0.855 was an unexplained exception (see the broad

Table 2. Effect of taxifolin on aminopyrine *N*-demethylation in liver microsomes of untreated rats

Concentration (mM)	Activity*	
	nmol HCHO (mg prot)$^{-1}$ min^{-1}	%
0.00	4.02 ± 0.06	100
0.01	3.72 ± 0.08	92.5 ± 2.0
0.05	3.34 ± 0.07	83.1 ± 1.7
0.10	2.96 ± 0.08	73.6 ± 2.0
0.50	1.85 ± 0.03	46.0 ± 0.7
1.00	1.54 ± 0.02	38.3 ± 0.5

* N = 3; ±SD.

Regression: (100 − % metabolism) = 0.908(±0.034) log conc + 5.31(±0.04) N = 15; r^2 = 0.991.

log IC_{50} = −0.338 ± 0.108.

Table 3. Effect of flavonoids on aminopyrine *N*-demethylation in liver microsomes of untreated rats

No	Compound	IC_{50}*	95% Confidence limits
1	Flavanone (F)	1.12	(0.83–1.50)
2	5-OH-F	0.86	(0.27–2.74)
3	7-OH-F	0.19	(0.15–0.25)
4	5,7-$(OH)_2$-F	0.09	(0.06–0.13)
5	Naringenin	0.10	(0.08–0.13)
6	Eriodictyol	0.08	(0.07–0.09)
7	Taxifolin	0.46	(0.36–0.59)
11	Cianidanol (C)	0.83	(0.67–1.03)
12	3-Methyl-C	0.24	(0.21–0.28)
13	3-n-Propyl-C	0.05	(0.04–0.07)
14	3-n-Butyl-C	0.05	(0.04–0.07)
15	3-n-Heptyl-C	0.07	(0.05–0.10)
16	3-n-Dodecyl-C	1.19	(0.99–1.45)
17	3-Palmitoyl-C	1.0†	
18	3-Benzoyl-C	0.05	(0.03–0.09)
19	3-(3,4-Dihydroxybenzoyl)-C	0.06	(0.04–0.07)

* In mM.

† 49.7 ± 0.9% inhibition at 1.00 mM, see text.

confidence limits in Table 3), yet repeating some measurements confirmed the original values. Compound No. 17 (palmitoyl-cianidanol) was another exception. In the concentration range 0.01–0.1 mM, a slight activation of the reaction was seen; only two concentrations were inhibitory, namely 0.50 mM (17.7 ± 0.9% inhibition) and 1.00 mM (49.7 ± 0.9% inhibition). The concentration of 1.0 mM was thus taken as the IC_{50} value.

As shown in Table 3, the IC_{50} values span more than a 20-fold range. The flavanone derivatives suggest that the hydroxylation pattern markedly influences their inhibitory potency, while a contribution from lipophilicity can be inferred from the results of the cianidanol derivatives. Structure–inhibition relationships will be discussed in more details later.

Kinetics of inhibition

To better understand the mechanism(s) of monooxygenase inhibition, four compounds among the most active and water-soluble ones were selected, namely naringenin (No. 5) as a representative flavanone, taxifolin (No. 7) as a representative flavanolol (flavanolols = 3-hydroxyflavanones), and 3-butyl-cianidanol (No. 14) and 3-(3,4-dihydroxybenzoyl)-cianidanol (No. 19) as representative cianidanol ether and ester, respectively. These compounds were used to investigate the kinetics of inhibition of a few metabolic reactions in liver microsomes of untreated and 3-methylcholanthrene-(3MC) treated rats.

The effect of the four compounds on two reactions in liver microsomes of untreated rats are documented in Table 4. For aminopyrine *N*-demethylase, the inhibitors were tested at a 0.1 mM concentration which excepting taxifolin is very close to the IC_{50} values (see Table 3). Very clearly, all four compounds inhibit aminopyrine *N*-demethylase in a non-competitive manner (similar K_m, lower V_{max} as compared to controls). This is illustrated in Fig. 1 for the two cianidanol derivatives. We have shown previously that the same behaviour is displayed by cianidanol itself [27]. The four flavonoids are less potent in inhibiting biphenyl 4-hydroxylase than aminopyrine *N*-demethylase, but the decrease in potency varies from minor (taxifolin) to 9-fold (naringenin). Interestingly, the potency ranking of the four flavonoids is different in the two reactions ($r = 0.412$). While taxifolin inhibits biphenyl 4-hydroxylation in a non-competitive manner as does cianidanol [27], the three other compounds display competitive inhibition (Fig. 2).

