Biochemical Pharmacology, Vol. 45, No. 1, pp. 101–111, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

MODE OF ACTION OF THE NEW SELECTIVE LEUKOTRIENE SYNTHESIS INHIBITOR BAY X 1005 {(*R*)-2-[4-(QUINOLIN-2-YL-METHOXY)PHENYL]-2-CYCLOPENTYL ACETIC ACID} AND STRUCTURALLY RELATED COMPOUNDS

A. HATZELMANN,* R. FRUCHTMANN, K. H. MOHRS,† S. RADDATZ† and R. MÜLLER-PEDDINGHAUS‡

Institute for Cardiovascular and Arteriosclerosis Research and † Chemistry Science Laboratories, Bayer AG, Wuppertal, Federal Republic of Germany

(*Received* 28 *May* 1992; *accepted* 21 *September* 1992)

Abstract—BAY X 1005 {(*R*)-2-[4-(quinolin-2-yl-methoxy)phenyl]-2-cyclopentyl acetic acid} has been demonstrated to be a potent inhibitor of leukotriene B_4 (LTB_4) and 5-hydroxyeicosatetraenoic acid (5-HETE) synthesis in various *in vitro* systems. Using mainly human polymorphonuclear leukocytes (PMNL) this study elucidates the mechanism of inhibition of 5-lipoxygenase (5-LOX, EC 1.13.11.34)-derived arachidonic acid metabolites by BAY X 1005. At concentrations of BAY X 1005 which almost totally inhibited the formation of 5-LOX-derived metabolites, both arachidonic acid release and platelet-activating factor synthesis were only modestly affected. This suggests that the inhibitory effect of BAY X 1005 is not due to a limitation of substrate availability for 5-LOX. Compared to the inhibition of leukotriene synthesis in intact human PMNL about 800-fold higher concentrations of BAY X 1005 were required to inhibit leukotriene formation in a cell-free system suggesting that the inhibitory effect of BAY X 1005 cannot be explained by a direct effect on 5-LOX. In an attempt to identify possible target proteins of BAY X 1005, [^{14}C]BAY X 1005 was used in binding studies under equilibrium conditions. The quantitative analysis of specific binding in intact human PMNL revealed two binding sites for BAY X 1005. Upon subcellular fractionation of these cells the BAY X 1005 high affinity binding site was localized in the microsomal fraction whereas the low affinity binding site was localized in the granule fraction. The K_d for BAY X 1005 binding to the high affinity binding site (0.165 μmol/L) was almost identical to the IC_{50} value for inhibition of LTB_4 synthesis (0.22 μmol/L). Furthermore, the IC_{50} values for competition of BAY X 1005 binding at the high affinity binding site were almost identical to the IC_{50} values for inhibition of LTB_4 synthesis in the case of BAY X 1005, 12 other structurally related quinoline derivatives and the reference compounds REV-5901, WY-50,295 and MK-886, but not in the case of the direct 5-LOX inhibitors A-64077 and AA-861. The analysis of BAY X 1005 binding in rat PMNL also revealed two binding sites. Whereas the low affinity binding site in rat PMNL exhibited a K_d similar to the human, the rat high affinity binding site showed a 5.5-fold higher affinity for BAY X 1005 compared to the human. This correlates well with the 8.5-fold higher sensitivity of rat versus human PMNL concerning inhibition of LTB_4 synthesis. Competition experiments verified that this relationship holds true also for 12 other quinoline derivatives as well as for REV-5901, WY-50,295 and MK-886. Taken together, these results indicate a causal relationship between the binding of BAY X 1005 to the high affinity binding site and the inhibition of leukotriene synthesis in human and rat PMNL. In addition, the localization of this binding site in the microsomal fraction and the competition of BAY X 1005 binding by MK-886 suggest that the target protein of BAY X 1005 mediating leukotriene synthesis inhibition is identical to five lipoxygenase activating protein.

The concentration of free arachidonic acid is believed to be maintained at low levels in resting cells [1]. Upon appropriate stimulation of phagocytes, arachidonic acid is released mainly by the action of phospholipase A_2 (PLA_2,§ EC 3.1.1.4) catalysing the hydrolysis of certain phosphoglycerides at the *sn*-2 position. This catalytic step is tightly linked to the synthesis of platelet-activating factor (PAF) which originates from acetylation of the lyso-phosphoglyceride [2].

The metabolism of arachidonic acid by 5-lipoxygenase (5-LOX, EC 1.13.11.34) leads to the formation of 5-hydroperoxyeicosatetraenoic acid (5-HPETE) and leukotriene A_4 (LTA_4) due to the two

* Present address: Byk Gulden Pharmaceuticals, Konstanz, F.R.G.

‡ Corresponding author: Prof. Dr Reiner Müller-Peddinghaus, Bayer AG, Pharma Research Center, Institute for Cardiovascular and Arteriosclerosis Research, P.O. Box 101709, D-5600 Wuppertal 1, F.R.G. Tel. (49) 202 36 4090; FAX (49) 202 36 8009.

§ Abbreviations: BSA, bovine serum albumin; DMSO, dimethyl sulphoxide; EGTA, ethylene glycol-bis-[β-aminoethyl ether]*N*,*N*,*N*′,*N*′-tetraacetic acid; FLAP, five lipoxygenase activating protein; HEPES, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulphonic acid]; H(P)ETE, hydroxy(hydroperoxy)eicosatetraenoic acid; 5-LOX, 5-lipoxygenase; LT, leukotriene; PAF, platelet-activating factor; PBS, phosphate-buffered saline; PLA_2, phospholipase A_2; PMNL, polymorphonuclear leukocytes.

intrinsic catalytic activities of this enzyme, namely the 5-dioxygenase and dehydratase (LTA_4 synthase) activity [3]. While 5-HPETE may be reduced to 5-hydroxyeicosatetraenoic acid (5-HETE) by endogenous peroxidase(s), LTA_4 is further converted depending on the cell type either to leukotriene B_4 (LTB_4) by a specific hydrolase or to the cysteinyl leukotriene LTC_4 by a glutathione-*S*-transferase [4]; LTC_4 may be further metabolized to leukotrienes D_4 and E_4.

Because of their various pro-inflammatory properties the leukotrienes have attracted attention as mediators of several inflammatory and allergic conditions [5]. Thus, inhibition of the formation of these metabolites by selective 5-LOX inhibitors is expected to exert significant anti-inflammatory and anti-allergic effects.

Inhibition of 5-LOX activity may be achieved either directly, e.g. by iron chelators (A-64077) [6] or by antioxidants (AA-861) [7], or indirectly as has been reported for the leukotriene synthesis inhibitor MK-886 [8]. This latter compound has no direct effect on the purified 5-LOX enzyme, but binds with high affinity to a membrane bound 18 kD protein termed five lipoxygenase activating protein (FLAP) [9]. FLAP seems to be essential for leukotriene synthesis in intact cells [10], and it has been proposed that FLAP might serve as a 5-LOX-anchoring protein at a membrane site [11]. This hypothesis is based on the observation that 5-LOX Ca^{2+}-dependently translocates from the cytosol to a membrane site upon stimulation [12] and that MK-886 both prevents and reverses this translocation process [13].

