Pergamon

Biochemical Pharmacology, Vol. 47, No. 12, pp. 2259–2268, 1994.

0006-2952/94 $7.00 + 0.00

0006-2952(94)E0042-J

INVERSELY-CORRELATED INHIBITION OF HUMAN 5-LIPOXYGENASE ACTIVITY BY BAY X1005 AND OTHER QUINOLINE DERIVATIVES IN INTACT CELLS AND A CELL-FREE SYSTEM—IMPLICATIONS FOR THE FUNCTION OF 5-LIPOXYGENASE ACTIVATING PROTEIN

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(*Received* 15 *September* 1993; *accepted* 17 *January* 1994)

Abstract—A series of quinoline derivatives were analysed for the influence on leukotriene synthesis as a parameter for 5-LOX (EC 1.13.11.34) activity in a cell-free system of the 10,000 *g* supernatant of human PMNL (polymorphonuclear leukocytes). The ratios of the IC_{50} values for leukotriene synthesis inhibition in this cell-free system and in A23187-stimulated intact PMNL ranged from 1–1100. Consequently, plotting of the two values resulted in a random distribution ($r = -0.281$, $N = 18$) suggesting that no relationship between the inhibition of leukotriene synthesis in the cell-free system and in intact cells exists. At first sight this finding was not surprising since we have shown earlier that in intact cells this class of quinoline derivatives shares the same mode of action as MK-886, i.e. an indirect inhibition of 5-LOX activity by binding to FLAP. However, we found that the potency of these compounds in intact cells is strongly influenced by the *K* value (partition coefficient) which is a parameter for the ability of a substance to accumulate in a lipid (membrane) phase compared to the water phase. Therefore, the IC_{50} values for leukotriene synthesis inhibition in intact PMNL were corrected for the corresponding *K* value of the compounds and the resulting values again plotted against the IC_{50} values for inhibition of leukotriene synthesis in the cell-free system. As a result, a significant correlation ($r = -0.878$, $N = 18$) was obtained. In order to simplify this relationship the influence of the partition coefficient was eliminated by comparing compounds with about the same *K* value ($K = 7243 \pm 1646$, $N = 7$). As a result, the IC_{50} values for inhibition of leukotriene synthesis in the 10,000 *g* supernatant fraction (indicative for the affinity of the compounds to 5-LOX) and in intact cells (indicative for the affinity of the compounds to FLAP) were highly, but inversely correlated ($r = -0.992$). That means that a compound with a high affinity to 5-LOX will have a low affinity to FLAP and vice versa. We hypothesized that this pharmacologically obtained relationship could be indicative of a physiologically occurring equivalent. We therefore propose a model in which FLAP binds arachidonic acid as its physiological substrate with low affinity and allows 5-LOX to get access to its substrate (assuming a higher affinity of 5-LOX to arachidonic acid) after 5-LOX translocation from the cytosol to the membrane. In support of this model we provide evidence that arachidonic acid and other cis-unsaturated fatty acids, but neither a trans-unsaturated nor a saturated fatty acid, inhibit BAY X1005 binding to FLAP in intact human PMNL.

Key words: 5-lipoxygenase; 5-lipoxygenase activating protein; human polymorphonuclear leukocytes; partition coefficient; quinolines; BAY X1005

The leukotrienes have attracted attention as mediators of several inflammatory and allergic conditions because of their various pro-inflammatory properties [1]. The leukotrienes are formed along the 5-LOX¶ pathway of arachidonic acid metabolism, thus, 5-LOX has received considerable interest as a potential target for selective inhibitors which are expected to exert significant anti-inflammatory and anti-allergic effects [2]. Recently, we have described the quinoline derivative BAY X1005 as a potent and selective inhibitor of leukotriene synthesis *in vitro* [3] which proved to be effective in preclinical models of acute inflammation after oral application [4]. The inhibitory action of BAY X1005 and other quinoline derivatives on 5-LOX activity is mediated by the

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¶ Abbreviations: DMSO, dimethyl sulfoxide; EGTA, ethylene glycol-bis-[β-amino-ethyl ether]*N,N,N',N'*-tetra-acetic acid; FLAP, five lipoxygenase activating protein; HEPES, *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]; H(P)ETE, hydroxy(hydroperoxy) eicosatetraenoic acid; LOX, lipoxygenase; LTB_4, leukotriene B_4; PBS, Dulbecco's phosphate-buffered saline; PMNL, polymorphonuclear leukocytes; BAY X1005, (R)-2-[4-(quinolin-2-yl-methoxy)phenyl]-2-cyclopentyl acetic acid.

ability of these compounds to bind to a BAY X1005 high-affinity binding site which most probably is identical to FLAP based on the competition of BAY X1005 binding by MK-886 both in intact cells and in the microsomal fraction [5]. This latter leukotriene synthesis inhibitor served as a tool to isolate and identify FLAP [6] as an 18 kDa membrane protein necessary for leukotriene synthesis [7]. A critical step during the activation of 5-LOX in intact cells seems to be a Ca^{2+}-dependent translocation of the cytosolic enzyme to a membrane site [8] and since MK-886 both prevents and reverses this translocation step [9], FLAP has been proposed to serve as a 5-LOX anchoring protein at the membrane [10]. Therefore, MK-886 inhibits 5-LOX activity indirectly in contrast to the direct action of other well-known compounds like A-64077/zileuton [11] or AA-861 [12] on 5-LOX.

