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SHORT COMMUNICATION

Pharmacological Modulation of Human Platelet Leukotriene C_4-Synthase

Angelo Sala,‡ Giancarlo Folco,* Peter M. Henson† and Robert C. Murphy†*

*Center for Cardiopulmonary Pharmacology, Institute of Pharmacological Sciences, School of Pharmacy, University of Milan, Via Balzaretti 9, 20133 Milan, Italy; and †National Jewish Center for Immunology and Respiratory Medicine, 1400 Jackson St, Denver, CO 80202 U.S.A.

ABSTRACT. The aim of this study was to test if human platelet leukotriene C_4-synthase (LTC_4-S) is pharmacologically different from cloned and expressed LTC_4-S and, in light of the significant homologies between 5-lipoxygenase activating protein (FLAP) and LTC_4-S, if different potencies of leukotriene synthesis inhibitors acting through binding with FLAP (FLAP inhibitors) reflect in different potencies as LTC_4-S inhibitors. Leukotriene C_4 (LTC_4) synthesis by washed human platelets supplemented with synthetic leukotriene A_4 (LTA_4) was studied in the absence and presence of two different, structurally unrelated FLAP inhibitors (MK-886 and BAY-X1005) as well as a direct 5-lipoxygenase inhibitor (zileuton). LTC_4 production was analyzed by RP-HPLC coupled to diode array detection. We report that human platelet LTC_4-S was inhibited by MK-886 and BAY-X1005 (IC_{50} of 4.7 μM and 91.2 μM, respectively), but not by zileuton (inactive up to 300 μM); all 3 compounds were able to inhibit 5-lipoxygenase metabolite biosynthesis in intact human polymorphonuclear leukocytes (IC_{50} of 0.044 μM, 0.85 μM, and 1.5 μM, respectively). Platelet LTC_4-S does not appear pharmacologically different from expression cloned LTC_4-S. LTC_4 -S inhibition by FLAP inhibitors is in agreement with the significant homology reported for expression-cloned LTC_4-S with FLAP. Furthermore, functional homology of the binding sites for inhibitors on LTC_4-S and FLAP is suggested by the conservation of the relative potencies of MK-886 and BAY-X1005 vs FLAP-dependent 5-lipoxygenase activity and LTC_4-S inhibition: MK-886 was 19.3-fold more potent than BAY-X1005 as FLAP inhibitor and 19.6-fold more potent than BAY-X1005 as LTC_4-S inhibitor. BIOCHEM PHARMACOL 53;6:905–908, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. Five-lipoxygenase activating protein; human platelets; leukotriene C_4; leukotriene C_4-synthase; leukotriene synthesis inhibitors; 5-lipoxygenase inhibitors

LTC_4-S§ (EC 2.5.1.37), the specific enzyme responsible for the conversion of LTA_4 into LTC_4 has recently been purified to homogeneity [1–2] and expression-cloned from human monocytic leukemia (THP-1) and myelocytic leukemia (KG-1) cell lines [3–4]. The deduced aminoacid sequence showed no homology with other glutathione S-transferases, but significant identity (31%) was found with the FLAP. According to this observation, LTC_4-S was inhibited by increasing concentrations of compound MK-886, known to inhibit 5-LO activity in intact PMNL by binding with the integral membrane protein FLAP [5].

To test potential pharmacological differences between human platelet LTC_4-S and expression-cloned LTC_4-S, we studied the pharmacological modulation of human platelet LTC_4-S by two different, structurally unrelated leukotriene synthesis inhibitors acting through binding with FLAP (FLAP inhibitors), compound MK-886, and compound BAY-X1005 [6]. The results obtained showed that human platelet LTC_4-S is dose-dependently inhibited by both compounds, but not by a direct 5-lipoxygenase inhibitor.

MATERIALS AND METHODS

PMNL were prepared by dextrane sedimentation and centrifugation on discontinuous Percoll gradient (Pharmacia, Uppsala, Sweden), as previously described [7].

Washed human platelets were prepared according to Patscheke [8].

Cells were resuspended in PBS without Ca^{2+} and Mg^{2+} ($PBS^{=}$) and kept on ice until use.

LTA_4 free acid was obtained through base hydrolysis of LTA_4 methyl esther (Cayman Chemical Co., Ann Arbor, MI), using ice-cold acetone:sodium hydroxide 0.25 M (4:1, v:v) at room temperature for 60 min. LTA_4 free acid was

† Corresponding author. A. Sala, Center for Cardiopulmonary Pharmacology, Inst. of Pharmacological Sciences, University of Milan, Via Balzaretti 9, 20133 Milan, Italy. Telephone +39-2-20488312; FAX +39-2-29404961.

§ *Abbreviations:* LTC_4-S, leukotriene C_4-synthase; FLAP, 5-lipoxygenase activating protein; LTC_4, leukotriene C_4; leukotriene A_4, LTA_4; PMNL, polymorphonuclear leukocytes; 5-HETE, 5-hydroxy-eicosatetraenoic acid; fMLP, formyl-methionyl-meucyl-mhenylalanine.

added to 1 mL of human platelets (10^8 cells) at the final concentration of 1 μM. Metabolism of exogenous LTA_4 proceeded for 10 min at 37°C in the presence of increasing concentrations of the compounds tested in DMSO, or DMSO alone.

Human PMNL (1×10^6 cells mL^{-1}) were supplied with Ca^{++} (2 mM) and Mg^{++} (0.5 mM) and, following preincubation at 37°C for 5 min in the presence of increasing concentrations of the compounds tested in DMSO, or DMSO alone, the calcium ionophore A23187 (Calbiochem, La Jolla, CA; 1 μM) was added to trigger eicosanoid metabolism.