The quinoline derivative BAY X 1005 has been described as a potent and selective inhibitor of LTB_4- and 5-HETE-synthesis in various *in vitro* systems including human and rat PMNL,* and proved to be effective in preclinical models of acute inflammation after oral application.† The experiments described in this paper have been performed in order to elucidate the mechanism of action of 5-LOX inhibition by BAY X 1005.

MATERIALS AND METHODS

Chemicals

Arachidonic acid, A23187, bovine serum albumin (BSA) (No. A-7030), 4-bromophenacyl bromide, chlorpromazine, dextran (M_r 500,000), EDTA, EGTA (ethylene glycol-bis-[β-aminoethyl ether] *N,N,N',N'*-tetraacetic acid), HEPES (*N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulphonic acid]), β-mercaptoethanol and prostaglandin B_2 were purchased from Sigma Chemie (Deisenhofen, F.R.G.); all other eicosanoids were from Paesel + Lorei (Frankfurt, F.R.G.). [1-^{14}C]Arachidonic acid (1.85–2.2 GBq/mmol) was obtained from Amersham Buchler (Braunschweig, F.R.G.); [^{3}H]acetic acid (sodium salt, 70.3 GBq/mmol) and [^{3}H]PAF (1-*O*-octadecyl, 5.2 TBq/mmol) from Du Pont de Nemours (Bad Homburg, F.R.G.). Ficoll-Paque was purchased from Pharmacia LKB (Freiburg, F.R.G.) and PBS (Dulbecco's phosphate-buffered saline) tablets either from Flow Laboratories (Meckenheim, F.R.G.) or from Unipath (Wesel, F.R.G.). All other chemicals and solvents utilized were of analytical grade and were purchased from Merck (Darmstadt, F.R.G.).

The inhibitors used were synthesized at the Chemistry Department of Bayer AG (Wuppertal, F.R.G.). The specific activity of [^{14}C]BAY X 1005 ranged from 0.7 to 1.5 GBq/mmol. Stock solutions from the compounds were prepared in dimethyl sulphoxide (DMSO) so that the final DMSO concentration in the assays never exceeded 0.5% (v/v).

Cell isolation

The isolation and characterization (purity, viability) of rat peritoneal as well as PMNL from human blood (anticoagulated with sodium citrate) via dextran sedimentation, centrifugation on Ficoll-Paque and hypotonic lysis of red blood cells [14] have been essentially performed as described.

For the preparation of red blood cells, human blood (anticoagulated with sodium citrate) was supplemented with 1 mmol/L EDTA (final concentration) and centrifuged for 15 min at 300 *g*. The resulting sediment was diluted 1:2 (v/v) with 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) supplemented with 1 mmol/L EDTA, and 30 mL of the cell suspension were layered on top of 10 mL Ficoll-Paque. Red blood cells were sedimented by centrifugation for 30 min at 300 *g* and washed in PBS/HEPES. The red blood cells were contaminated with the physiologically occuring percentage of granulocytes (≤0.1%).

All cells were stored in PBS/HEPES at 4°.

Subcellular fractions of human PMNL

About 10–20 mL of the cells (3×10^7/mL) were suspended in PBS/HEPES (pH 8.2) supplemented with EGTA, Mg^{2+} and β-mercaptoethanol (1 mmol/L each) and sonicated for about 4 min at 4° in a Branson sonifier/model 250 (Heinemann, Schwäbisch Gmünd, F.R.G.) at an output control of 1 and a duty cycle of 10.

The cell homogenate was successively centrifuged for 10 min at 1,000 *g*, then for 15 min at 10,000 *g* (Sigma 2K15, Osterode, F.R.G.) and finally for 30 min at 280,000 *g* in a L7-55 ultracentrifuge (Beckman, München, F.R.G.). All centrifugation steps were performed at 4°. The resulting sediments were resuspended to homogeneity in an equivalent volume of buffer using manual glass homogenizers.

Arachidonic acid release

Human PMNL (2×10^7 cells/mL, 50 mL tubes) were incubated in PBS/HEPES containing Ca^{2+} and Mg^{2+} (1 mmol/L each) with 2 μmol/L [1-^{14}C]-arachidonic acid for about 30 min at 35°. All further

* Fruchtmann R, Mohrs K-H, Hatzelmann A, Raddatz S, Fugmann B, Junge B, Müller-Peddinghaus R and Horstmann H, *In vitro* pharmacology of BAY X 1005, a new inhibitor of leukotriene synthesis, submitted.

† Müller-Peddinghaus R, Kohlsdorfer C, Theissen-Popp P, Fruchtmann R, Perzborn E, Beckermann B, Bühner K, Ahr HJ and Mohrs K-H, BAY X 1005, a new inhibitor of leukotriene synthesis: *in vivo* inflammation pharmacology and pharmacokinetics, submitted.

steps were performed at room temperature. The cell suspension was centrifuged for 5 min at 130 *g*, and the cells were then resuspended in the above-mentioned buffer supplemented with 2% BSA and allowed to stand for about 10 min. Thereafter, the cells were again centrifuged for 5 min at 130 *g*.

PMNL ($1.5–2 \times 10^7$ cells/mL) were then suspended in PBS/HEPES containing Ca^{2+} (1.5 mmol/L), Mg^{2+} (1 mmol/L) and 2% BSA. Aliquots of the cell suspensions were preincubated in the absence or presence of the test compounds for 5 min at 30° and then stimulated with A23187 (resulting in a final concentration of 5 μmol/L) for 20 min. The incubations were stopped by centrifugation of the cells for 10–15 sec at 15,000 *g* in an Eppendorf centrifuge, and 0.9–0.95 mL aliquots of the supernatants were removed.

Arachidonic acid metabolites were extracted from the supernatants of the samples with 4 mL ethyl acetate at pH 3–4 by vigorous mixing (Vortex mixer) in 10 mL glass tubes. After centrifugation for 5 min at 550 *g* the organic phases were removed and 0.5 mL aliquots were counted for radioactivity in a liquid scintillation counter (LS 5000 TD/C, Beckman). The remaining extracts were evaporated to dryness under nitrogen and the residues dissolved in 30–50 μL chloroform/methanol (80:20, v/v) for TLC analysis (aluminium plates 20 x 20 cm, silica gel 60; Merck) as described [15]. Chromatograms were counted on a TLC linear analyser (LB 284, Berthold, Wildbad, F.R.G.), and the relative amounts of arachidonic acid metabolites calculated by the peak areas and corrected according to the radioactivity in the aliquots.

PAF synthesis

The incubation conditions for measuring PAF synthesis were identical to those described above except that all assays were preincubated in the presence of 0.74 MBq/mL [^{3}H]acetate.

The incubations were stopped by transferring 1 mL aliquots of the cell suspensions into 10 mL glass tubes containing 3 mL chloroform/methanol (1:1, v/v). After vigorous mixing (Vortex mixer) the glass tubes were centrifuged for 5 min at 550 *g*, then the chloroform phases (lower phase) were collected and evaporated to dryness under nitrogen. The recovery of PAF was ≥80% under these conditions. The residues were dissolved in 50 μL chloroform/methanol (80:20, v/v) and 25 μL aliquots were taken for TLC analysis of [^{3}H]acetyl containing PAF (plates see above) in a solvent system of chloroform/methanol/acetic acid/water (50:25:8:4, by vol.) as described by Gomez-Cambronero *et al.* [16]. Chromatograms were evaluated using a TLC linear analyser as described above and PAF was identified by comparison with the R_f value of a reference standard.

