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Selectivity of Neutrophil 5-Lipoxygenase and Cyclo-oxygenase Inhibition by an Anti-inflammatory Flavonoid Glycoside and Related Aglycone Flavonoids

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Abstract—A newly described plant-derived flavonoid, hypolaetin-8-glucoside, which has anti-inflammatory and gastroprotective actions in-vivo, and its corresponding aglycone, hypolaetin, have been compared with 14 other flavonoids for inhibition of eicosanoid generation via the 5-lipoxygenase and cyclo-oxygenase pathways in elicited rat peritoneal leukocytes stimulated with calcium ionophore. Comparable results for the inhibitory profiles of the compounds were obtained using either radioimmunoassay of released eicosanoids or radio-TLC of metabolites formed from labelled arachidonate, but there were differences in absolute potency of the inhibitors. Hypolaetin-8-glucoside was a weak but selective inhibitor of 5-lipoxygenase (IC50 56 μM vs 5-lipoxygenase; > 1000 μM vs cyclo-oxygenase), whereas the aglycone hypolaetin was a more potent and selective 5-lipoxygenase inhibitor (IC50 4·5 μM vs 70 μM). Results with three other glycoside/aglycone pairs confirmed that addition of sugar residues greatly reduces inhibitory potency whilst retaining selectivity against 5-lipoxygenase. Analysis of 12 aglycone flavonoids showed that inhibitory potency and selectivity against 5-lipoxygenase is conferred by the presence of 3'4'-vicinal diol (catechol) in ring B as part of a 3,4-dihydroxycinnamoyl structure as proposed by others and by incorporation of additional hydroxyl substituents. In contrast, "cross-over" of inhibitory selectivity is observed in compounds containing few hydroxyl substituents (with none in ring B) which are selective against cyclo-oxygenase. These results are discussed in relation to possible mechanisms of hypolaetin-8-glucoside's protective actions and the concept that these inhibitory effects of flavonoids cannot be ascribed to a unitary free radical scavenging action.

Flavonoids form a large class of plant-derived phenolic substances present in the diet and which have low mammalian toxicity (see Fig. 1 for general structures). They also exert many potentially beneficial actions at both organ and biochemical levels (Havsteen 1983). Of note are the anti-inflammatory actions of several related glycoside flavonoids (see Gabor 1979). For example, hypolaetin-8-glucoside (Fig. 1) has recently been identified as a biologically active component of *Sideritis mugronensis*, decoctions of which are used as an antirheumatic and digestive agent in Spanish folk medicine (Villar et al 1982). This flavonoid has been shown to exert both anti-inflammatory and antigastric ulcer actions in rats (Villar et al 1984a). Further studies of this compound revealed that neither the glycoside or its corresponding aglycone, hypolaetin, exerted significant cyclo-oxygenase inhibitory effects in microsomal systems in-vitro, thus establishing that the anti-inflammatory actions of the novel glycoside are not of the aspirin-like type (Alcaraz & Hoult 1985a). The two compounds also exerted inhibitory effects on the plant 15-lipoxygenase enzyme (Alcaraz & Hoult 1985b).

These results prompted a more extensive consideration of the possible inhibitory effects of these flavonoids on the pro-inflammatory leukocyte 5-lipoxygenase system, since it has now been well established that several members of the flavonoid class, notably quercetin, are relatively powerful inhibitors of the generation of leukotrienes and the slow reacting substance of anaphylaxis (eg, Baumann et al 1980a; Yoshimoto et al 1983; Corvazier & Maclouf 1985; Kimura et al 1985; Michel et al 1986; Horie et al 1986; Welton et al 1986; Wheeler & Berry 1986; Kimura et al 1987).