The selected flavonoids were further investigated for their ability to inhibit biphenyl hydroxylation in liver microsomes of 3MC-treated rats. Table 5 shows that the IC_{50} values towards biphenyl 2-hydroxylation are consistently 2–2.5 times lower than those towards biphenyl 4-hydroxylation ($r = 0.986$) while maximal velocities are 7.5 times slower and K_m values are similar. This suggests the involvement of comparable populations of isocytochromes P-450 in both reactions. The four inhibitors display identical IC_{50} values towards biphenyl 4-hydroxylation in untreated and 3MC-treated rats ($r = 0.998$), but the differing affinities here exclude the involvement of identical populations of isozymes in both reactions (see also the Discussion).

The kinetics of inhibition were evaluated at a 0.5 mM concentration of the inhibitors, i.e. close to the IC_{50} values. The three flavonoids investigated all inhibit biphenyl 4-hydroxylation in a non-competitive manner. In contrast, not all inhibitors behave non-competitively towards biphenyl 2-hydroxylation

Table 4. Effects of selected flavonoids on aminopyrine *N*-demethylation and biphenyl 4-hydroxylation in liver microsomes of untreated rats

	Naringenin	Taxifolin	3-Butyl-cianidanol	3-(3,4-Dihydroxybenzoyl)-cianidanol
Aminopyrine N-demethylation				
V_{max}*				
control values	5.65 ± 0.15	5.65 ± 0.15	4.94 ± 0.18	4.94 ± 0.18
0.1 mM inhibitor	2.08 ± 0.12	4.04 ± 0.20	1.78 ± 0.14	2.02 ± 0.22
K_m†				
control values	0.46 ± 0.03	0.46 ± 0.03	0.66 ± 0.05	0.66 ± 0.05
0.1 mM inhibitor	0.44 ± 0.05	0.49 ± 0.05	0.69 ± 0.11	0.61 ± 0.15
Biphenyl 4-hydroxylation				
IC_{50}†	0.89	0.67	0.23	0.32
(95% CL)	(0.63–1.25)	(0.15–2.88)	(0.19–0.29)	(0.27–0.39)
V_{max}*				
control values	1.10 ± 0.14	1.17 ± 0.17	1.10 ± 0.14	1.10 ± 0.14
inhibitor‡	0.90 ± 0.29	0.44 ± 0.04	0.98 ± 0.09	0.94 ± 0.16
K_m†				
control values	0.39 ± 0.10	0.38 ± 0.10	0.39 ± 0.10	0.39 ± 0.10
inhibitor‡	1.39 ± 0.83	0.37 ± 0.08	0.83 ± 0.13	1.14 ± 0.37

* nmol HCHO (mg prot)$^{-1}$ min^{-1}.

† mM.

‡ The concentrations were: naringenin and taxifolin, 1.00 mM; 3-butyl-C 0.10 mM; 3-(3,4-dihydroxybenzoyl)-C 0.50 mM.

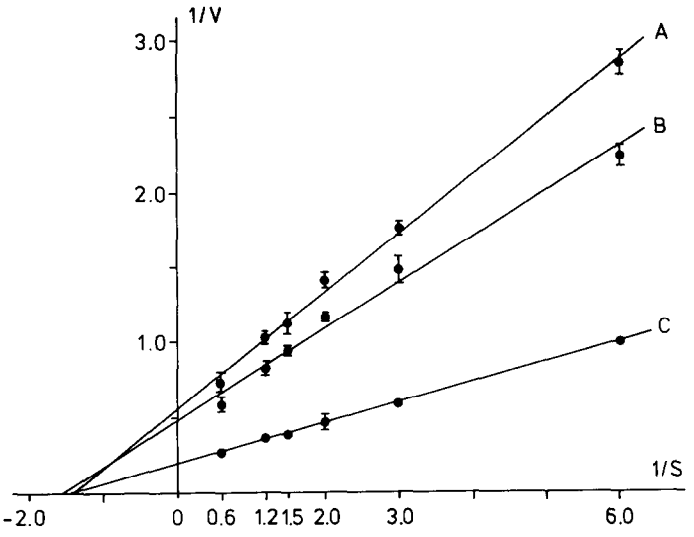


Fig. 1. Inhibition of aminopyrine *N*-demethylase by (A) 3-butyl-cianidanol (0.10 mM) and (B) 3-(3,4-dihydroxybenzoyl)-cianidanol (0.10 mM) in liver microsomes from untreated rats (C: control).

