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Interactions of inhibitors of the lipoxygenase and cyclo-oxygenase pathways with a supplementary binding site on soybean lipoxygenase

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1 The oxygenation of [1-^{14}C]-arachidonic acid by a soluble soybean lipoxygenase (E.C.1.13.11.12) preparation was determined in the presence of various cyclo-oxygenase and lipoxygenase inhibitors. The results showed that several non-inhibitory compounds drastically blunted the inhibitory potency of potent lipoxygenase inhibitors.

2 Studies on the combined effects of a variety of structurally unrelated inhibitors of lipoxygenase, cyclo-oxygenase or both oxygenation pathways provided strong evidence for the existence of a supplementary binding site on soybean lipoxygenase which reduces the effective interactions of inhibitors with the catalytic site.

3 Thus several cyclo-oxygenase inhibitors (which do not inhibit at the lipoxygenase catalytic site), as well as low concentrations of lipoxygenase inhibitors, interact with this putative supplementary site and blunt the inhibitory efficacy of potent lipoxygenase inhibitors.

4 Although the degree of interaction with the catalytic site determines the absolute potency of inhibitors, the additional interaction at the putative supplementary binding site is also obligatory for inhibitory potency.

5 In this new multiple-site model the potent lipoxygenase inhibitors (e.g. acetone phenylhydrazone, phenidone) possess high affinities for both sites, whereas weak inhibitors and certain cyclo-oxygenase inhibitors (e.g. benoxaprofen, phenylbutazone, indomethacin) interact predominantly with the supplementary site on the lipoxygenase but lack affinity for the catalytic site.

Introduction

Besides prostaglandin biosynthesis another important pathway of oxygenation of arachidonic acid is the lipoxygenase reaction cascade which occurs both in mammalian and plant tissue (Bailey & Chakrin, 1981; Taylor & Morris, 1983). A comparison of these two enzymatic pathways shows a remarkable similarity of the initial reactions. The first step of oxygenation of the fatty acid precursor by soybean lipoxygenase involves abstraction of a hydrogen atom at C-13 and the introduction of a hydroperoxy group at carbon 15 (Hamberg & Samuelsson, 1967). In contrast, the first oxygenation catalyzed by cyclo-oxygenase involves hydrogen abstraction at C-13 but results in the formation of a peroxy radical at C-11 followed by isomerization and the introduction of a hydroperoxy group at C-15 to yield prostaglandin G (PGG) (Samuelsson, 1972). Although both soybean lipoxygenase and mammalian cyclo-oxygenase are lipoxygenase-type reactions, only cyclo-oxygenases are selectively inhibited by non-steroidal anti-inflammatory drugs (NSAIDs) whereas lipoxygenase activity is not affected by these agents (Baumann *et al.*, 1980; Bailey & Chakrin, 1981).

Recently, Kuehl *et al.* (1981) and Humes *et al.* (1981) demonstrated that certain analgesic-antipyretic agents apparently interact with two sites on the cyclo-oxygenase. The interaction of these inhibitors with the proposed supplementary binding site was suggested to explain the interactions of weak and potent cyclo-oxygenase inhibitors: drugs interacting more effectively with the supplementary site, which is obligatory for inhibitory action, than with the catalytic site, i.e. those of weak to moderate anti-cyclo-oxygenase activity, were capable of preventing inhibition of the enzyme by indomethacin. Since, in contrast to cyclo-oxygenases, soybean and

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mammalian lipoxygenases are not sensitive to indomethacin or almost all other NSAIDs, these authors suggested that the unique potency of 'aspirin-like drugs' in their inhibitory action on the cyclo-oxygenase is attributable to the presence of the putative supplementary binding site on the cyclo-oxygenase and its absence in the lipoxygenase.

In contrast to Kuehl *et al.* (1981) and Humes *et al.* (1981), in this paper evidence is presented to show that soybean lipoxygenase (E.C.1.13.11.12) does contain supplementary binding sites which, together with the catalytic site, determine the pharmacological profile of selective lipoxygenase inhibitors, selective cyclo-oxygenase inhibitors and dual inhibitors of both arachidonic acid peroxidating pathways.