5-LOX activity in the 10,000 g supernatant fraction of human PMNL

The incubations were performed at pH 7.3. For this purpose the 10,000 *g* supernatant (equivalent to 3×10^7 cells/mL) obtained as described above was diluted 1:2 (v/v) in an equivalent PBS/HEPES buffer, pH 6.5, which resulted in a final pH of 7.3. Aliquots (0.85 mL) of the fractions were preincubated for 5 min at 30° in the presence of ATP (3 mmol/L), Ca^{2+} (1.1–1.2 mmol/L) and the inhibitor under investigation and then stimulated by the addition of arachidonate for 5 min. A stock solution of arachidonic acid was prepared in NaOH (50 mmol/L) and diluted 1:100 (v/v) in the assays resulting in a final arachidonate concentration of 10 μmol/L.

The assays were stopped by the addition of 0.85 mL cold methanol containing EGTA (2 mmol/L), HCl (20 mmol/L) and prostaglandin B_2 (500 ng) as internal standard, and allowed to stand for at least 30 min at −20°. Thereafter, the samples were centrifuged for 5 min at 15,000 *g* in an Eppendorf centrifuge and the supernatants were purified on disposable C_{18} extraction cartridges (Bakerbond SPE, 3 mL) using a vacuum extraction system (SPE-21) from Baker Chemikalien (Groß-Gerau, F.R.G.). The cartridges had been conditioned by rinsing first with 3 mL of methanol and then with 3 mL of water. After adding the samples the cartridges were washed first with 2 mL of water and then with 2 mL of methanol/water (30:70, v/v), and the metabolites were eluted with 2.5 mL of methanol. The samples were evaporated to dryness under nitrogen, then metabolites were dissolved in 0.15–0.2 mL of ethanol/water (50:50, v/v), and the samples were stored at −20° until HPLC analysis.

Reverse phase HPLC analysis of arachidonic acid metabolites has been performed essentially as described [14]. The "System Gold" from Beckman consisting of a Solvent Module 126, an Autosampler 507 and a Detector Module 166 was used. Visualization and quantification of the metabolites were achieved using an Epson PC AX computer. For measuring ultraviolet absorbance of the metabolites the wavelength was set to 270 nm both for leukotrienes and for prostaglandin B_2, and to 236 nm for HETE and HPETE. Absorbance coefficients of 50,000 $M^{-1} cm^{-1}$ for leukotrienes and of 23,000 $M^{-1} cm^{-1}$ for HETE/HPETE and prostaglandin B_2 were used. The identification of the different metabolites was made by comparison with the retention times of reference substances. For separation a Nucleosil 120-5 C_{18} column (250/8/4 mm) and an adequate guard column (Nucleosil 120-7 C_{18}, 11/4 mm) from Macherey-Nagel (Düren, F.R.G.) were used. After injection of the samples (0.1 mL) the column was eluted at room temperature at a flow rate of 1 mL/min. Starting with solvent A (acetonitrile/methanol/water/acetic acid/phosphoric acid/ammonia, 23:35:41:0.3:0.2:0.3, by vol.) for 3 min, the gradient was linearly increased to solvent B (acetonitrile/methanol/water/acetic acid/phosphoric acid/ammonia, 35:28:36:0.5:0.2:0.3, by vol.) within 12 min and the elution continued with solvent B up to 34 min. The column was then washed with methanol for 6 min. The solvents were filtered through 0.45 μm membrane filters (Type HV) from Millipore (Eschborn, F.R.G.).

BAY X 1005 binding and competition studies in intact cells

Unless otherwise indicated the binding and competition assays were performed in duplicate in

PBS/HEPES supplemented with 1 mmol/L Ca^{2+}/Mg^{2+} at 30°.

Binding assay. Aliquots (1 mL) of the cell suspensions were incubated for 5–8 min with the indicated concentrations of [^{14}C]BAY X 1005. Afterwards, either a 100-fold excess of unlabelled BAY X 1005 in DMSO or a corresponding volume of DMSO alone was added and incubated for another 5–8 min. The cells were then spun down for 15 sec at 15,000 *g* in an Eppendorf centrifuge and aliquots of the supernatants were taken for measuring radioactivity in a liquid scintillation counter (LS 5000 TD/C, Beckman). Total binding of [^{14}C]BAY X 1005 (C_{total}) was calculated from the radioactivity in the supernatants. The portion of [^{14}C]BAY X 1005 cell bound in the presence of a 100-fold excess of unlabelled BAY X 1005 was the unspecific binding (C_{unspec}). The specific BAY X 1005 binding was calculated according to: $C_{spec} = C_{total} - C_{unspec}$. The determination of the binding constants according to Scatchard was performed as described by Bisswanger [17].

Competition assay. To 0.1 mL aliquots of [^{14}C]-BAY X 1005 (resulting in a final concentration of 0.1 μmol/L in human and of 0.02 μmol/L in rat PMNL, respectively) 0.9 mL of the cell suspension was added (final cell concentration 1.2–1.5 × 10^7/mL) and incubated for 7–11 min. Afterwards, the assays were supplemented with the indicated concentrations of the various compounds and incubated for another 7–11 min. The assays were stopped by centrifugation as described above and aliquots of the supernatants were counted for radioactivity. The competition of BAY X 1005 binding by the various compounds was calculated according to: competition (%) = 100 − (100 × C_x/C_{spec}), with C_x being the cell-bound amount of [^{14}C]-BAY X 1005 in the presence of the compound.

BAY X 1005 binding in subcellular fractions of human PMNL

The binding experiments were performed in PBS/HEPES (pH 8.2) supplemented with EGTA, Mg^{2+} and β-mercaptoethanol (1 mmol/L each) at 4°.

Aliquots (1.5 mL) of the subcellular fractions were incubated for 10–20 min with the indicated concentrations of [^{14}C]BAY X 1005. Afterwards, either a 100-fold excess of unlabelled BAY X 1005 or a corresponding volume of DMSO was added and incubated for another 10–20 min. Two 0.2 mL aliquots were taken for measuring radioactivity in a liquid scintillation counter, and the remaining 1.1 mL were transferred into microconcentrators (Centricon-10, Amicon, Witten, F.R.G.). Ultrafiltration of the samples was performed by centrifugation of the microconcentrators for 30–60 min (depending on the subcellular fraction used) at 5000 *g* in a Sigma 2K15 centrifuge until about 0.5 mL of filtrate was obtained. Two 0.2 mL aliquots of the filtrate were taken for counting radioactivity. Total binding of [^{14}C]BAY X 1005 (C_{total}) was calculated from the difference in radioactivity before and after ultrafiltration corrected for the loss of radioactivity (due to unspecific binding of BAY X 1005 to the ultrafiltration system material) in corresponding buffer controls. The portion of [^{14}C]BAY X 1005 bound within the retentate in the presence of a 100-fold excess of unlabelled BAY X 1005 was the unspecific binding (C_{unspec}).