This study was undertaken to compare the influence of BAY X1005 and a series of structurally-related quinoline derivatives on 5-LOX activity in a cell-free system of human PMNL and in intact cells. The results obtained will be discussed, taking into account the observation that the potency of these compounds in intact cells is strongly influenced by their partition coefficients phospholipid/water which served as parameter for the affinity of the compounds to the lipid phase of the cell membrane.

MATERIALS AND METHODS

Chemicals. Ammonium thiocyanate, arachidonic acid, dextran (molecular mass 500,000 kDa), EGTA, ferric chloride hexahydrate, HEPES, linoleic acid, linolelaidic acid, linolenic acid, stearic acid and β-mercaptoethanol were purchased from Sigma Chemie (Deisenhofen, F.R.G.). LTB_4, 5-H(P)ETE and 15-HETE were obtained from Paesel and Lorei (Frankfurt, F.R.G.). Lecithine (egg yolk phosphatidylcholine) was from Lipoid KG (Ludwigshafen, F.R.G.). Ficoll-Paque was purchased from Pharmacia LKB (Freiburg, F.R.G.) and PBS tablets were either from Flow Laboratories (Meckenheim, F.R.G.) or Unipath (Wesel, F.R.G.). All other chemicals utilized were of analytical grade and were purchased from Merck (Darmstadt, F.R.G.). The reference compounds, A-64077 and AA-861, were synthesized at the Chemistry Department of Bayer AG (Wuppertal, F.R.G.).

Assay conditions. The isolation of PMNL from human blood (anticoagulated with sodium citrate) via dextran sedimentation, centrifugation on Ficoll-Paque and hypotonic lysis of red blood cells has been essentially performed as described [13]. Cells were stored in PBS/HEPES (137 mmol/L NaCl, 2.7 mmol/L KCl, 8.1 mmol/L Na_2HPO_4, 1.5 mmol/L KH_2PO_4, 10 mmol/L HEPES, pH 7.4) at 4°. The disruption of the cells by sonication and the preparation of subcellular fractions by differential centrifugation of the cell homogenate were essentially performed as described [5]. The experimental conditions testing the influence of the substances on 5-LOX activity in the 10,000 *g* supernatant fraction, as well as on BAY X1005 binding both in intact cells and in the cell-free system have been described in detail elsewhere [5]. Inhibition of A23187-stimulated LTB_4 synthesis in intact human PMNL was measured as described [3].

Stock solutions from the compounds were prepared in DMSO and from all fatty acid derivatives tested in ethanol so that the final concentration of the solvents in the assays never exceeded 0.5% (v/v). This concentration of solvents did not influence the parameters investigated.

Determination of partition coefficients. For the determination of the partition coefficients (K values) the compounds were dissolved in 1 N NaOH and diluted to the desired concentrations (5–100 μmol/L) using phosphate-buffered saline (10 mmol/L KH_2PO_4, 10 mmol/L NaCl, pH 7.0). The solutions were allowed to stand overnight and thereafter filtrated through 0.22 μm membrane filters (Millex GS, Millipore, Eschborn, F.R.G.) to remove any precipitate. The solutions were then added to the desired amount of lecithine, incubated first for 30 min, then stirred for 30 min on a magnetic stirrer at 700 rpm (to generate the liposomes) and incubated for 24 hr at 25° under an argon atmosphere. The concentrations of the lipid and the compound have to be chosen in such a way that the lecithine liposomes contain less than 1% (w/w) of the compound under equilibrium conditions, therefore making K independent of the concentration of the compound in the liposomes. The samples were then centrifuged first for 1 hr at 380,000 *g* at 25° and the resulting supernatants centrifuged for 30 min at 74,000 *g* at 4°. The supernatants after the second centrifugation were taken for measuring ultraviolet absorbance of the compounds using a diode array photometer (HP-8452A, Hewlett-Packard, Bad Homburg v.d.H., F.R.G.) and 5 cm quartz cuvettes. The obtained values were corrected for the absorbance in corresponding samples prepared without any compound addition (blanks). The K values of the compounds were calculated according to:

$$K = [A_o - (A_s - A_b)] \times M_w/(A_s - A_b) \times M_l;$$

where A_o = absorbance of the solution before incubation with lecithine; A_s = absorbance of the supernatant of the centrifuged sample; A_b = absorbance of the blank; M_w = wt of the water phase in the sample and M_l = wt of the lecithine in the sample.

Quantification of phospholipids. The analysis of phospholipids using a colorimetric determination was performed according to Stewart [14] with some minor modifications. Briefly, 0.9 mL aliquots (equivalent to 3×10^7 cells/mL) of the subcellular fractions to be analysed were transferred into 10 mL glass tubes containing 3.1 mL chloroform/methanol/0.1 N hydrochloric acid (1.5:1.5:0.1, by volume). After vigorous mixing (Vortex mixer), the glass tubes were centrifuged for 5 min at 550 *g*, then the chloroform phase (lower phase) was collected and evaporated to dryness under nitrogen. The residues of two identical samples were dissolved in 1.5 mL chloroform and mixed with 1.5 mL working solution (2.7 g ferric chloride hexahydrate and 3 g ammonium thiocyanate dissolved in 1 L distilled water). Then the glass tubes were again centrifuged for 5 min at 550 *g*. The absorbance of the chloroform phases was

read at 460 nm using an UV/VIS spectrophotometer (model Du-64, Beckman, München, F.R.G.). Under these conditions the absorbance of the corresponding buffer blank was very low and was substracted from all other samples. Using egg yolk lecithine as a reference phospholipid, the absorbance linearly increased (0–2.5) from 0–500 μg lecithine/1.5 mL chloroform.