We have chosen rat peritoneal leukocytes for the test system as it has the additional relevant advantages that cellular activation (for example by the calcium ionophore A23187) causes the generation of both 5-lipoxygenase products (eg, leukotriene B_4) and cyclo-oxygenase products (eg, thromboxane (TX) B_2, prostaglandin (PG) E_2), and that two different methodological approaches can be used with equal facility for assay of enzymatic activities (see below). We have therefore compared hypolaetin-8-glucoside and hypolaetin with selected glycoside and aglycone flavonoids in order to investigate further the possible basis for hypolaetin-8-glucoside's anti-inflammatory action and to attempt to

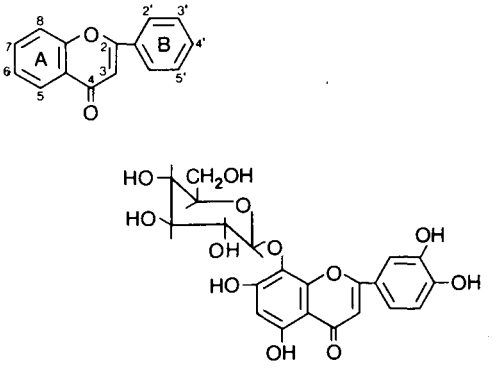

FIG. 1. Basic flavone nucleus showing numbering and lettering of the ring systems. The flavonol series contains -OH at position 3; the flavan and flavanone series have saturation at 2,3. The structure of hypolaetin-8-glucoside is shown below.

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define those structural requirements amongst the flavonoids which confer potency or selectivity as inhibitors of 5-lipoxygenase or cyclo-oxygenase.

Methods

Isolation of rat peritoneal leukocytes

Mixed peritoneal leukocytes were elicited from 200g female Wistar rats by an i.p. injection of 10 mL of a solution of 6% glycogen in sterile saline, followed 20 h later by 60 mL ice-cold modified Hanks balanced salt solution (HBSS) free of Ca^{2+} and Mg^{2+}. After 45 s massage, the peritoneal washing was removed, centrifuged at 400*g* for 10 min at 4°C and the contaminating erythrocytes in the pellet lysed after resuspension in a small volume of HBSS by adding 9 vol isotonic Tris-buffered ammonium chloride (0·83%, pH 7·2) for 10 min at 37°C. After a further centrifugation, the cells were resuspended in HBSS containing 1·26 mM Ca^{2+} and 0·9 mM Mg^{2+} plus 10 mM L-cysteine. Cell smears prepared using May-Graunwald/Giemsa stains showed that 70–75% of the cells were PMNs, and the remainder mononuclear cells. Viability based on trypan blue exclusion was better than 95%.

Stimulation of release of eicosanoids and their radioimmunoassay (RIA)

Aliquots of 0·5 mL mixed peritoneal leukocytes at 5×10^6 cells mL^{-1} were placed in 1·5 mL polypropylene microcentrifuge tubes and preincubated for 10 min at 37°C with the drug(s) of interest or its vehicle (generally methanol) added in 10 μL. To this was added 10 μL of ionophore A23187 dissolved in dimethylsulphoxide (DMSO) (final concentration of 1μM in most experiments, DMSO vehicle added to controls) for a further 10 min incubation. After the cells had been pelleted, the supernatants were decanted and frozen at −20°C. Aliquots of 15–50 μL of the thawed samples were assayed for PGE_2, TXB_2 (stable hydrolysis product of TXA_2), $PGF_{2\alpha}$ and 6-keto $PGF_{1\alpha}$ (stable hydrolysis product of prostacyclin) without extraction using antibodies and double antibody precipitation procedures described in Berry et al (1986). Amounts of leukotriene (LT)B_4 were measured by direct RIA of 2·5 to 50 μL aliquots using an antibody raised in rabbits with 5·4 nCi (11·2 pg) [^3H_n] LTB_4 tracer (Amersham International; sp. act. 168 Ci $mmol^{-1}$), and dextran-coated charcoal was used to separate bound from free ligand (Carey & Forder 1986). This antibody gave 0·3 and < 0·1% cross-reactivity to the 20-OH and 20-COOH metabolites of LTB_4; cross-reactivity to other hydroxyarachidonate metabolites and LTB_4 isomers was less than 0·5%, with the exception of all *trans* LTB_4 isomer I (2·2%).