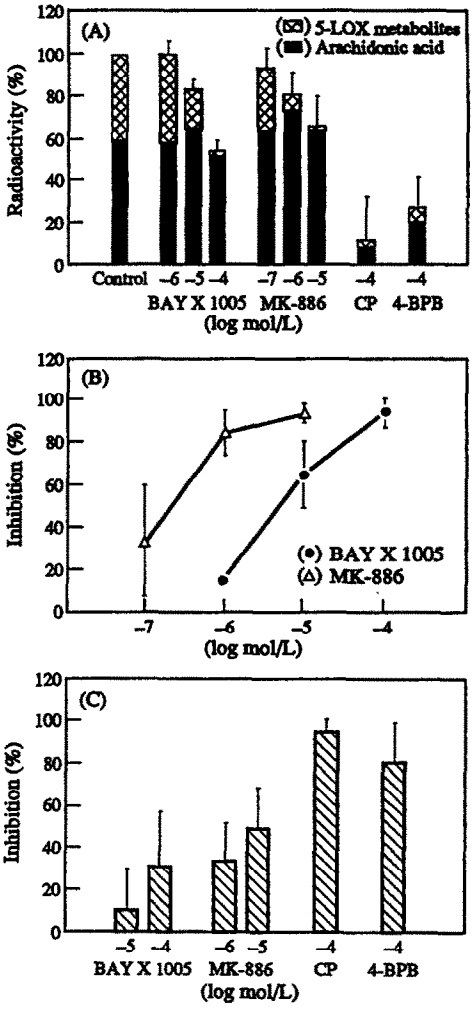


Fig. 1. Influence of BAY X 1005 and MK-886 on A23187-stimulated arachidonic acid release (A), formation of 5-LOX metabolites (B) and PAF synthesis (C). (A, B) Cells prelabelled with [1-^{14}C]arachidonic acid were incubated as described in "Performance of studies". Values of cpm from unstimulated cells (blank) were substracted. One hundred per cent radioactivity corresponds to 13010 ± 4095 cpm/assay (N = 9). The composition of radioactivity was analysed by TLC. Results are given as means ± SD from 3–8 independent experiments. (C) Cells were incubated as described in "Performance of Studies". A23187 triggered the synthesis of 440 ± 148 cpm/assay. Results are given as means ± SD from five independent experiments. CP, chlorpromazine; 4-BPB, 4-bromophenacyl bromide.

* Fruchtmann R, Mohrs K-H, Hatzelmann A, Raddatz S, Fugmann B, Junge B, Müller-Peddinghaus R and Horstmann H, *In vitro* pharmacology of BAY X 1005, a new inhibitor of leukotriene synthesis, submitted.

† Müller-Peddinghaus R, Kohlsdorfer C, Theissen-Popp P, Fruchtmann R, Perzborn E, Beckermann B, Bühner K, Ahr HJ and Mohrs K-H, BAY X 1005, a new inhibitor of leukotriene synthesis: *in vivo* inflammation pharmacology and pharmacokinetics, submitted.

Inhibition of LTB_4 synthesis in intact PMNL

Isolation of the cells, incubation conditions, extraction and quantification of LTB_4 by reverse phase HPLC were performed as described.*

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 order to investigate the influence of BAY X 1005 on arachidonic acid release, human PMNL were allowed to incorporate [1-^{14}C]arachidonic acid into cellular lipids before stimulation which allowed the quantitation of both free arachidonic acid and 5-LOX-derived arachidonic acid metabolites in a one-step analysis applying TLC. Prelabelled cells were stimulated in the presence of BSA which is known to bind arachidonic acid efficiently, so that the reacylation of released arachidonic acid into cellular lipids was minimized. Since BAY X 1005 has a very high albumin binding,† the potency of this inhibitor was markedly decreased in the presence of BSA compared to under BSA-free assay conditions (see below). As shown in Fig. 1A about 40% of total released arachidonic acid was metabolized into 5-LOX metabolites, the formation of which was concentration-dependently inhibited by BAY X 1005 and MK-886 (Fig. 1B). At concentrations of both inhibitors at which the formation of 5-LOX metabolites was almost totally inhibited, both the release of total arachidonic acid (Fig. 1A) and the synthesis of PAF (Fig. 1C) were only modestly affected. In contrast, chlorpromazine and 4-bromophenacyl bromide inhibited both parameters substantially at a concentration of 100 μmol/L (Fig. 1A and C).

Using the 10,000 *g* supernatant fraction of human PMNL as a source to examine 5-LOX activity under cell-free conditions, about 800-fold higher concentrations of BAY X 1005 were required to inhibit leukotriene synthesis compared to using intact cells (Table 1). In contrast, in the case of A-64077 and AA-861 only 10–20-fold higher concentrations of the inhibitors were necessary to inhibit leukotriene formation in the cell-free system compared to intact cells.

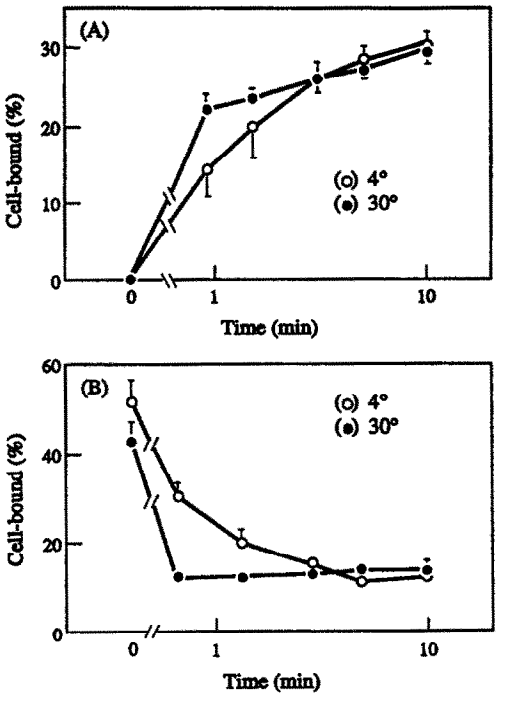


Fig. 2. Kinetics of BAY X 1005 binding and competition in human PMNL. (A) [^{14}C]BAY X 1005 (1 μmol/L final concentration) was added to the cells and the percentage of cell-bound compound determined at the indicated times. (B) Cells were preincubated with [^{14}C]BAY X 1005 (0.1 μmol/L final concentration) for 5 min before addition of a 100-fold excess of the unlabelled compound (time 0). Thereafter the percentage of cell-bound compound was determined at the indicated times. Results are given as means ± SD from three independent experiments.

Both the binding of BAY X 1005 (Fig. 2A) and the competition of cell-bound BAY X 1005 (Fig. 2B) were largely independent of temperature. The somewhat delayed kinetics of BAY X 1005 binding at 4° compared to 30° may be due to temperature-dependent differences in the fluidity of membrane lipids (membrane permeability). A steady-state concerning BAY X 1005 binding and competition was reached after about 3 min. The incubation times for further experiments were well above this time period.

Both the binding of BAY X 1005 in human PMNL and its pharmacological effect, namely the inhibition

Table 1. Inhibition of leukotriene synthesis in the 10,000 *g* supernatant fraction of human PMNL and in intact human PMNL

Compound	10,000 *g* supernatant* IC_{50} (μmol/L)	Intact cells† IC_{50} (μmol/L)	Ratio
BAY X 1005	170 (20)	0.22 (4)	773
A-64077	40 (6)	3.0 (5)	13
AA-861	5.4 (6)	0.24 (4)	22

* Inhibition of the synthesis of LTB_4 and its 6-trans isomers.
† Inhibition of LTB_4 synthesis.
Number of experiments given in brackets.