since 3-butyl-cianidanol shows a mixed type of inhibition (Fig. 3). Thus, each of the three inhibitors has a distinct inhibition pattern towards biphenyl 4-hydroxylation in liver microsomes of untreated and 3MC-treated rats, and towards biphenyl 2-hydroxylation in liver microsomes of 3MC-treated rats. Taxifolin is a non-competitive inhibitor in the three reactions, 3-(3,4-dihydroxybenzoyl)-cianidanol inhibits competitively the first reaction and non-competitively the other two reactions, while 3-butyl-cianidanol behaves as a competitive, non-competitive, and mixed inhibitor, respectively.

Effect of flavonoids on spectrally determined cytochrome P-450

To examine whether flavonoids inhibit cytochrome P-450 mediated reactions by degradation of the enzyme, we have measured the influence of selected compounds (those listed in Tables 4 and 5) on spectrally determined concentrations of cytochrome P-450 following a 30 min incubation in the presence of NADPH. The results (Table 6) show that incubating microsomes in the sole presence of 1 mM NADPH results in a 1/3 loss of cytochrome P-450. This loss is believed to be caused by lipoperoxidation, and Table 6 shows that it is prevented by each of the flavonoids investigated in an apparently dose-related manner. It is thus apparent that the flavonoids, far from degradating cytochrome P-450, protect it against NADPH-initiated lipoperoxidation. The same behaviour was already noted for cianidanol [27] and is certainly linked to their well-known antioxidant activity [e.g. 31, 32].

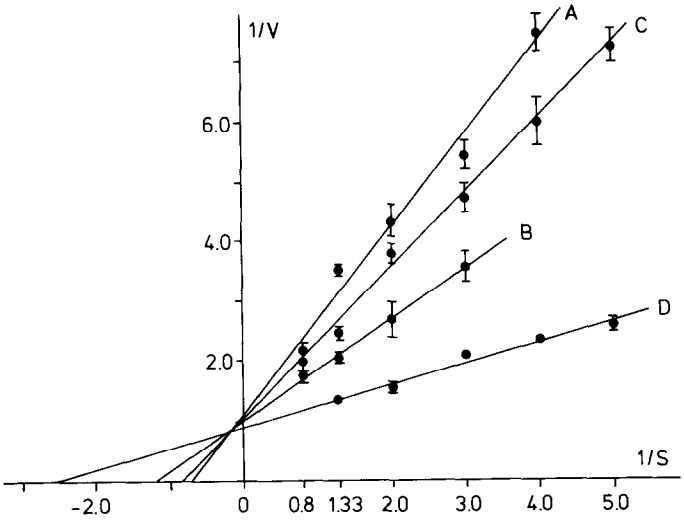


Fig. 2. Inhibition of biphenyl 4-hydroxylase by (A) naringenin (1.00 mM), (B) 3-butyl-cianidanol (0.10 mM), and (C) 3-(3,4-dihydroxybenzoyl)-cianidanol (0.50 mM) in liver microsomes from untreated rats (D: control).

Table 5. Effects of selected flavonoids on biphenyl 2-hydroxylation and 4-hydroxylation in liver microsomes of 3MC-treated rats