Statistics. Results are expressed as means ± SD of the number of experiments (N) indicated. IC_{50} values were determined graphically or by computerized regression analysis of log concentrations (mol/L) versus inhibition (%).

RESULTS

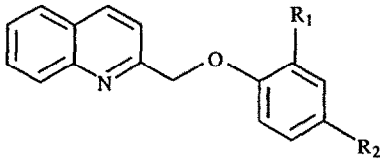
In a previous report [5], we have shown that the inhibition of 5-LOX activity by the quinoline derivative BAY X1005 in intact human PMNL and a cell-free system seems to be unrelated because the corresponding IC_{50} values differ by a factor of about 800. We now have extended these studies on a series of phenyl-substituted (quinolin-2-yl-methoxy)phenyl derivatives. For this purpose, we have investigated the influence of the compounds both on A23187-stimulated LTB_4 synthesis in intact human PMNL and on arachidonic acid-stimulated leukotriene synthesis in the 10,000 *g* supernatant of disrupted cells in the presence of Ca^{2+} and ATP. The amount of LTB_4 (about 0.5 nmol/10^7 cells) formed in intact cells comprises only about 20% of total 5-LOX metabolites. Nevertheless, the inhibition of formation of this metabolite reflects inhibition of 5-LOX activity since LTB_4 synthesis is inhibited in parallel with 5-HETE synthesis as demonstrated for BAY X1005 [3] and other structurally-related quinoline derivatives (data not shown). For the inhibition experiments in the 10,000 *g* supernatant an arachidonic acid concentration of 10 μmol/L was used. Under these conditions, the amount of leukotrienes formed (reflected by the synthesis of LTB_4 and its 6-*trans* isomers) comprises about 40% of total 5-LOX metabolites and is in the same order of magnitude (about 1 nmol/10^7 cell equivalents) compared to the amount of LTB_4 synthesized in A23187-stimulated intact cells.

The results of the inhibition studies are summarized in Table 1. With respect to the direct 5-LOX inhibitors AA-861 and A-64077, the IC_{50} values for inhibition of leukotriene synthesis in the two test systems differed roughly by one order of magnitude. For half of the quinoline derivatives tested (compound Nos. 1–9) and similarly, for BAY X1005, 100–1000-fold higher concentrations were required to inhibit 5-LOX activity in the cell-free system compared to intact cells. However, the same number of compounds was identified which inhibited leukotriene synthesis in the two test systems either in a comparable fashion to A-64077 and AA-861 (compound Nos. 10–16) or even with the same potency (compound Nos. 17 and 18). Actually, we do not know to what extent, if at all, the relatively potent direct effect of these compounds on 5-LOX activity in the cell-free system contributes to the inhibition of 5-LOX activity in intact cells. In the case of BAY X1005, the lack of effect on 5-LOX activity in the cell-free system was originally the starting point of the search for another mechanism of leukotriene synthesis inhibition in intact cells [5]. The main result of this effort was the finding that the IC_{50} values for LTB_4 synthesis inhibition in intact PMNL corresponds to the K_d value for binding to a high-affinity binding site identical to FLAP. This is true for BAY X1005 [5] and about fifty other structurally-related quinoline derivatives tested so far. With respect to the compounds investigated in this paper, it is important to stress that we could confirm the identity of the IC_{50} values for the above mentioned two parameters (Table 2) irrespective of the magnitude of the ratio of IC_{50} values for leukotriene synthesis inhibition in the cell-free system and in intact cells, respectively. That means that for the quinoline derivatives with "characteristics" of direct 5-LOX inhibitors (see compound Nos. 12 and 16–18, Tables 1 and 2) the IC_{50} value for LTB_4 synthesis inhibition in intact cells also reflects the affinity of the compounds to FLAP. By this ability, these compounds clearly differ from the reference direct 5-LOX inhibitors A-64077 and AA-861 which do not interact with FLAP [5].

The results shown so far suggest that for the quinoline derivatives, no relationship exists for inhibition of 5-LOX activity in either intact cells or the cell-free system, respectively. This finding is reflected by a random distribution ($r = -0.281$, $N = 18$) found by plotting the IC_{50} values obtained for leukotriene synthesis inhibition in these two test systems (Fig. 1A). At this stage an observation was decisive, namely that the potency of these compounds to inhibit leukotriene synthesis in intact human PMNL was positively influenced by the ability of the compounds to accumulate within the lipid matrix of the cells. This parameter was quantified by measuring the partition coefficient (K value) of the compounds in an artificial membrane (phospholipid)/water system (Table 1). We multiplied the IC_{50} value for inhibition of LTB_4 synthesis in intact cells by the corresponding K value and, as a result, the products significantly ($r = -0.878$, $N = 18$) correlated with the IC_{50} values for inhibition of leukotriene synthesis in the cell-free system (Fig. 1B). The participation of the K value in this relationship could be eliminated by selecting compounds, with about the same K value. Figure 1C demonstrates one group of compounds with similar K values. The IC_{50} values for inhibition of leukotriene synthesis either in intact cells or in the cell-free system were highly ($r = -0.992$, $N = 7$), but inversely correlated.