Release of radiolabelled arachidonate metabolites and their measurement by radio-TLC

Aliquots of 0·5 mL leukocytes at 2×10^7 cells mL^{-1} were placed in 7 mL polypropylene tubes and preincubated with drugs or vehicle as described above; after this, 10 μL A23187 or the DMSO vehicle was added together with 10 μL of [1-^{14}C] arachidonic acid (Amersham International; sp. act. 58mCi $mmol^{-1}$) containing 0·125 μCi (0·66 μg, 4·3 μM). After 10 min incubation at 37°C the samples were acidified with 10 μL 0·5M citric acid to pH 3·5 and extracted twice with 1 mL ethyl acetate. Residues resuspended in methanol were applied to Merck aluminium foil-backed silica gel TLC sheets, type 5554, and chromatographed for 60 min (15 cm migration) in a solvent containing ethyl acetate–formic acid (80:1, v/v). Authentic standards were co-chromatographed (R_F values in brackets): arachidonic acid (0·68), 5-HETE (0·57), LTB_4 (0·46), TXB_2 (0·28), PGE_2 (0·23) and 6-keto $PGF_{1\alpha}$ (0·12). Phospholipids and di/triglycerides were present at the origin and solvent front, respectively. After localization of the relevant areas, the entire channel was sectioned and counted so that the proportions of metabolites corresponding to each type could be measured after correction to dpm values and deduction of appropriate control values deduced from blanks.

Materials

Hypolaetin-8-glucoside was extracted from *Sideritis mugronensis* as previously described (Villar et al 1984a). Its aglycone was prepared by enzymatic hydrolysis using rapidase C-40 (Societe Rapidase, Seclin, France) with final purification by preparative TLC on cellulose (Merck) using 30% (v/v) acetic acid as solvent. Gossypin was a gift from Dr A. G. R. Nair (Pondicherry University, India), and its aglycone, gossypetin, was prepared by enzymatic hydrolysis as above. Dazoxiben and BW755c (3-amino-1-[(*m*-trifluoromethyl)phenyl]-2 pyrazoline) were kind gifts from Pfizer Limited and Wellcome Research Laboratories, respectively. All other fine chemicals were from the Sigma Chemical Company, with the exception of flavone, 3-hydroxyflavone, galangin, fisetin and myricetin which were all from Aldrich Chemical Company.

Results

Profile of eicosanoids released from stimulated leukocytes

Table 1 shows that rat peritoneal leukocyte suspensions generate small amounts of both 5-lipoxygenase and cyclo-oxygenase products under resting (basal) conditions and much larger quantities after A23187 stimulation. Effects of A23187 are dose-dependent and maximal at 0·5 μM (Table 1), peaking within 2 min. Analysis by both RIA and radio-TLC methods shows similar profiles of the amounts of metabolites with $LTB_4 > TXB_2 > PGE_2 \approx$ 6-keto $PGF_{1\alpha}$. However, the proportional increases in amounts of eicosanoid release are greater when measured by RIA (e.g. 75- and 12-fold increases of LTB_4 and TXB_2) than when measured by radio-TLC (6-fold and 3-fold increases, respectively). In some measure this reflects the fact that there is already some stimulation of the cells caused by the addition of the exogenous radiolabelled arachidonate needed for the radio-TLC method (see "basal" values, top line Table 1); thus the proportional increase due to A23187 is likely to be smaller than in the case where the analysis is performed by RIA. In this latter situation, the basal values represent cells subjected to no additions at all, and release of eicosanoids is very low. Thus, addition of labelled arachidonate does measurably perturb the cells, although their ability to respond to further stimuli which activate eicosanoid generation is not impaired.