	Naringenin	Taxifolin	3-Butyl-cianidanol	3-(3,4-Dihydroxybenzoyl)-cianidanol
Biphenyl 2-hydroxylation				
IC_{50}*	0.40	0.29	0.15	0.13
(95% CL)	(0.31–0.52)	(0.14–0.62)	(0.10–0.23)	(0.08–0.21)
V_{max}†				
control values		0.288 ± 0.008	0.288 ± 0.008	0.288 ± 0.008
0.50 mM inhibitor	ND‡	0.135 ± 0.007	0.133 ± 0.028	0.115 ± 0.003
K_m*				
control values		0.18 ± 0.02	0.18 ± 0.02	0.18 ± 0.02
0.50 mM inhibitor	ND‡	0.18 ± 0.03	0.74 ± 0.31	0.18 ± 0.02
Biphenyl 4-hydroxylation				
IC_{50}*	0.90	0.71	0.28	0.33
(95% CL)	(0.68–1.20)	(0.37–1.40)	(0.14–0.56)	(0.25–0.45)
V_{max}†				
control values		2.18 ± 0.09	2.18 ± 0.09	2.18 ± 0.09
0.50 mM inhibitor	ND‡	1.27 ± 0.07	0.86 ± 0.05	1.28 ± 0.05
K_m*				
control values		0.15 ± 0.02	0.15 ± 0.02	0.15 ± 0.02
0.50 mM inhibitor	ND‡	0.14 ± 0.03	0.15 ± 0.03	0.16 ± 0.01

* mM.
† nmol HCHO (mg prot)$^{-1}$ min^{-1}.
‡ Not determined.

Spectrally examined interactions of flavonoids with cytochrome P-450

The interaction of a number of flavonoids with microsomal cytochrome P-450 has been examined spectrally. The investigated compounds are naringenin (No. 5), taxifolin (No. 7), the methyl, propyl- and butyl-cianidanol ethers (No. 12–14), and the benzoyl and 3,4-dihydroxybenzoyl-cianidanol esters (No. 18 and 19). For each of the cianidanol derivatives, a modified type II (reverse type I) spectrum was seen (peak at *ca.* 420 nm, trough at *ca.* 388 nm), in full analogy with cianidanol itself [27]. Naringenin and taxifolin in the presence of microsomes exhibited an induced absorption below 410 nm which masked the 388 nm trough. Nevertheless, the 420 nm peak was clearly visible, also indicating a modified type II binding spectrum for naringenin and taxifolin. We conclude that the investigated flavonoids, namely cianidanol and its ethers and esters, and the flavanones naringenin and taxifolin, all bind to native rat liver microsomal cytochrome P-450 as weak Fe(III) ligands, a behaviour typical of many alcohols and phenols [26].

The apparent spectral dissociation constant (K_s)

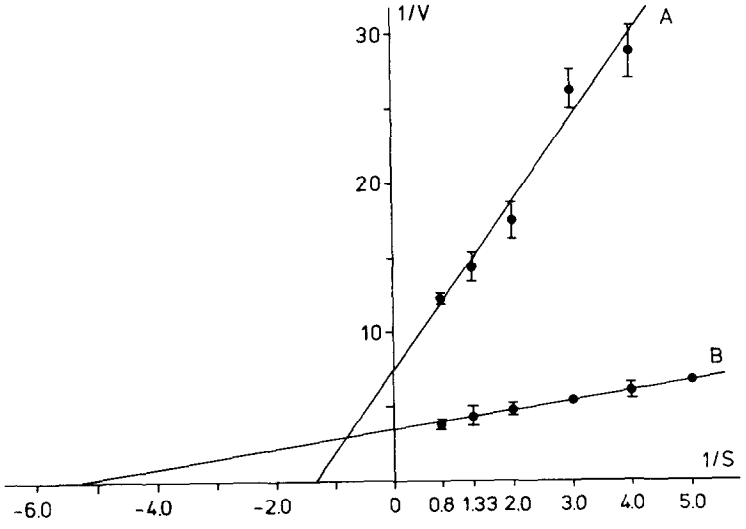


Fig. 3. Inhibition of biphenyl 2-hydroxylase by (A) 3-butyl-cianidanol (0.50 mM) in liver microsomes from 3MC-treated rats (B: control).

Table 6. Influence of flavonoids on spectrally determined concentrations of cytochrome P-450 in liver microsomes of untreated rats

Conditions	Cytochrome P-450 (% ± SD)*
No incubation	100.0 ± 1.3†
30 min incubation, no NADPH	96.8 ± 1.5
30 min incubation, 1 mM NADPH	67.5 ± 3.2
30 min incubation, 1 mM NADPH, naringenin 0.1 mM	71.3 ± 3.2
1.0 mM	82.2 ± 1.8
30 min incubation, 1 mM NADPH, taxifolin 0.1 mM	83.4 ± 1.3
1.0 mM	100.6 ± 6.6
30 min incubation, 1 mM NADPH, 3-butyl-C‡ 0.1 mM	96.8 ± 3.8
1.0 mM	114.6 ± 6.4
30 min incubation, 1 mM NADPH, 3-(3,4-dihydroxybenzoyl)-C‡ 0.1 mM	92.3 ± 6.5
1.0 mM	98.7 ± 7.0

* N = 3.
† 100% = 1.090 ± 0.014 mmol (mg prot)$^{-1}$.
‡ C = cianidanol.

has been measured for three well-soluble cianidanol derivatives, namely two ethers and one ester. As shown in Table 7, the K_s values of these compounds are lower than that of cianidanol, as discussed later.