These results suggested a reciprocal affinity of the compounds to FLAP and 5-LOX, respectively. We were concerned, however, that the IC_{50} values for inhibition of leukotriene synthesis in the 10,000 *g* supernatant could have been influenced by the presence of FLAP in this fraction [5]. In addition, it was possible that the presence of lipids would influence the potency of the compounds to inhibit 5-LOX activity via the difference of the K values. We therefore selected four compounds covering the whole range of K values (see Table 1) and compared their influence on leukotriene synthesis in the 10,000 and 280,000 *g* supernatant, respectively. Although the latter fraction is devoid of FLAP [5] and

Table 1. Relationship between K values and inhibition of leukotriene synthesis in intact human PMNL (A) and in the 10,000 g supernatant fraction of human PMNL (B) concerning phenyl-substituted (quinolin-2-yl-methoxy)phenyl derivatives



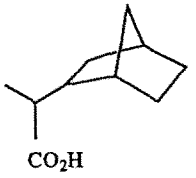
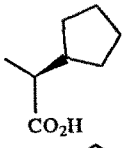
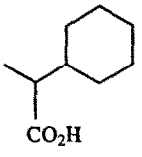
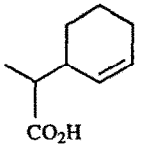
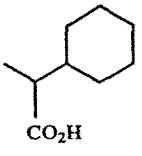
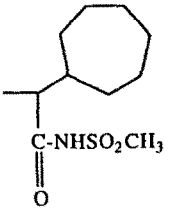
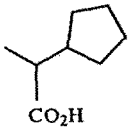
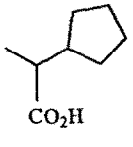
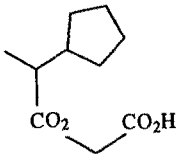
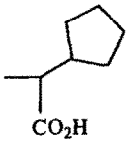
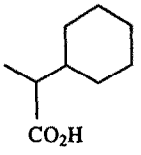
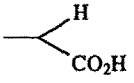
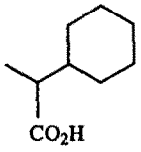
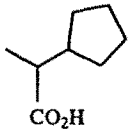
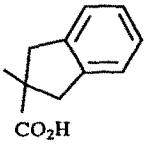
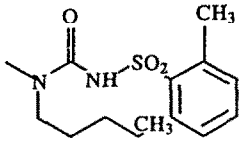
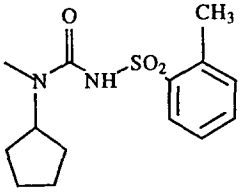
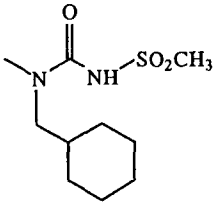
Compound	R_1	R_2	Inhibition of leukotriene synthesis A IC_{50} (μmol/L)	B IC_{50} (μmol/L)	K value	ratio IC_{50} B/A	$K \times IC_{50}$ (A)
1	H		0.1	112	5600	1120	560
2 BAY X1005	H		0.22	170	1820	773	400
3	H		0.11	71	7200	645	792
4	H		0.24	130	2300	542	552
5	F		0.15	46	6000	307	900
6	H		0.07	21	22,000	300	1540
7	F		0.69	106	2000	154	1380
8	Cl		0.25	32	8500	128	2125

Table 1.—*Continued.*

Compound	R_1	R_2	Inhibition of leukotriene synthesis A IC_{50} (μmol/L)	B IC_{50} (μmol/L)	*K* value	ratio IC_{50} B/A	$K \times IC_{50}$ (A)
9	H		0.64	73	2000	114	1280
10	nC_3H_7		0.14	8.4	56,000	60	7840
11	Br		0.35	11.5	38,000	33	13,300
12	nC_3H_7		3.6	87	825	24	2970
13	OCH_3		2.2	49	2800	22	6160
14	Br		0.81	11.4	8800	14	7128
15	H		0.88	10	5300	11	4664
16	H		0.6	4	50,000	7	30,000

IC_{50} values were calculated based on the inhibition of LTB_4 synthesis in A23187-stimulated human PMNL (A) and the inhibition of LTB_4 plus its 6-*trans* isomers in the arachidonate-stimulated 10,000 *g* supernatant fraction of disrupted human PMNL (B), respectively. Results are given as the mean of at least three independent experiments.

Table 1.—*Continued.*

Compound	R_1	R_2	Inhibition of leukotriene synthesis A IC_{50} (μmol/L)	B IC_{50} (μmol/L)	*K* value	ratio IC_{50} B/A	$K \times IC_{50}$ (A)
17	H		3.1	9	1200	3	3720
18	H		2.2	3.1	9300	1	20,640
A-64077			3.0	40	700	13	2109
AA-861			0.24	5.4	10,000	22	2400

compared to the 10,000 *g* supernatant, contains only trace amounts (about 15%) of phospholipids (Fig. 2), no difference in the IC_{50} values for inhibition of leukotriene synthesis was detected (Table 3).