The mixed cell suspension taken 20 h after administration of glycogen and prepared as described under Methods contains 70–75% polymorphonuclear leukocytes and 25–30% mononuclear cells. Separation of these cells by density gradient centrifugation on Histopaque 1077 adapted from

Table 1. Generation of eicosanoids by A23187-stimulated rat peritoneal leukocytes.

	LTB_4	5-HETE	TXB_2	PGE_2	6K-$PGF_{1\alpha}$
Analysis by radio-TLC (% conversion of *AA)					
Basal release	1·4± 0·1	1·3±0·1	1·5±0·1	0·8±0·1	0·9±0·1
A23187, 1 μM	8·5± 0·4	10·2±0·8	4·2±0·1	1·4±0·1	1·5±0·1
Analysis by RIA (ng released/5×10^6 cells)					
Basal release	2·2± 0·7	—	1·9±0·5	0·6±0·3	0·4±0·2
A23187, 1 μM	165±19·8	—	23·1±3·4	5·1±1·2	3·6±0·2
basal	1·6± 0·5	—	2·7±0·3	—	—
A23187, 0·01 μM	13·7± 3·7	—	5·7±0·5	—	—
A23187, 0·05 μM	193·3±14·0	—	16·7±2·3	—	—
A23187, 0·1 μM	170·0±11·0	—	23·2±2·7	—	—
A23187, 0·5 μM	221·1±23·6	—	30·0±2·6	—	—
A23187, 1·0 μM	222·7±20·4	—	34·7±3·0	—	—
A23187, 5·0 μM	210·0±20·4	—	44·4±6·0	—	—

Results show mean±s.e.m. for at least 5 separate experiments (top two blocks), each performed in triplicate with replicates assayed singly (radio-TLC) or in duplicate (RIA). The dose-response experiment (lower block) is representative: triplicate incubations, each replicate assayed by RIA in duplicate. — = not tested.

the method of Boyum (1974) afforded pure (>95%) suspensions of the two cell types. Both kinds of cell responded to A23187 stimulation with similar profiles of eicosanoid generation (i.e. $LTB_4 > TXB_2 > PGE_2$).

Inhibition of eicosanoid generation by flavonoids

Table 2 and Fig. 2 show data for the dose-dependent inhibition of 5-lipoxygenase and cyclo-oxygenase in the mixed peritoneal leukocyte system by various flavonoids including hypolaetin-8-glucoside and its corresponding aglycone, hypolaetin. Both of these compounds were better inhibitors of the 5-lipoxygenase enzyme (Fig. 2A, Table 2), but the glycoside was considerably less potent. Three other glycoside/aglycone pairs were tested (rutin/quercetin, see Fig. 2B; gossypin/gossypetin; naringin/naringenin), confirming the pattern of weaker inhibition by the glycosides. However, with the exception of naringin, the glycosides retained the selectivity against 5-lipoxygenase shown by the aglycones.

Further analysis of the aglycone series (Table 2) showed that certain polyhydroxylated compounds, notably quercetin, hypolaetin, gossypetin and fisetin, had the most marked 5-lipoxygenase inhibitory actions in terms of potency and specificity. Position-specific substitution of the B ring appears to be critical: 3′4′ substitution enhances 5-lipoxygenase inhibitory activity, whereas 2′-substitution (e.g. morin) is detrimental. An interesting feature of the compounds with fewer hydroxyl substituents is the "cross-over" of selectivity: galangin, chrysin, 3-hydroxyflavone and flavone, whilst retaining some 5-lipoxygenase inhibitory activity, are more potent and therefore relatively selective inhibitors of cyclo-oxygenase.

Table 2. Inhibition of 5-lipoxygenase and cyclo-oxygenase by flavonoids.