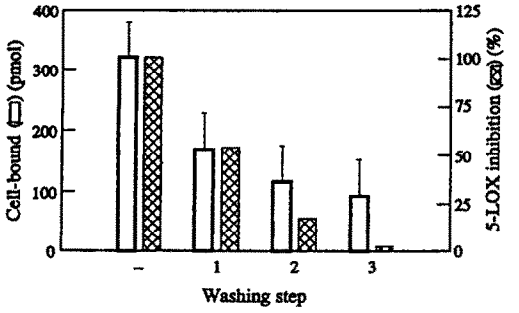


Fig. 3. Reversibility of BAY X 1005 binding and action in human PMNL. Cells (2×10^7/mL) were incubated with [^{14}C]BAY X 1005 (1 μmol/L final concentration) for about 3 min. Then, aliquots of the cell suspension were used to determine cell-bound BAY X 1005 and to stimulate leukotriene synthesis by A23187. The rest of the cells were washed by centrifugation, the resulting cell pellet resuspended in a corresponding volume of buffer and the cell suspension allowed to stand for about 3 min. This procedure was repeated another two times. Results are given as means ± SD from three independent experiments.

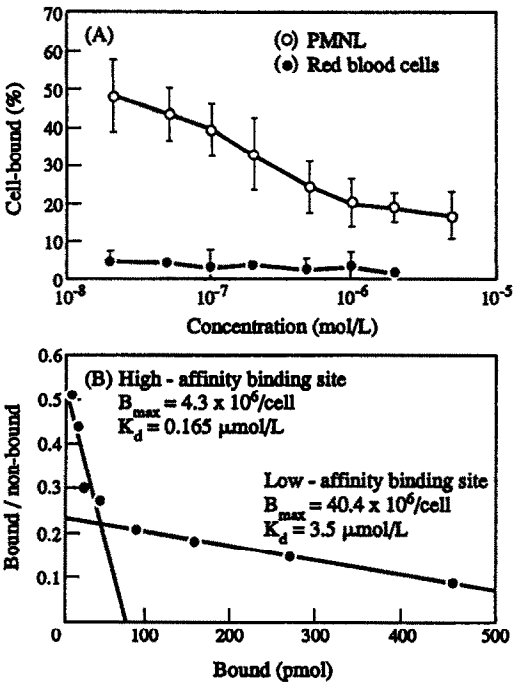


Fig. 4. BAY X 1005 binding in human PMNL and red blood cells. (A) PMNL (1.2×10^7 cells/mL) or red blood cells (2×10^7 cells/mL) were incubated for 6–8 min with the indicated concentrations of [^{14}C]BAY X 1005, then for another 6–8 min either in the presence or absence of a 100-fold excess of unlabelled BAY X 1005. (B) Scatchard plot of specifically bound BAY X 1005 in human PMNL. Results are given as means ± SD from three to five independent experiments.

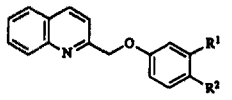
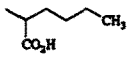
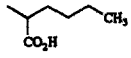
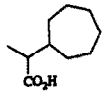
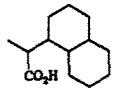
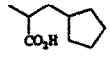
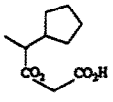
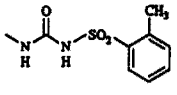
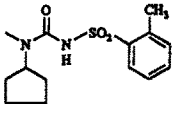
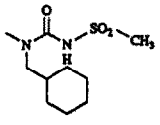
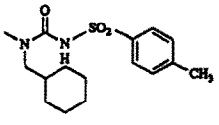
of the formation of 5-LOX metabolites (Fig. 3), were reversible. The portion of BAY X 1005 which could not be washed out (about 30%) was due to non-specific binding.

There was a concentration-dependent binding of BAY X 1005 in human PMNL whereas under comparable conditions no significant binding could be detected in human red blood cells (Fig. 4A). The portion of unspecific binding was almost independent of the BAY X 1005 concentration and appeared to be 30–35% of total binding (data not shown). The analysis of specific BAY X 1005 binding according to Scatchard (Fig. 4B) resulted in the identification of two binding sites: a high affinity binding site with a K_d value of 0.165 μmol/L and a B_{max} value of 4.3×10^6 binding sites/cell, and a low affinity binding site with a K_d value of 3.5 μmol/L and a B_{max} value of 40.4×10^6/cell. It was noteworthy that the K_d value of the high affinity binding site was almost identical to the IC_{50} value (0.22 μmol/L) for inhibition of LTB_4 synthesis (see Table 2).

These binding constants were used to calculate that at a concentration of 0.1 μmol/L about 60–70% of the specific binding of BAY X 1005 was due to binding to the high affinity binding site. This concentration was used to investigate the inhibition of BAY X 1005 binding by various other compounds. This provides an indirect estimate of the potency of these compounds to bind to the high affinity binding site assuming that inhibition reflects competition of bound BAY X 1005 by a certain compound. The obtained IC_{50} values were compared with the IC_{50} values for inhibition of LTB_4 synthesis. For a series of 13 quinoline derivatives and the reference compounds REV-5901, Wy-50,295 and MK-886 a very good correlation ($r = 0.969$) was obtained between the IC_{50} values for inhibition of BAY X 1005 binding and LTB_4 synthesis (Fig. 5). In contrast, in the case of the direct 5-LOX inhibitors A-64077 and AA-861 about 100-fold higher concentrations were necessary to inhibit BAY X 1005 binding compared to the inhibition of LTB_4 synthesis (Table 2).

For comparison, binding of BAY X 1005 was investigated in rat peritoneal PMNL (Fig. 6A). The percentage of unspecific binding (30–50%) depended on the concentration of BAY X 1005 used and generally tended to be somewhat higher than in human PMNL. Compared to human PMNL different binding constants were obtained by Scatchard analysis (Fig. 6B). For the high affinity binding site in rat PMNL a K_d value of 0.03 μmol/L and a B_{max} value of 0.89×10^6/cell were calculated, whereas the low affinity binding site had a K_d value of 5.4 μmol/L and a B_{max} value of 28×10^6/cell. The order of magnitude of the binding constants for the low affinity binding site was comparable between rat and human PMNL. In contrast, the K_d value of the high affinity binding site indicated a 5.5-fold higher affinity for BAY X 1005 in rat versus human PMNL whereas the B_{max} value was 5-fold lower in rat compared to human PMNL. In agreement with this, the IC_{50} value for inhibition of LTB_4 synthesis was 8.5-fold lower in rat versus human PMNL (Table 2). In addition, a series of other compounds were tested all of which are more potent (to various degrees) with respect to inhibition of LTB_4 synthesis in rat compared to human PMNL (Table 2). The

Table 2. Relationship between inhibition of BAY X 1005 binding at the BAY X 1005 high affinity binding site and inhibition of LTB_4 synthesis in human and rat PMNL