Arachidonic acid is the physiological substrate of 5-LOX. Because of the above mentioned finding that the quinoline derivatives influence FLAP and 5-LOX in an inverse fashion, we were interested to see whether arachidonic acid is able to interact with FLAP in addition to serve as 5-LOX substrate. We tested this indirectly by competition of BAY X1005 binding to the high-affinity binding site. Figure 3 shows that in intact human PMNL BAY X1005 binding was concentration-dependently inhibited by the *cis*-unsaturated fatty acids arachidonic acid, linolenic acid and linoleic acid. There appeared to be a trend that the extent of inhibition increased with the degree of fatty acid unsaturation. In contrast, stearic acid as a representative saturated fatty acid had no effect whereas in the case of the *trans*-unsaturated linolelaidic acid no concentration dependency was obtained in the concentration range investigated. Under comparable conditions the IC_{50} value for inhibition of BAY X1005 binding by BAY X1005 itself was 0.24 μmol/L [5] which means that about 100-fold higher concentrations of arachidonic acid were necessary to inhibit BAY X1005 binding compared to BAY X1005 itself.

Under comparable conditions arachidonic acid had (relative to BAY X1005) almost the same inhibitory effect on BAY X1005 binding to the high-affinity binding site in the cell-free system (10,000 *g* supernatant) compared to intact cells (Fig. 4). In addition, at a concentration of 10 μmol/L all of the tested arachidonic acid metabolites (LTB_4, 5- and 15-H(P)ETE) showed a weaker inhibitory effect than arachidonic acid itself.

Table 2. Influence of a series of quinoline derivatives on LTB_4 synthesis and BAY X1005 binding in intact human PMNL

Compound No.	Inhibition of LTB_4 synthesis IC_{50} (μmol/L)	Inhibition of BAY X1005 binding IC_{50} (μmol/L)
2 (BAY X1005)	0.22	0.24
3	0.11	0.08
6	0.07	0.04
8	0.25	0.12
9	0.64	0.43
12	3.6	2.8
16	0.6	0.19
17	3.1	1.3
18	2.2	1.7

IC_{50} values were calculated based on the inhibition of A23187-stimulated LTB_4 synthesis, and the inhibition of BAY X1005 binding to the high-affinity binding site by using a [^{14}C]BAY X1005 concentration of 0.1 μmol/L. Compound numbers are taken from Table 1. Results are given as the mean of at least three independent experiments.

DISCUSSION

Recently, we have demonstrated that the inhibitory effect of BAY X1005 on the formation of 5-LOX-derived arachidonic acid metabolites in intact human

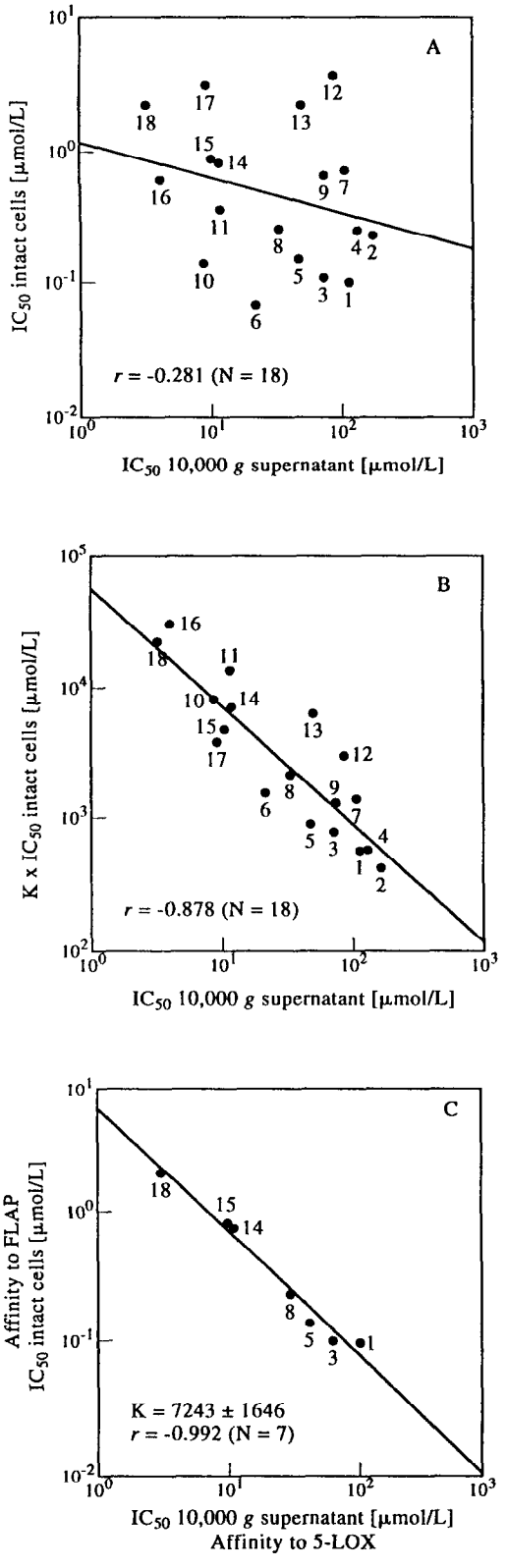


Fig. 1. Relationship between inhibition of leukotriene synthesis by a series of quinoline derivatives in a cell-free system versus intact cells (A), versus intact cells considering the *K* values of the compounds (B) and versus intact cells for compounds with about the same *K* value (C). (Data were taken from Table 1.)