Methods

Inhibition of lipoxygenase activity

Arachidonic acid lipoxygenation was carried out at 25°C for 10 min with shaking in a mixture containing 50 mM potassium phosphate (pH 8.0), [1-^{14}C]-arachidonic acid (38 nCi, 2.5 μM), soybean lipoxygenase (5 μg, 750 units) and test drugs in a final volume of 300 μl. If necessary, inhibitors were predissolved in ethanol, whereby the final concentration of the solvent did not exceed 1% (v/v) in the assay system. Appropriate blanks (heat-denaturated enzyme) and controls (ethanolic buffer instead of test drugs) were run through the same procedures. The combined test drugs were preincubated for 10 min at 25°C with the buffered enzyme before lipoxygenation was started by the addition of the radiolabelled fatty acid. The lipoxygenase reaction was stopped by the addition of 2.5 vol (750 μl) n-hexane into which the fatty acid is selectively extracted leaving the slightly more polar lipoxygenation products (predominantly 15-H(P)ETE according to Hamberg & Samuelsson, 1967) in the aqueous incubation mixture. This selective extraction procedure was repeated twice before the total amount of lipoxygenation products was quantified by liquid scintillation counting of the remaining buffer phase as previously reported (Knippel *et al.*, 1981). The validity of the extraction procedure was routinely monitored by comparative t.l.c. on silica gel (0.5 mm thick, E. Merck, Darmstadt, F.R.G.). Chromatograms were usually developed in diethylether/light petroleum/acetic acid (50:50:1, v/v). The relative yield of lipoxygenation products formed from a fixed amount of labelled arachidonic acid in the presence of test compounds was used to determine the efficacy of the lipoxygenase inhibitors. All IC_{50}-values and dose-response curves were calculated from at least four separate observations.

Chemicals

[1-^{14}C]-arachidonic acid (50 Ci mol^{-1}) was purchased from The Radiochemical Centre (Amersham, U.K.). Acetylsalicylic acid was a gift from Bayer AG, F.R.G., phenylbutazone was provided by Ciba-Geigy, Wehr, F.R.G., and benoxaprofen was from E. Lilly, U.K. Phenidone, nordihydroguaiaretic acid, aminopyrine, antipyrine, indomethacin, paracetamol, salicylic acid, methyl salicylate as well as lyophilized soybean lipoxygenase (150 units mg^{-1}) were obtained from Sigma Chemical Corp., St. Louis, Missouri, U.S.A. All other chemicals including the organic solvents and phenylhydrazine were provided by E. Merck AG, Darmstadt, F.R.G.

Acetonylacetone bisphenylhydrazone and acetone phenylhydrazone were synthesised in our laboratory from reagent grade materials according to standard procedures as recently described (Baumann & Wurm, 1982).

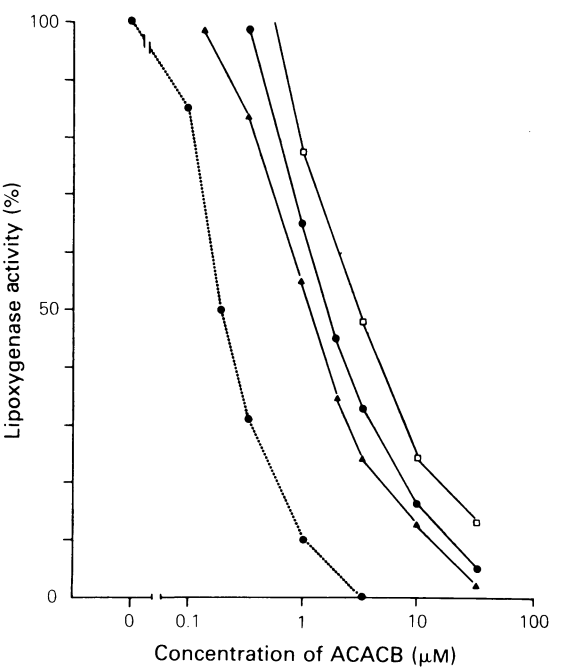

Figure 1 Nordihydroguaiaretic acid (0.01 mM, □——□), benoxaprofen (0.1 mM, ●——●) and phenylbutazone (1 mM, ▲——▲) reduce acetonylacetone bisphenylhydrazone (ACACB, ●.....●) inhibition of soybean lipoxygenase activity. Substrate and enzyme concentrations as given in Table 1; symbols represent means of five experiments.