DISCUSSION

Structure–inhibition relationships

All the 8 flavonoids investigated display a modified type II (reverse type I) spectrum when interacting with uninduced rat liver cytochrome P-450, i.e. they behave as weak Fe(III) ligands. It thus seems reasonable to postulate that this behaviour accounts for at least part of the inhibition of monooxygenase activities mediated by flavonoids. Indeed, for the 4 compounds which were soluble enough to be investigated, the pK_s and pIC_{50} values are fairly well intercorrelated ($N = 4$; $r = 0.795$). When all the 16 flavonoids investigated are considered, a 20-fold range is apparent in their inhibitory potency towards aminopyrine *N*-demethylation as assessed by IC_{50} values (Table 3). A plot of log *P* versus pIC_{50} values is informative in terms of structure–inhibition relationships (Fig. 4).

For the flavanone derivatives (No. 1–7), the inhibitory potency appears to be only marginally influenced by lipophilicity. This suggests that the differences in activity are mainly accounted for by the hydroxylation pattern. Thus, 5-hydroxylation (compound No. 2) does not increase the inhibitory potency of flavanone, in contrast to 7-hydroxylation (No. 3) which markedly increases it. The 5-hydroxy group forms a very strong intramolecular H-bond with the carbonyl group in the 4-position [e.g. 33, 34] and is thus less available for acting as an Fe(III) ligand. This is obviously not the case of the 7-hydroxy group, which occupies a sterically unhindered position and is thus believed to freely interact with the Fe(III) atom of cytochrome P-450. Interestingly, a 5-hydroxy group potentiates the inhibitory effect of the 7-hydroxy group (see No. 4), probably by slightly decreasing its acidity as seen in the compared UV spectra of 5-hydroxylated and 5,7-dihydroxylated flavanones [35]. Further hydroxylation in the 3′- and 4′-positions (No. 5 and 6) does not influence activity, suggesting that these hydroxyl groups do not contribute to the binding to cytochrome P-450. An additional hydroxylation in the 3-position is detrimental to activity, possibly because the resulting compound (taxifolin) is too hydrophilic. In short, these results suggest that the 7-hydroxy group is the principal contributor to the inhibitory effect of flavanones, and hence must be the hydroxyl group which preferentially interacts with Fe(III) due to its steric availability and adequate acidity.

In the case of cianidanol and its 3-*O*-alkyl and 3-*O*-acyl derivatives, a different picture emerges from Fig. 4. Indeed, activity increases with lipophilicity,

Table 7. Apparent spectral dissociation constant of some cianidanol derivatives for hepatic cytochrome P-450 from untreated rats

No.	Compound	K_s (mM ± SD)
11	Cianidanol (C)	0.50 ± 0.15*
12	3-Methyl-C	0.32 ± 0.07
13	3-Propyl-C	0.23 ± 0.05
19	3-(3,4-Dihydroxybenzoyl)-C	0.08 ± 0.01

* From Ref. 27.

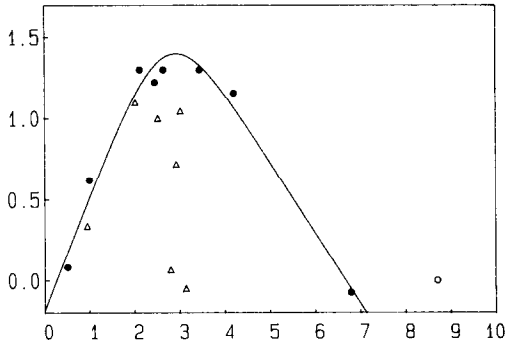


Fig. 4. Relationship between pIC_{50} (aminopyrine *N*-demethylase, Table 3) and lipophilicity for flavanones (△) and the cianidanol derivatives (●) except 3-palmitoyl-cian- (●) is described by equation 1 in the text (log *P* values in abscissa axis).