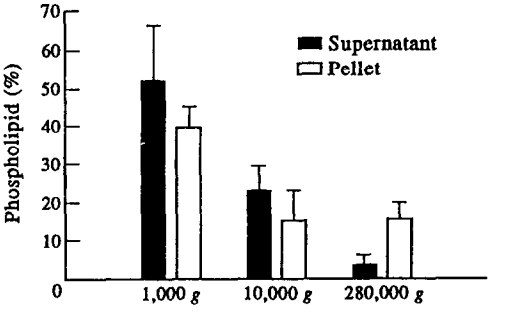


Fig. 2. Relative distribution of phospholipids in subcellular fractions of human PMNL. Human PMNL (3×10^7 cells/mL) were disrupted by sonication and the subcellular fractions generated by differential centrifugation of the homogenate. Phospholipid (100%) in the homogenate corresponds to 21 μg/10^7 cells taking egg yolk lecithine for calibration. The recovery of phospholipid after the individual centrifugation steps was greater than 80%. Results are given as means ± SD from four to seven independent experiments.

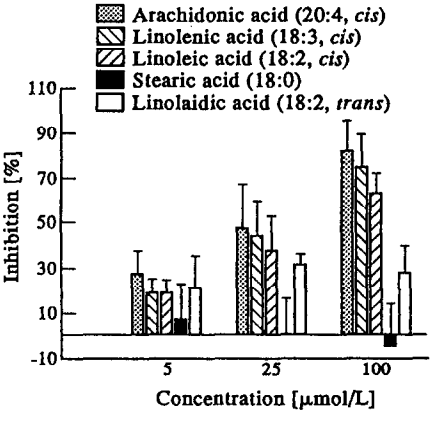


Fig. 3. Influence of fatty acids on BAY X1005 binding at the high-affinity binding site in intact human PMNL. Cells (1.25×10^7/mL) were incubated first for 5 min at 4° in the presence of 0.1 μmol/L [^{14}C]BAY X1005, then for a further 5 min in the absence or presence of the indicated concentrations of the fatty acids. The assays were stopped by centrifugation and aliquots of the supernatants were counted for radioactivity. Results are given as means ± SD from three independent experiments.

PMNL cannot be explained by a direct effect on 5-LOX. This conclusion was based on the observation that the concentration of BAY X1005 required to inhibit leukotriene formation in a cell-free system compared to intact cells was about 800-fold higher [5]. We have now extended these studies to a series of structurally-related quinoline derivatives (Table 1).

The 10,000 *g* supernatant fraction of human PMNL was chosen as an easily accessible source of 5-LOX. In addition, 5-LOX activity in this fraction was highly reproducible. For the direct 5-LOX inhibitors A-64077 and AA-861 (Table 1) the IC_{50} values for inhibition of leukotriene synthesis in intact cells and

Table 3. Comparison of leukotriene synthesis inhibition by a series of quinoline derivatives in the 10,000 and 280,000 *g* supernatant of disrupted human PMNL

Compound No.	*K* value	IC_{50} (μmol/L) 10,000 *g* supernatant	IC_{50} (μmol/L) 280,000 *g* supernatant
12	825	110	130
2 (BAY X1005)	1820	140	110
6	22,000	29	38
11	38,000	15	11

Aliquots (0.85 mL) of the supernatant fractions (equivalent to 10^7 cells/mL) were stimulated for leukotriene synthesis (LTB_4 plus its 6-*trans* isomers) by the addition of 10 μmol/L arachidonate in the presence of Ca^{2+} and ATP for 5 min at 30°. Under these conditions 0.98 ± 0.33 nmol and 0.46 ± 0.23 nmol metabolites were formed in the 10,000 and 280,000 *g* supernatant, respectively, as determined by reverse phase HPLC analysis. Compound numbers are taken from Table 1. IC_{50} values were determined from four to five independent experiments.

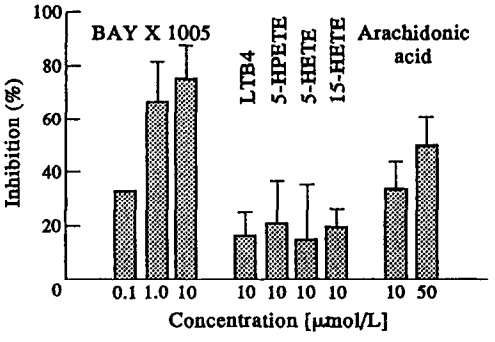


Fig. 4. Inhibition of BAY X1005 binding in the 10,000 *g* supernatant fraction of human PMNL by various arachidonic acid metabolites. Aliquots of the 10,000 *g* supernatant (equivalent to 3 × 10^7 cells/mL) were incubated first for 15 min at 4° with [^{14}C]BAY X1005 (0.1 μmol/L final concentration), then for a further 15 min in the absence or presence of the indicated concentrations of BAY X1005 or arachidonic acid metabolites, respectively. The separation of unbound and bound [^{14}C]BAY X1005 was achieved by ultrafiltration of the assays in Amicon centrifree micro-concentrators. Results are given as means ± SD from three independent experiments.