Compound	Class	OH gp positions	5-Lipoxygenase (approx. IC50 value, μM)	Cyclo-oxygenase (approx. IC50 value, μM)	Comment
Aglycones					
Morin	flavonol	3,5,7,2′,4′	160	180	non-selective
Myricetin	flavonol	3,5,7,3′,4′,5′	13	56	LO-selective
Fisetin	flavonol	3,7,3′,4′	11	80	LO-selective
Kaempferol*	flavonol	3,5,7,4′	20	20	non-selective
Quercetin*	flavonol	3,5,7,3′,4′	3·5	16	LO-selective
Gossypetin	flavonol	3,5,7,8,3′,4′	10	not active	LO-selective
Naringenin*	flavanone	5,7,4′	16	100	LO-selective
Hypolaetin*	flavone	5,7,8,3′,4′	4·5	70	LO-selective
Galangin	flavonol	3,5,7	20	7	CO-selective
Chrysin	flavone	5,7	18	5	CO-selective
3-Hydroxy-flavone	flavonol	3	16	1	CO-selective
Flavone	flavone	(none)	32	8	CO-selective
Glycosides					
Rutin*	(flavonol)	3-sugar,5,7,3′,4′	45	ca450	LO-selective
Gossypin	flavonol	8-sugar,3,5,7,3′,4′	250	630	LO-selective
Naringin*	flavanone	7-sugar,5,4′	>500	320	non-selective
Hypolaetin-8-glucoside*	flavone	8-sugar,5,7,3′,4′	56	>1000	LO-selective

IC50 values based on tests at 6 concentrations; each in triplicate.
* indicates compounds tested also using radio-TLC methodology.
Inhibition of 5-lipoxygenase in terms of production of leukotriene B_4; inhibition of cyclo-oxygenase in terms of thromboxane B_2 (see legend to Fig. 2).