Compound	 R^1	R^2	IC_{50} inhibition of LTB_4 synthesis (μmol/L)		IC_{50} inhibition of BAY X 1005 binding (μmol/L)		Ratio human/rat	
			Human	Rat	Human	Rat	LTB_4	Binding
1		H	8.4	0.14	4.2	0.25	60.0	16.8
2	H		0.84	0.055	0.71	0.056	15.3	12.7
3 (BAY X 1005)	H		0.22	0.026	0.24	0.046	8.5	5.2
4	H		0.11	0.019	0.08	0.015	5.8	5.3
5	H		0.1	0.007	0.05	0.008	14.3	6.3
6	H		0.1	0.018	0.04	0.006	5.5	6.7
7	H		0.46	0.026	0.28	0.02	17.7	14.0
8		H	3.1	1.1	3.5	0.9	2.8	3.9
9	H		0.64	0.038	0.43	0.054	16.8	8.0
10	H		7.4	0.41	2.4	0.55	18.0	4.4
11	H		3.1	0.29	1.3	0.34	10.7	3.8
12	H		2.2	0.068	1.7	0.076	32.3	22.4
13	H		1.5	0.047	0.44	0.053	31.9	8.3
MK-886			0.09	0.027	0.07	0.025	3.3	2.8
REV-5901			5.2	0.18	2.0	0.12	28.9	16.7
WY-50,295			3.1	0.13	1.65	0.18	23.8	9.2
A-64077			3.0	0.98	300.0	NT	3.1	NT
AA-861			0.24	0.052	22.0	NT	4.6	NT

IC_{50} values are given as means from at least three independent experiments.
NT, not tested.

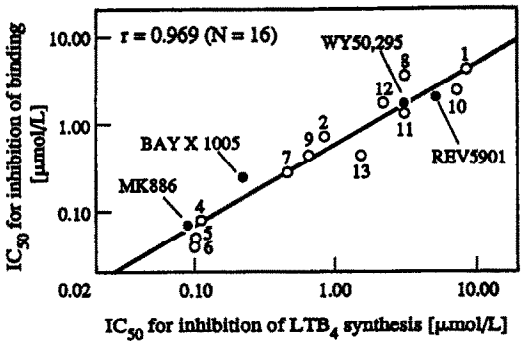


Fig. 5. Correlation of inhibition of LTB_4 synthesis and competition at the BAY X 1005 high affinity binding site by various compounds in human PMNL. Data are taken from Table 2.

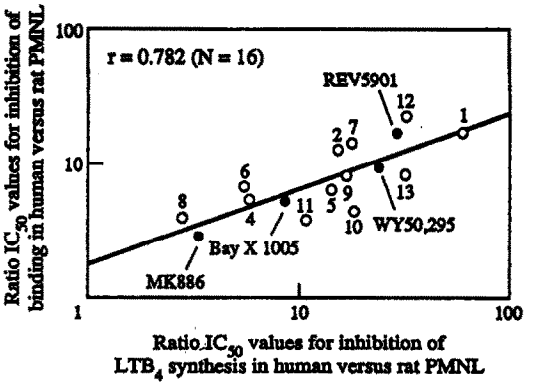


Fig. 7. Correlation of the ratios for inhibition of LTB_4 synthesis and competition at the BAY X 1005 high affinity binding site by various compounds in human versus rat PMNL. Data are taken from Table 2.

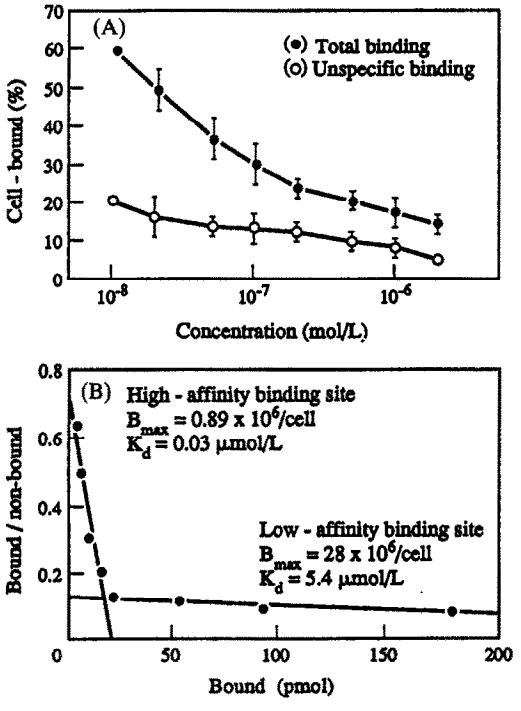


Fig. 6. Binding of BAY X 1005 in rat PMNL. (A) Cells (1.5×10^7/mL) were incubated for 8 min with the indicated concentrations of [^{14}C]BAY X 1005. The cell suspensions were then incubated for another 8 min either in the presence or absence of a 100-fold excess of unlabelled BAY X 1005. (B) Scatchard plot of specifically bound BAY X 1005. Results are given as means ± SD from four independent experiments.

ratios of the IC_{50} values for inhibition of LTB_4 synthesis and competition of BAY X 1005 binding in human versus rat PMNL correlated significantly ($r = 0.782$, N = 16) (Fig. 7).

By sonication of human PMNL followed by several centrifugation steps subcellular fractions of the cells were obtained which were characterized by specific marker proteins (data not shown). By using an ultrafiltration method a high affinity BAY X 1005 binding site could be identified in the microsomal fraction (280,000 *g* pellet) whereas a BAY X 1005 low affinity binding site was restricted to the granule fraction (10,000 *g* pellet); no specific binding could be detected in the cytosolic fraction (280,000 *g* supernatant) containing 5-LOX (Fig. 8A). The binding constants for specific binding calculated according to Scatchard (Fig. 8B) were comparable to those obtained for the two BAY X 1005 binding sites in intact cells (Fig. 7B). In addition, BAY X 1005 binding in the 280,000 *g* pellet fraction could be inhibited by MK-886 with a (compared to BAY X 1005) relative potency corresponding to the potency of this compound to inhibit LTB_4 synthesis in intact human PMNL (data not shown).

DISCUSSION

The quinoline derivative BAY X 1005 has been described as a potent and selective inhibitor of LTB_4 and 5-HETE synthesis in various *in vitro* systems,* and has been proved to be effective in preclinical models of acute inflammation after oral application.† The experiments described in this paper were performed in order to elucidate the mode of action of 5-LOX inhibition by BAY X 1005.

The two most likely assumptions would have been either an inhibition of PLA_2 activity or a direct effect of BAY X 1005 on 5-LOX itself; however, both

* Fruchtmann R, Mohrs K-H, Hatzelmann A, Raddatz S, Fugmann B, Junge B, Müller-Peddinghaus R and Horstmann H, *In vitro* pharmacology of BAY X 1005, a new inhibitor of leukotriene synthesis, submitted.

† Müller-Peddinghaus R, Kohlsdorfer C, Theissen-Popp P, Fruchtmann R, Perzborn E, Beckermann B, Bühner K, Ahr HJ and Mohrs K-H, BAY X 1005, a new inhibitor of leukotriene synthesis: *in vivo* inflammation pharmacology and pharmacokinetics, submitted.