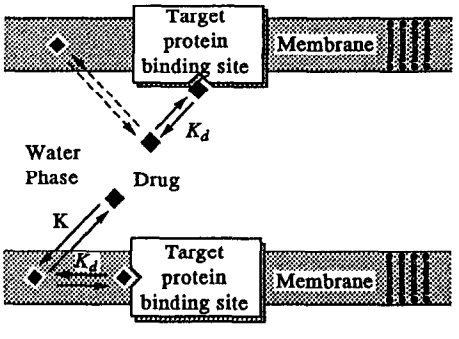


Fig. 5. Influence of the membrane affinity on the drug concentration at the binding site of the target protein. *K*, Membrane affinity = partition coefficient membrane/water; K_d, affinity to the target protein = dissociation constant. (Figure reproduced from Ref. 20 with permission from Raven Press)

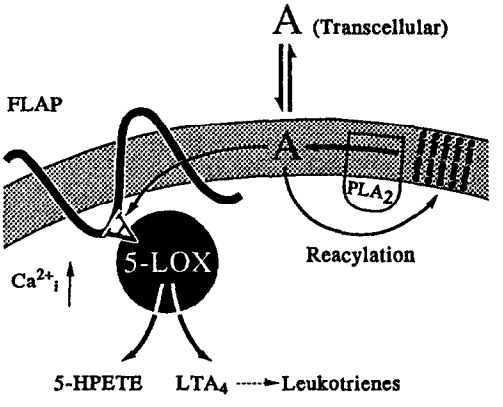


Fig. 6. Proposed scheme for FLAP-regulated leukotriene synthesis. PLA_2, phospholipase A_2; and A, arachidonic acid.

in the 10,000 *g* supernatant differed roughly by one order of magnitude. In contrast, with respect to the quinoline derivatives, for many of the compounds 100- or even 1000-fold higher concentrations were required to inhibit leukotriene formation in the cell-free system compared to intact cells (Table 1). This result was not surprising since we have shown earlier [5] that this class of compounds likely shares the same mode of action as MK-886 in intact cells, namely an indirect inhibition of 5-LOX activity by binding to FLAP. However, we also identified an equal number of quinoline derivatives which exhibited similar IC_{50} values for inhibition of leukotriene synthesis in intact cells and the cell-free system, respectively. This behaviour resembles that of the reference direct 5-LOX inhibitors A-64077 and AA-861, and raised the possibility that a direct effect of these quinoline derivatives on 5-LOX might contribute to leukotriene synthesis inhibition in intact cells. Although we cannot totally rule out this possibility, the fact (Table 2) that the IC_{50} values for inhibition of LTB_4 synthesis in intact cells are almost identical to the IC_{50} values for inhibition of BAY X1005 binding to the high-affinity binding site (FLAP) argues for the main involvement of FLAP in the inhibition of 5-LOX activity by the compounds.

Consequently, we regarded the IC_{50} values for inhibition of leukotriene synthesis in intact cells and in the 10,000 *g* supernatant as parameter for the affinity of the compounds to FLAP and 5-LOX, respectively. However, plotting of these two values resulted in a random distribution ($r = -0.281$, N = 18; Fig. 1A).

At this stage one observation was decisive, namely that the potency of these compounds in intact cells is strongly influenced by their partition coefficients (*K* values) in such a manner that the potency increases with higher *K* values (data not shown). The importance of the *K* values for the binding of a drug to its target protein is illustrated in Fig. 5. If the binding site of the target protein is within the water phase, the concentration of the drug in the water phase is in equilibrium with the drug at the binding site. In this case the drug concentration in the water phase is of relevance. If, however, the binding site of the target protein is located within the membrane or at the interfacial region between the membrane and the water phase the drug at the binding site will be in equilibrium with the concentration of the drug within the lipid phase of the membrane. This situation then would explain the positive influence of a high *K* value on the potency of the drug, and such a situation seems to hold true for the compounds binding to FLAP. Recent site-directed mutagenesis experiments performed in order to identify the amino acids critically involved in the binding of MK-886 to FLAP indicate that an aspartic acid residue located at the membrane/cytosol interface is indeed critical for the affinity of MK-886 to FLAP [15].

The most important biochemical consequence of this situation is that the *real* affinity of the quinoline derivatives to FLAP must be expressed by the *apparent* affinity to FLAP multiplied by the corresponding *K* value. As mentioned above, the apparent K_d values of these compounds are identical to the IC_{50} values for inhibition of LTB_4 synthesis in intact cells, therefore, the product $K \times IC_{50}$ shown in Fig. 1B, represents the real affinity of the compounds to FLAP. That the inhibitory effect of the compounds on 5-LOX activity in the cell-free system indeed reflects a direct interference with the substrate binding site of 5-LOX is supported by the fact that the inhibition of 5-LOX activity by BAY X1005 at a low arachidonate concentration can be partly overcome at higher arachidonate concentrations (data not shown) indicative for a competitive type of inhibition. Of course, kinetic measurements are necessary to prove this assumption, however, it is important to note that applying kinetic measurements the inhibition of soybean lipoxygenase by BAY X1005 seems to be largely competitive (Paul Marshall, personal communication). In addition, one should note that other components present in the 10,000 *g* supernatant fraction which might have been expected to influence the inhibition of 5-LOX activity by the mentioned compounds, namely FLAP and phospholipids, do not seem to play a role in this context since the IC_{50} values for inhibition of leukotriene synthesis in the 10,000 and 280,000 *g* supernatant fractions were almost identical for a couple of compounds tested (Table 3), although the latter fraction only contains minor amounts of both FLAP [5] and phospholipids (Fig. 2).