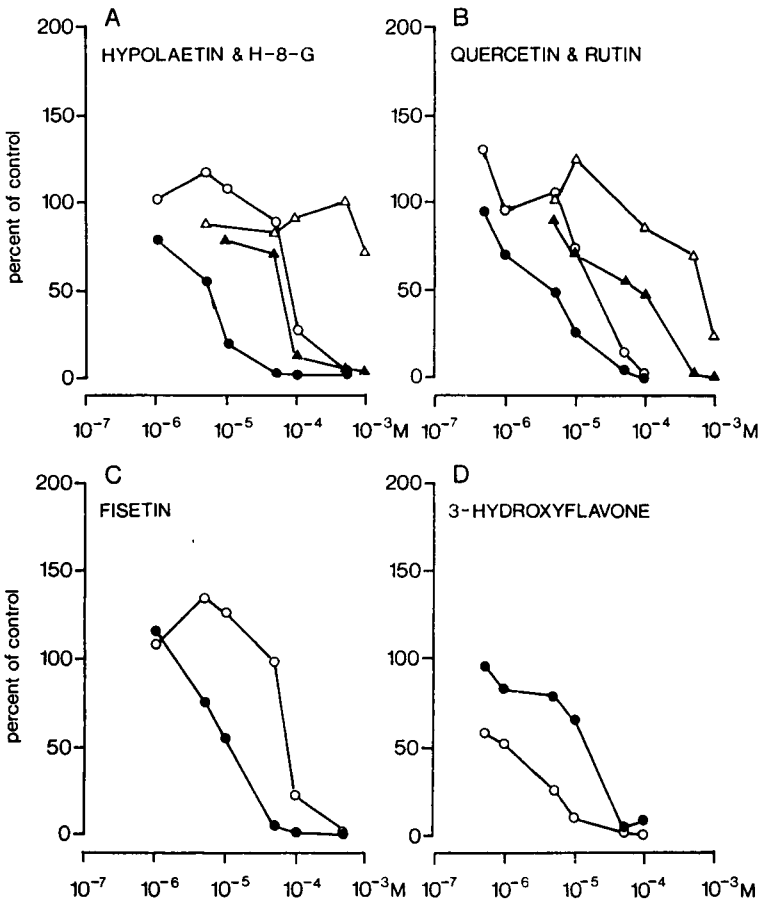

FIG. 2. Inhibition of rat peritoneal leucocyte 5-lipoxygenase (●, ○) and cyclo-oxygenase (▲, △) by aglycone flavonoids (filled symbols) and glycosylated flavonoids (open symbols). Inhibition of 5-lipoxygenase assayed in terms of production of leukotriene B_4; inhibition of cyclo-oxygenase assayed in terms of production of thromboxane B_2. Points show mean values obtained from triplicated determinations at each concentration, and the results are expressed as a percentage of the amount of immunoreactive LTB_4 (●, ○) or TXB_2 (▲, △) generated in control tubes not containing inhibitor. For clarity, s.e.m. bars not shown. H-8-G = hypolaetin-8-glucoside.

Tests with standard inhibitors yielded the expected results (approximate IC50 values for 5-lipoxygenase and cyclo-oxygenase, respectively): BW 755c (14 μM, 1 μM: cyclo-oxygenase selective); NDGA (0·8 μM, 5 μM: 5-lipoxygenase selective); indomethacin (inactive, 0·9 μM: cyclo-oxygenase selective); dazoxiben (> 100 μM, 0·5 μM: thromboxane synthetase selective).

Further studies on several of the flavonoids using the radio-TLC method confirmed the general conclusions about their inhibitory effects (those compounds tested in this way are indicated with * in Table 2). For example, hypolaetin-8-glucoside and hypolaetin selectively inhibited 5-lipoxygenase with the aglycone showing greater potency (approximate IC50 values versus 5-lipoxygenase and cyclo-oxygenase: hypolaetin-8-glucoside 1000 μM and > 1000 μM; hypolaetin 16 and 150 μM). Similar results were obtained for rutin/quercetin: rutin 325 μM and > 1000 μM; quercetin 7 and 40 μM. There was good agreement between inhibition of LTB_4 and of 5-HETE production using the radio-TLC method (5-HETE is a major product of rat leukocyte 5-lipoxygenase, see Table 1, which can be measured using radio-TLC by reference to an authentic standard, but for which we do not possess an immunoassay). However, it was notable that although the profiles of action of the inhibitors were similar using the radio-TLC methodology to those data obtained using RIA, the inhibitory potencies were always lower (compare these IC50 values with those derived from RIA experiments and shown in Table 2).

Discussion

Elicited rat peritoneal leukocytes serve as a convenient and useful system to investigate inhibition of both the 5-lipoxygenase and cyclo-oxygenase pathways of arachidonate metabolism. We have measured the inhibitory profiles of hypolaetin-8-glucoside and several related compounds. Hypolaetin-8-glucoside is a newly described flavonoid which has been extracted from *Sideritis mugronensis*, a herb used in Spanish folk medicine as an anti-inflammatory, antirheumatic and gastroprotective agent. The glycoside may not be the only biologically active constituent of the plant: for example, it contains a diterpenoid (borjatriol) which also has anti-inflammatory actions (Villar et al 1984b). Nevertheless, as hypolaetin-8-glucoside exerts a preferential, albeit weak, 5-lipoxygenase inhibitory action, this could play a part in its anti-inflammatory, antirheumatic actions in-vivo because products of arachidonate 5-lipoxygenation display powerful pro-inflammatory properties (Samuelsson 1983). Further,

hypolaetin, the aglycone derived from the naturally occurring glycoside, is a much more active 5-lipoxygenase inhibitor (Fig. 2, Table 2). If by analogy with other glycosylated flavonoids hydrolysis of the parent glycoside takes place after oral administration (Hackett 1986), then the released hypolaetin may contribute importantly to inhibition of 5-lipoxygenase in-vivo. This cannot be verified at present because the pharmacokinetic profile of hypolaetin-8-glucoside in mammals has not been established.