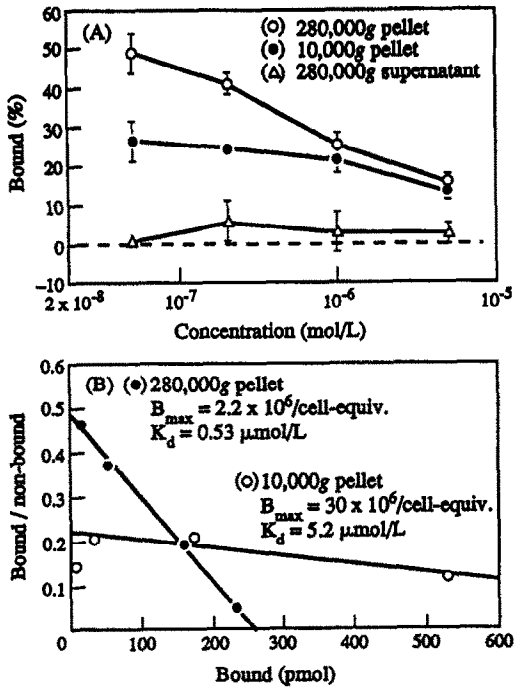


Fig. 8: Binding of BAY X 1005 in subcellular fractions of human PMNL. (A) Cells (3.2×10^7/mL) were disrupted by sonication. Comparable protein concentrations of the subcellular fractions were obtained by diluting the 280,000 *g* supernatant 1:2 (v/v) (0.26 ± 0.02 mg/mL), concentrating the 280,000 *g* pellet 3-fold (0.29 ± 0.05 mg/mL) and resuspending the 10,000 *g* pellet in the original volume of buffer (0.31 ± 0.03 mg/mL). Fractions were incubated first for 15–20 min with the indicated concentrations of [^{14}C]-BAY X 1005, and then for another 15–20 min either in the presence or absence of a 100-fold excess of unlabelled BAY X 1005. (B) Scatchard Plot of specifically bound BAY X 1005. Results are given as means ± SD from three to four independent experiments.

possibilities could be excluded. First, as shown in Fig. 1, BAY X 1005 neither significantly affected arachidonic acid release nor PAF synthesis at concentrations at which the formation of 5-LOX metabolites were almost totally inhibited. This was also true for the leukotriene synthesis inhibitor MK-886 [8] while the (rather unspecific) PLA_2 inhibitors chlorpromazine and 4-bromophenacyl bromide [18] almost totally inhibited both responses as expected.

Second, as shown in Table 1, about 800-fold higher concentrations of BAY X 1005 were required to inhibit leukotriene synthesis in a cell-free system compared to intact cells. In contrast, in the case of the direct 5-LOX inhibitors A-64077 [6] and AA-861 [7] the IC_{50} values for inhibition of leukotriene synthesis were within one order of magnitude in both test systems indicating that BAY X 1005 does not affect the formation of 5-LOX metabolites in intact cells by a direct effect on 5-LOX.

In an attempt to identify other putative target proteins of BAY X 1005 its binding in human PMNL was investigated. It turned out that BAY X 1005 is mainly bound to two binding sites in these cells (Fig. 4B); however, it cannot be excluded that other binding sites exist which might have been below the detection limit of the binding assay used. In contrast to human PMNL no significant binding of BAY X 1005 could be detected in human red blood cells (Fig. 4A) which suggests that BAY X 1005 which is a rather hydrophobic compound does not simply accumulate within the membrane lipid matrix. Interestingly, red blood cells are not capable of synthesizing leukotrienes on their own which can be taken as an indication of the specificity of BAY X 1005 binding to protein(s) involved in the leukotriene biosynthesis pathway.

The identity of the BAY X 1005 low affinity binding site located in the granule fraction (Fig. 8) and a relationship to any physiological response of human PMNL are unknown at present. However, the K_d value for the binding of BAY X 1005 to the high affinity binding site (0.165 µmol/L) is almost identical to the IC_{50} value for inhibition of LTB_4 synthesis (0.22 µmol/L) suggesting that the basis for inhibition of 5-LOX activity could be the binding of BAY X 1005 to this binding site. This assumption is further corroborated by the observation that in the case of a series of other quinoline derivatives a convincing correlation exists between the competition at the BAY X 1005 high affinity binding site and the inhibition of LTB_4 synthesis (Table 2, Fig. 5). This is also true for the reference quinolines REV-5901 [19] and Wy-50,295 [20], and in particular also for the indole derivative MK-886. This compound has been demonstrated to bind to a 18 kD membrane protein termed FLAP [9] which has been proposed to function as an anchoring protein for 5-LOX at the membrane [11]. BAY X 1005 seems to share the same working mechanism as MK-886, and probably the high affinity BAY X 1005 binding site characterized in this paper will turn out to be identical to FLAP. In agreement with this assumption are the localization of this binding site in the 280,000 *g* pellet fraction (Fig. 8) which corresponds to the localization reported for FLAP [9], and the parallelism of BAY X 1005 and MK-886 inhibition curves in the binding assay, as well as the additive effect of BAY X 1005 and MK-886 with respect to inhibition of 5-LOX activity in intact human PMNL (data not shown). Furthermore, qualitatively similar to MK-886, BAY X 1005 both inhibits and reverses 5-LOX translocation in A23187-stimulated human PMNL; however, these experiments indicate that inhibition of 5-LOX translocation by BAY X 1005 does not fully relate to inhibition of 5-LOX acitivity which has to be discussed in terms of the biological function of FLAP for 5-LOX translocation and 5-LOX activation.* Nevertheless, the mechanism of action of MK-886, BAY X 1005 and the other quinoline derivatives investigated is therefore clearly distinct from the so-called direct 5-LOX inhibitors like AA-861 or A-64077, the primary target of which is 5-LOX itself. In agreement with this we could not

* Hatzelmann A, Mohrs K-H and Müller-Peddinghaus R, Translocation of 5-lipoxygenase in A23187-stimulated human PMNL: influence of BAY X 1005 and other leukotriene synthesis inhibitors, manuscript in preparation.

detect any significant competition of these latter compounds at the BAY X 1005 high affinity binding site (Table 2).

While the inhibitory effect of MK-886 cannot readily be removed by elimination of the free drug from the incubation medium [8], both the action and the binding of BAY X 1005 (most probably by a diffusion controlled process, Fig. 2) occur in a reversible manner in human PMNL (Fig. 3), which in fact was the prerequisite for the established binding and competition assays described in this paper.

Stimulated by the observation that in rat PMNL all of the mentioned compounds are more effective at inhibiting LTB_4 synthesis than in human PMNL, we also investigated BAY X 1005 binding in rat PMNL. With respect to the two binding sites a qualitatively similar picture emerged but the binding constants, especially for the high affinity binding site, appeared to be different in rat compared to human PMNL (Fig. 6). The affinity of BAY X 1005 to the high affinity binding site is about 5.5-fold higher in rat versus human PMNL which correlates well with the 8.5-fold higher potency of BAY X 1005 to inhibit LTB_4 synthesis in rat versus human PMNL. In addition, this correlation can be extended to the other quinoline derivatives tested as well as to REV 5901, WY 50,295 and MK-886 (Table 2, Fig. 7). This suggests that the affinity of FLAP for these compounds is different in rat and human PMNL which is surprising in view of the reported high homology (92%) between rat and human FLAP [9]. Although other explanations cannot be totally excluded, the binding/competition data indicate that differences in the binding site(s) of FLAP largely contribute to the greater potency of various leukotriene synthesis inhibitors to inhibit LTB_4 synthesis in rat compared to human PMNL. In agreement with this, in the case of the direct 5-LOX inhibitors A-64077 and AA-861 the ratios of the IC_{50} values for LTB_4 synthesis inhibition in human versus rat PMNL are rather small [3–5] (Table 2). As a consequence, predictions about the potency of leukotriene synthesis inhibitors in man based on the potency of these compounds in animal species like the rat (may also be true for the mouse and guinea-pig) are difficult and tend to overestimate leukotriene synthesis inhibitor potencies.