Therefore the graphs shown in Fig. 1B and C indicate an inverse relationship between binding of the quinoline derivatives to FLAP and 5-LOX, respectively. For example, a compound having a relatively high affinity to 5-LOX will have a relatively low affinity to FLAP and vice versa. In addition, there should be compounds with an intermediate affinity for both 5-LOX and FLAP, respectively, which are able to shuttle between these two proteins. This latter situation may be exemplified by nordihydroguaiaretic acid (NDGA) which inhibits LTB_4 synthesis in A23187-stimulated human PMNL with an IC_{50} value of 1.5 μmol/L but also inhibits BAY X1005 binding to the high-affinity binding site (FLAP) with an IC_{50} value of 4.5 μmol/L (data not shown). NDGA is the only so-called direct 5-LOX inhibitor identified so far which is able to interact with FLAP in addition to 5-LOX, which may be explained by the symmetric nature of this molecule resulting in an intermediate affinity for either protein.

Considering this situation, we think that there may exist a physiological equivalent, and propose a model (Fig. 6) according to which FLAP binds arachidonic acid released by phospholipases from membrane phospholipids. After the translocation of 5-LOX from the cytosol to the membrane has occurred, FLAP transfers arachidonic acid to 5-LOX which then metabolizes arachidonic acid into leukotrienes. According to this model 5-LOX must be supposed to have a high and FLAP a low affinity to arachidonic acid in order to allow 5-LOX to pick up arachidonic acid from FLAP. As a consequence of this model FLAP specifically would regulate the access of 5-LOX to its substrate. In support of this model we demonstrated that BAY X1005 binding at the high-affinity binding site was inhibited by arachidonic acid both in intact cells and in a cell-free system (Figs 3 and 4). This effect was not specific compared to other *cis*-unsaturated fatty acids (like linoleic or linolenic acid) but compared to a *trans*-unsaturated or a saturated fatty acid (Fig. 3). At present we cannot rule out the possibility that the inhibitory effect of the *cis*-unsaturated fatty acids is due to the membrane perturbating capability of these substances. Furthermore, the concentrations of arachidonic acid necessary to effectively inhibit BAY X1005 binding were rather high (25–100 μmol/L) and one may ask whether such concentrations occur under physiological conditions. No data are available for intracellular arachidonic acid concentrations after stimulation of human PMNL. However, recent measurements of arachidonic acid generation after pancreatic islet cell stimulation revealed intracellular concentrations of 50–100 μmol/L [16] which correspond to the concentrations of arachidonic acid used in this study. The observation that arachidonic acid itself is more effective than the tested 5- and 15-LOX-metabolites at inhibiting BAY X1005 binding (Fig. 4) argues against a preferential handling of any of these metabolites by FLAP. One could, for example, have hypothesized that FLAP is involved in the carrier system mediating leukotriene export from leukotriene synthesizing cells [17].

In summary, we have provided evidence that the

binding of BAY X1005 and other leukotriene synthesis inhibitors of the quinoline class to FLAP and 5-LOX occurs in an inverse manner. As a prerequisite for this finding, the recognition of the importance of the partition coefficient for the binding of these compounds to FLAP was crucial. Based on this pharmacologically obtained relationship and the ability of arachidonic acid to compete for BAY X1005 binding to FLAP, we propose FLAP as an arachidonic acid binding protein which may specifically regulate the access of 5-LOX to its substrate under physiological conditions. At the time of preparation of the present manuscript, two papers by Vickers *et al.* appeared which are totally in line with the conclusion drawn from our experiments. Firstly, by applying a novel photoaffinity analog of arachidonic acid, Mancini *et al.* [18] could demonstrate the labelling of FLAP expressed to high levels in Sf9 insect cells. Secondly, using the same cellular system, Abramovitz *et al.* [19] showed that FLAP stimulates the utilization of arachidonic acid by 5-LOX. Further work is necessary to proof the role of FLAP as an arachidonic acid transfer protein for 5-LOX, as well as to clarify the postulated participation of FLAP in the regulation of the 5-LOX translocation process [10].

Acknowledgements—Parts of this paper have been presented on the *Second International Symposium on Trends in Eicosanoid Biology* in September 1992, Interlaken, Switzerland. The proceedings of this meeting will be published in a forthcoming volume of *Advances in Prostaglandin, Thromboxane and Leukotriene Research* (Eds. Samuelsson B, Dahlen S-E, Fritsch J and Hedquist P) Raven Press. The authors thank Dr Pleiss (Bayer AG, Wuppertal, F.R.G.) for the synthesis of [^{14}C]BAY X1005 and Dr Fugmann (Bayer AG, Wuppertal, F.R.G.) for the synthesis of A-64077. The expert technical assistance of Mrs Frielingsdorf and Mrs Huber is highly appreciated.

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