Table 1 Action of lipoxygenase inhibitors on soybean lipoxygenase activity in the presence and absence of other cyclo-oxygenase and lipoxygenase inhibitors

Lipoxygenase inhibitor	*Drug added*	*Dose* (μM)	*Soybean lipoxygenase* IC_{50} (μM)
ACACB	—	—	0.19
	Phenidone*	10	1.10
	NDGA*	10	3.00
	Benoxaprofen	10	0.50
		33	0.60
		100	1.58
		1000	1.61
	Indomethacin	200	0.40
		1000	1.40
	Paracetamol	500	0.55
		1000	1.60
	Phenylbutazone	200	0.80
		1000	1.12
	Salicylic acid	500	1.00
		1000	1.80
	Methyl salicylate	500	1.20
		1000	2.00
	Acetylsalicylic acid	1000	0.21
	Aminopyrine	500	0.22
		1000	0.32
	Antipyrine	1000	0.18
ACPH	—	—	1.10
	Phenidone*	10	4.86
	NDGA*	10	1.80
	Benoxaprofen	200	2.00
		1000	2.11
	Indomethacin	200	5.30
		1000	9.44
	Paracetamol	500	6.21
		1000	7.94
	Salicylic acid	500	3.00
		1000	4.20
	Methyl salicylate	1000	5.30
Phenidone	—	—	50
	Benoxaprofen	200	52
		1000	112
	NDGA*	10	55
	Indomethacin	200	51
		1000	59
	Phenylbutazone	1000	199
	Paracetamol	1000	61
	Salicylic acid	1000	60
	Methyl salicylate	1000	71
NDGA	—	—	70
	Salicylic acid	500	75
		1000	100
	Methyl salicylate	500	210
		1000	300
	Benoxaprofen	500	280
		1000	350

Several lipoxygenase inhibitors and non-steroidal anti-inflammatory drugs reduce the inhibition of soybean lipoxygenase activity by acetonylacetone bisphenylhydrazone (ACACB), acetone phenylhydrazone (ACPH), phenidone or nordihydroguaiaretic acid (NDGA). Enzyme and substrate concentrations were 2500 units ml^{-1} and 2.5 μM arachidonic acid.

*Phenidone, NDGA IC_{50} values are 50 and 70 μM, respectively; all other drugs were not inhibitory ($IC_{50} > 2000$ μM). Values shown are the mean of four separate observations.

Results

The final aim of our studies was to elucidate whether selective cyclo-oxygenase inhibitors or selective lipoxygenase inhibitors or dual inhibitors of both pathways are capable of influencing the inhibitory actions of potent lipoxygenase inhibitors via interactions at a putative supplementary binding site on soybean lipoxygenase. We, therefore, first selected the two highly potent and rather selective lipoxygenase inhibitors, acetonylacetone bisphenylhydrazone (ACACB) and acetone phenylhydrazone (ACPH), which in previous experiments were shown to suppress soybean lipoxygenase activity 15 and 25 times more effectively than rat renal medulla cyclooxygenase (Baumann & Wurm, 1982). We also studied the effects of test compounds on the inhibition of lipoxygenase by phenidone as it has been used previously as a reliable inhibitor of both oxygenation pathways (Blackwell & Flower, 1978; Bailey & Chakrin, 1981).

As expected, ACACB, ACPH and phenidone in decreasing order of potency inhibited soybean lipoxygenase activity and therefore possess high affinities for the catalytic site of the enzyme (Table 1).

The IC_{50} of the lipoxygenase inhibitor ACACB was shifted to higher concentrations when the cyclooxygenase inhibitors indomethacin, paracetamol, salicylic acid, methyl salicylate and phenylbutazone (see Figure 1) were added to the lipoxygenation system, whereas aminopyrine, antipyrine and acetylsalicylic acid had only slight effects (Table 1). All these compounds lack any anti-lipoxygenase activity, since at concentrations of up to 2000 μM none of them significantly altered lipoxygenase activity (Table 1, footnote).

We then studied the influence of some well-known lipoxygenase inhibitors on the inhibitory efficacy of ACACB against the lipoxygenase enzyme. Since all such compounds suppress lipoxygenase activity by themselves, we studied the actions of phenidone, nordihydroguaiaretic acid (NDGA) and benoxaprofen at concentrations which had no detectable effects on soybean lipoxygenase activity in our assay system. Preliminary inhibition studies revealed that concentrations of up to 10 μM phenidone ($IC_{50} = 50\,\mu M$), 10 μM NDGA ($IC_{50} = 70\,\mu M$) and 100–1,000 μM benoxaprofen ($IC_{50} > 2000\,\mu M$) did not inhibit lipoxygenase activity. Benoxaprofen which has been claimed to be an inhibitor of both cyclo-oxygenases and lipoxygenases (Walker & Dawson, 1979; Dawson, 1980) did not exhibit any anti-soybean lipoxygenase activity; similar observations confirming the lack of an anti-lipoxygenase effect of benoxaprofen have recently been made by Verrando *et al.* (1983).