Our results also identify or confirm some other features concerning the inhibition of arachidonate metabolism by flavonoids. It is evident that their effects on 5-lipoxygenase and cyclo-oxygenase depend upon precise structural features, and that the relative effects of substitutions on inhibiting the two enzymes do not follow in tandem. Thus flavonoids with 3′4′-substitution in the B ring (catechols) with additional hydroxyl substituents in the body of the molecule are the most potent and selective inhibitors of 5-lipoxygenase, in the sense that compounds lacking these features are either less active 5-lipoxygenase inhibitors or are relatively more active against cyclo-oxygenase. In fact, all those flavone compounds with few hydroxyl substituents and lacking the 3′4′-vicinal diol grouping proved to be more active against cyclo-oxygenase (Table 2). This "cross-over" of inhibitory selectivity indicates that the compounds do not inhibit the two enzymes by a single mechanism.

The requirement for 3′4′-diol substitution in the B ring (giving rise in the case of the flavones to the 3,4-dihydroxycinnamoyl structure) has already been established as important for the inhibition by flavonoids of arachidonate lipoxygenases such as the plant 15-lipoxygenase (Wurm et al 1982), rat basophil 5-lipoxygenase (Yoshimoto et al 1983; Yamamoto et al 1984), platelet 12-lipoxygenase (Michel et al 1985; Welton et al 1986), as well as for inhibiting lipoxygenase action on linoleate in epidermal cell cytosols (Wheeler & Berry 1986). Moreover, 3′4′-substitution confers optimal inhibitory potency on the ability of members of the closely-related chalcone series to inhibit the epidermal 12-lipoxygenase (Nakadate et al 1985). Our results are consistent with all these studies. Further conclusions about flavonoid structure-activity relationships on lipoxygenase inhibition are that 3-hydroxylation (the flavonol series) is not obligatory for high potency (eg, hypolaetin, this study; cirsiliol and its derivatives, Yoshimoto et al 1983; Horie et al 1986); that methoxylation (a common feature of many plant flavonoids) is compatible with high activity if occurring on the A ring, but not if it modifies the B ring vicinal diol (Yoshimoto et al 1983; Yamamoto et al 1984); that 2′-hydroxyl substitution is unfavourable (see morin in this study; 2′-substituted Scutellariae flavonoids, Kimura et al 1985), and that glycosylation considerably reduces potency regardless of other structural features (this study; Landolfi et al 1984; Swies et al 1984; Michel et al 1985). All this points to specific structure-dependent interactions of these molecules with the lipoxygenase enzymes as the basis for inhibition, rather than a straightforward physiochemical effect as has sometimes been supposed.

Different structural requirements for cyclo-oxygenase inhibition operate (Table 2), in that B ring hydroxyl substitution reduces potency. It has been noted previously that substituted phenols, including vicinal diols, can substitute as cofactors for prostaglandin generation in cell-free systems (e.g. Baumann et al 1979) and that those compounds can enhance prostaglandin generation, especially if the subtrate concentration is high (Swies et al 1984). Potentiation of prostanoid output is also evident for the glycosylated compounds which are very weak cyclo-oxygenase inhibitors (Table 2), but which in certain intact tissue systems can augment prostacylcin output (Alcaraz & Hoult 1985a). This may contribute to the gastroprotective effect of such glycosides (e.g. Villar et al 1984a).

These effects of the flavonoids may depend upon their ability to behave as free radical scavengers in the context of the lipid peroxide intermediates which are involved both as initiators of the cyclo-oxygenase and lipoxygenase reactions, and as potentially toxic products of them (Hanel & Lands 1982; Egan et al 1976; Cleland 1984). Flavonoids are known to be capable of inhibiting lipid peroxidation (Younes & Siegers 1981; Slater et al 1987) and acting as scavengers of superoxide, hydroxyl and azide radicals (Baumann et al 1980b; Husain et al 1987; Bors & Saran 1987). The extent to which these properties may account for the divergent actions of the flavonoids on the two pathways of arachidonate metabolism considered in this paper, and the relationships to their protective effects in-vivo requires further rigorous analysis.

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