reaches a plateau, decreases and levels off for the most lipophilic compounds. Excluding the highly lipophilic 3-palmitoyl-cianidanol (No. 17), a statistically highly significant bilinear relationship (Fig. 4) is calculated:

$$pIC_{50} = 0.705(\pm 0.179) \log P - 1.14(\pm 0.25) \log(\beta P + 1) - 0.187(\pm 0.342)$$

$$N = 8; r = 0.988; s = 0.199; F = 53.8$$

$$\log \beta = 2.718; \log P_{optimum} = 2.92 \quad (1)$$

These results suggest that within the explored skeleton, substitution pattern and lipophilicity range, no compound with much increased inhibitory potency should exist.

Mechanism(s) of monooxygenase inhibition

The results of this study can also be interpreted in terms of the mode of interaction of investigated flavonoids with cytochrome P-450. As discussed above, the 7-hydroxy group is believed to be the one preferentially binding to the Fe(III) atom. The bilinear lipophilicity dependence displayed by the cianidanol derivatives is interpreted to mean that (a) inhibition is influenced by membranal transport processes, and/or (b) the inhibitors bind not only as Fe(III) ligands but simultaneously also to a neighbouring lipophilic site in the enzyme, presumably a substrate binding site. Hypothesis (b) is precisely the mechanism best explaining the inhibition of aniline hydroxylase by alcohols [36], i.e. a combination of electronic effects due to the interaction between the oxygen and Fe(III) atoms, and of lipophilic/steric influences due to the binding to an accessory site. Figure 5 summarizes these conclusions by presenting a hypothetical binding mode of cianidanol derivatives to cytochrome P-450. While Fig. 5 as such only applies to cianidanol derivatives, a combination of electrostatic and lipophilic interactions also appears reasonable for the binding of flavanones to cytochrome P-450.

The concept of dual interactions could explain the differences in the mechanism of inhibition (non-competitive vs competitive) seen between inhibitors and between monooxygenase activities (Tables 4 and 5). Indeed, a major difference between cytochrome P-450 isozymes lies in the properties of their lipophilic binding site as evidenced by differences in substrate selectivities. In the case of flavonoids, when the substrate/lipophilic interaction does not prevent a simultaneous strong ligand binding (O–Fe(III) interaction), non-competitive inhibition is expected. In some cases, an essentially lipophilic/substrate binding mode with little O–Fe(III) interaction must occur, resulting in a kinetically competitive inhibition. This is seen in the inhibition of uninduced biphenyl 4-hydroxylation by naringenin, 3-butyl- and 3-(3,4-dihydroxybenzoyl)-cianidanol (Table 4). Intermediate cases (mixed inhibition kinetics) are also to be expected according to this model, as indeed seen with the inhibition of 3MC-induced biphenyl 2-hydroxylation by 3-butyl-cianidanol (Table 5). Note however that a fundamentally different explanation is suggested by the work of Winzor *et al.* [37], who interpreted apparently non-competitive inhibition of cytochrome P-450 as resulting from the competitive inhibition of multiple isozymes.

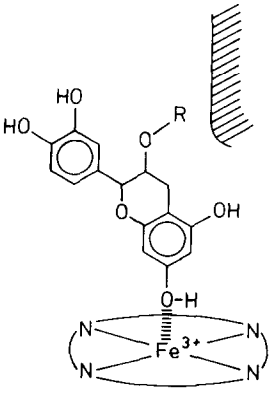


Fig. 5. Proposed mode of binding of cianidanol and its derivatives with cytochrome P-450, showing an electrostatic interaction (ligand binding) and a lipophilic interaction.

Therapeutic outlook

Considered globally, the IC_{50} values reported here are relatively weak ones (0.1–1 mM), and no noteworthy *in vivo* inhibitory effects are to be expected. IC_{50} values in the range 0.1–10 μM are usually found to be necessary for compounds to be effective *in vivo* inhibitors of toxication reactions leading to chemical carcinogenesis or toxicity. Exploratory studies confirm that the investigated flavonoids are essentially inactive in inhibiting *in vivo* drug oxidation.

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