Low concentrations of phenidone or NDGA as well as relatively larger amounts of benoxaprofen which do not inhibit lipoxygenase activity were capable of drastically blunting the inhibitory efficacy of the potent lipoxygenase inhibitor ACACB (Figure 1, Table 1). When we extended our studies to the other ketone hydrazone lipoxygenase inhibitor ACPH and to the less potent inhibitors phenidone and NDGA, similar effects were found. Several selective cyclooxygenase inhibitors (paracetamol, indomethacin, phenylbutazone) and suitable derivatives (salicylic acid, methyl salicylate) which do not inhibit soybean lipoxygenase, as well as other well known lipoxygenase inhibitors (phenidone, NDGA, benoxaprofen) at doses which alone had no detectable effects on enzyme activity, shifted the IC_{50} values of ACACB, ACPH, phenidone and NDGA to higher concentrations (Table 1).

Discussion

Our data showed that a variety of selective cyclooxygenase inhibitors which do not interact with the catalytic site of soybean lipoxygenase as well as low, i.e. non-inhibitory, concentrations of either lipoxygenase inhibitors or dual inhibitors of both pathways were capable of blunting the inhibitory efficacy of potent (ACACB, ACPH) and weak lipoxygenase inhibitors (phenidone, NDGA).

According to the hypotheses of Kuehl *et al.* (1981) and Humes *et al.* (1981) who postulated supplementary sites on cyclo-oxygenases for the binding of inhibitors, but in contrast to these authors' conclusions that such additional sites are an exclusive feature of cyclo-oxygenases and are absent in the lipoxygenase, we postulate the existence of such supplementary sites at least on soybean lipoxygenase. Firstly, our unpublished studies (I. & J. Baumann) on a highly purified soluble lipoxygenase preparation of plant origin (homogeneous according to polyacrylamide gel electrophoresis) exclude the possibility that the second-added test compounds might compete for the transport of ACACB, ACPH, phenidone or NDGA to the active site on the enzyme. Secondly, because the interactions take place at levels of added compounds which do not inhibit lipoxygenation by themselves, mere competition at the catalytic site is rather improbable but cannot be absolutely excluded.

According to the arguments developed by Kuehl *et al.* (1981), which were later confirmed by other investigators for the inhibition of cyclo-oxygenases (Rajtar *et al.*, 1981; Cerletti *et al.*, 1981; 1983; Livio *et al.*, 1982; Rotilio *et al.*, 1984), our preferred model for the present results is one in which interaction of a second compound with the supplementary site reduces the action of a given anti-lipoxygenase drug as a lipoxygenase inhibitor. Since these interactions

take place at concentrations of test compounds which do not inhibit lipoxygenase activity at all, an allosteric alteration of the catalytic site due to the binding of suitable drugs to the supplementary site is not excluded but is, nevertheless, rather improbable.

Interestingly, benoxaprofen did not exhibit any anti-lipoxygenase activity in our assay system which contained a 15-lipoxygenase of plant origin. Whether mammalian 5- or 12-lipoxygenases are more sensitive to this drug has not been clarified at the present time, but studies on other better known lipoxygenase inhibitors, i.e. the ketone hydrazones, show that the soybean 15-lipoxygenase is quite similar to the human platelet 12-lipoxygenase (Wallach & Brown, 1981). Thus, we think that there remains considerable doubt about the anti-lipoxygenase efficacy of benoxaprofen.

Besides benoxaprofen, paracetamol and other cyclo-oxygenase inhibitors, especially non-inhibitory methyl salicylate, effectively reduced the inhibitory potency of the lipoxygenase inhibitors tested (Table 1). Since this compound has recently been shown to possess a high affinity for the supplementary binding site of the cyclo-oxygenase (Rotilio *et al.*, 1984), we tend to speculate that the supplementary binding sites of both cyclo-oxygenases and lipoxygenases show certain structural resemblances.

Surveying our results and the data provided by Kuehl *et al.* (1981) and Humes *et al.* (1981) we suppose that cyclo-oxygenases as well as lipoxygenases contain additional, perhaps rather similar, supplementary binding sites in addition to the well-recognized, rather different, catalytic sites. In such a model the pattern of interaction with both sites determines the final pharmacological profile of an individual agent resulting in either selective inhibition of cyclo-oxygenases, lipoxygenases or of both enzymes.

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