Taken together, the binding and competition experiments described in this paper provide indirect evidence that BAY X 1005 and other quinoline derivatives share the same mode of action as MK-886. Additional indirect evidence supporting this conclusion comes from recent reports showing that another series of quinolines compete for MK-886 photoaffinity labelling of human FLAP [21] and inhibit 5-LOX translocation in HL-60 cells [22]. Further experiments will be carried out to identify directly the target proteins which correspond to the two BAY X 1005 binding sites described in this paper.

Acknowledgements—The authors thank Dr Pleiss (Bayer AG, Wuppertal, F.R.G.) for the synthesis of [^{14}C]BAY X 1005 and Dr Fugmann (Bayer AG, Leverkusen, F.R.G.) for the synthesis of A-64077, as well as Dr Junge (Bayer AG, Wuppertal, F.R.G.) for helpful discussions. The expert technical assistance of Mrs Frielingsdorf and Mrs Huber is highly appreciated.

REFERENCES

1. Irvine RF, How is the level of free arachidonic acid controlled in mammalian cells? *Biochem J* **204**: 3–16, 1982.
2. Chilton FH and Murphy RC, Remodeling of arachidonate-containing phosphoglycerides within the human neutrophil. *J Biol Chem* **261**: 7771–7777, 1986.
3. Rouzer CA, Matsumoto T and Samuelsson B, Single protein from human leukocytes possesses 5-lipoxygenase and leukotriene A_4 synthase activities. *Proc Natl Acad Sci USA* **83**: 857–861, 1986.
4. Shimizu T, Enzymes functional in the syntheses of leukotrienes and related compounds. *Int J Biochem* **20**: 661–666, 1988.
5. Lewis RA, Austen KF and Soberman RJ, Leukotrienes and other products of the 5-lipoxygenase pathway. *N Engl J Med* **323**: 645–655, 1990.
6. Carter GW, Young PR, Albert DH, Bouska J, Dyer R, Bell RL, Summers JB and Brooks DW, 5-Lipoxygenase inhibitory activity of zileuton. *J Pharmacol Exp Ther* **256**: 929–937, 1991.
7. Yoshimoto T, Yokoyama C, Ochi K, Yamamoto S, Maki Y, Ashida Y, Terao S and Shiraishi M, 2,3,5-Trimethyl-6-(12-hydroxy-5,10-dodecadiynyl)-1,4-benzoquinone (AA861), a selective inhibitor of the 5-lipoxygenase reaction and the biosynthesis of slow-reacting substance of anaphylaxis. *Biochim Biophys Acta* **713**: 470–473, 1982.
8. Gillard J, Ford-Hutchinson AW, Chan C, Charleson S, Denis D, Foster A, Fortin R, Leger S, McFarlane CS, Morton H, Piechuta H, Riendeau D, Rouzer CA, Rockach J, Young R, MacIntyre DE, Peterson L, Bach T, Eiermann G, Hopple S, Humes J, Hupe L, Luell S, Metzger J, Meurer R, Miller DK, Opas E and Pacholok S, L-663,536 (MK-886) (3-[1-(4-chlorobenzyl)-3-*t*-butyl-thio-5-isopropylindol-2-yl]-2,2-dimethylpropanoic acid), a novel, orally active leukotriene biosynthesis inhibitor. *Can J Physiol Pharmacol* **67**: 456–464, 1989.
9. Miller DK, Gillard JW, Vickers PJ, Sadowski S, Leveille C, Mancini JA, Charleson P, Dixon RAF, Ford-Hutchinson AW, Fortin R, Gauthier JY, Rodkey J, Rosen R, Rouzer CA, Sigal IS, Strader CD and Evans JF, Identification and isolation of a membrane protein necessary for leukotriene production. *Nature* **343**: 278–281, 1990.
10. Dixon RAF, Diehl RE, Opas E, Rands E, Vickers PJ, Evans JF, Gillard JW and Miller DK, Requirement of a 5-lipoxygenase-activating protein for leukotriene synthesis. *Nature* **343**: 282–284, 1990.
11. Ford-Hutchinson AW, FLAP, a novel drug target for inhibiting the synthesis of leukotrienes. *Trends Pharmacol Sci* **12**: 68–70, 1991.
12. Rouzer CA and Kargman S, Translocation of 5-lipoxygenase to the membrane in human leukocytes challenged with ionophore A23187. *J Biol Chem* **263**: 10980–10988, 1988.
13. Rouzer CA, Ford-Hutchinson AW, Morton HE and Gillard JW, MK886, a potent and specific leukotriene biosynthesis inhibitor blocks and reverses the membrane association of 5-lipoxygenase in ionophore-challenged leukocytes. *J Biol Chem* **265**: 1436–1442, 1990.
14. Hatzelmann A and Ullrich V, Regulation of 5-lipoxygenase activity by the glutathione status in human polymorphonuclear leukocytes. *Eur J Biochem* **169**: 175–184, 1987.
15. Hatzelmann A, Haurand M and Ullrich V, Involvement of calcium in the thimerosal-stimulated formation of

leukotrienes by fMLP in human polymorphonuclear leukocytes. *Biochem Pharmacol* **39**: 559–567, 1990.

16. Gomez-Cambronero J, Durstin M, Molski TFP, Naccache PH and Sha'afi RI, Calcium is necessary but not sufficient for platelet-activating factor release in human neutrophils stimulated by physiological stimuli. *J Biol Chem* **264**: 21699–21704, 1989.
17. Bisswanger H, *Theorie und Methoden der Enzymkinetik*. Verlag Chemie, Weinheim, 1979.
18. Chang J, Musser JH and McGregor H, Phospholipase A_2: function and pharmacological regulation. *Biochem Pharmacol* **36**: 2429–2436, 1987.
19. Coutts SM, Khandwala A, Van Inwegen R, Chakraborty U, Musser J, Bruens J, Jariwala N, Dally-Meade V, Ingram R, Pruss T, Jones H, Neiss E and Weinryb I, Arylmethyl phenyl ethers, a new class of specific inhibitors of 5-lipoxygenase. In: *Prostaglandins, Leukotrienes and Lipoxins* (Ed. Martyn Bailey J), pp. 627–637. Plenum Press, New York, 1985.
20. Musser JH and Kreft AF, Substituted-[2-quinolinyl-(bridged)aryl] compounds, modulators of eicosanoid biosynthesis and action. *Drugs Future* **15**: 73–80, 1990.
21. Evans JF, Leveille C, Mancini JA, Prasit P, Therien M, Zamboni R, Gauthier JY, Fortin R, Charleson P, MacIntyre DE, Luell S, Bach TJ, Meurer R, Guay J, Vickers PJ, Rouzer CA, Gillard JW and Miller DK, 5-Lipoxygenase-activating protein is the target of a quinoline class of leukotriene synthesis inhibitors. *Mol Pharmacol* **40**: 22–27, 1991.
22. Kargman S, Prasit P and Evans JF, Translocation of HL-60 cell 5-lipoxygenase—inhibition of A23187- or *N*-formyl-methionyl-leucyl-phenylalanine-induced translocation by indole and quinoline leukotriene synthesis inhibitors. *J Biol Chem* **266**: 23745–23752, 1991.