Incubations were terminated after 10 min with 2 volumes of ice-cold methanol (Merck, Darmstadt, Germany), containing the HPLC internal standard PGB_2 (25 ng, Cayman) and samples were stored at −20°C.

Samples from PMNL and platelet incubations were analyzed by reverse-phase HPLC coupled with diode array-UV detection, as previously described [9]. Briefly, samples were diluted with water to a final methanol concentration lower than 20% and extraction was quickly carried out using a solid phase cartridge (Supelclean LC-18, Supelco, Bellafonte, PA); the retained material was eluted using 90% aqueous MeOH. After evaporation, the dried extract was reconstituted in HPLC mobile phase A (600 μL) and injected into an HPLC gradient pump system (Mod. 126, Beckman Analytical, Palo Alto, CA) connected to a diode-array UV detector (Mod. 168, Beckman Analytical), using a microprocessor-controlled autosampler (Jasco 851-AS, Tokyo, Japan), with sample kept at 4°C. UV absorbance was monitored at 280 and 235 nm, and full UV spectra (210–340 nm) acquired at a rate of 0.5 Hz.

A multilinear gradient from solvent A (methanol/acetonitrile/water/acetic acid 10/10/80/0.02, v/v/v/v, pH 5.5 with ammonium hydroxide) to solvent B (methanol/acetonitrile 50/50, v/v) at a flow rate of 0.5 mL/min, was used to elute a 3 × 150 mm column (RP-18 endcapped Superspher, 4 μm, Merck). Solvent B was increased to 35% over 6 min, to 65% over 25 min, and to 100% over 3 min. This method allows resolution of all 5-lipoxygenase metabolites, from 20-carboxy-LTB_4 to 5-HETE, in less than 35 min, with excellent sensitivity (1–3 pmol for LTA_4-derived metabolites and 20 pmol for 5-HETE), ensured by the midbore RP column. Quantitation was carried out on UV spectral analysis, positively identified peaks only, using their HPLC peak areas relative to that of PGB_2 at 280 nm, and calculated from the responses of standard compounds.

The results were expressed as mean ± standard deviation (SD). IC_{50} values were calculated using nonlinear regression fitting.

RESULTS AND DISCUSSION

Human platelets were able to convert exogenous LTA_4 into LTC_4, in agreement with data obtained in several laboratories [10]. Under the experimental conditions used (LTA_4 1 μM, 10 min incubation, 37°C, in the absence of albumin), the average LTC_4 production by 10^8 platelets was 143 ± 7 pmol. Subsaturating LTA_4 concentration was chosen according to a study reporting the activity of MK-886 as LTC4-S inhibitor [4], in light of the potential biological significance of this concentration. PMNL, the likely source of LTA_4 *in vivo*, are able to synthesize 250–350 pmol of LTA_4 per million cells upon A23187 challenge, and approximately 1/10th of this amount upon challenge with fMLP, after priming with granulocyte macrophage-colony stimulating factor [9, 11]. Although local concentrations of lipid mediators at inflammation sites might increase to relatively high levels, it is unlikely that concentrations higher than 1 μM might be achieved during biologically significant conditions.

Preincubation with increasing concentrations of MK-886 or BAY-X1005 caused a dose-dependent inhibition of LTC_4 formation with IC_{50} values of 4.7 μM and 91.2 μM, respectively (Fig. 1). Zileuton, a direct inhibitor of 5-lipoxygenase [12], did not cause inhibition of the LTC_4-S up to the concentration of 300 μM.

Challenge of peripheral human PMNL, with the calcium ionophore A23187 (1 μM, 10 min), resulted in the expected profile of 5-LO-derived metabolites. 20-hydroxy-LTB_4 was the main product, with significant amounts of 20-carboxy-LTB_4, LTC_4, LTB_4, 5-HETE, and nonenzymatic LTA_4 metabolites (namely Δ^6-*trans*-LTB_4 isomers and 5,6-diHETE isomers) being observed. In agreement

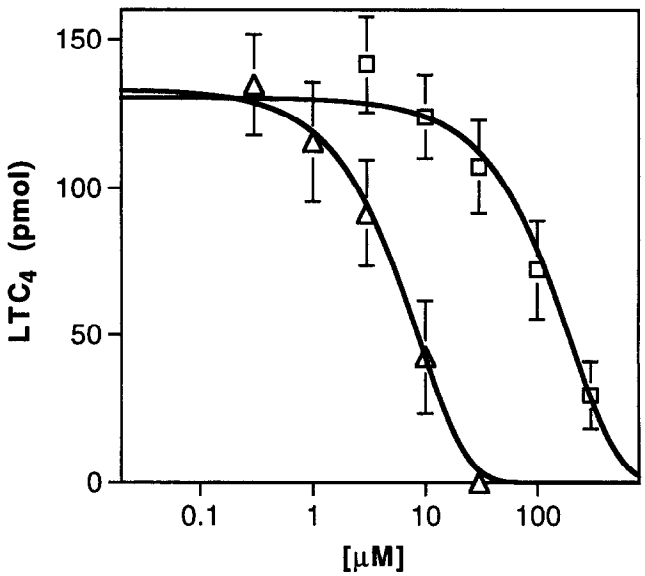

FIG. 1. Production of LTC_4 by human platelets incubated with synthetic LTA_4. Human platelets (10^8 cells) were incubated with synthetic LTA_4 free acid (1 μM, 10 min, 37°C) in the presence of increasing concentrations of MK-886 (△) or BAY-X1005 (□). LTC_4 (pmol) was quantitated by reverse-phase HPLC. Control platelets synthesized 143 ± 7 pmol of LTC_4. Values are expressed as means ± SD of 3 different platelet preparations.

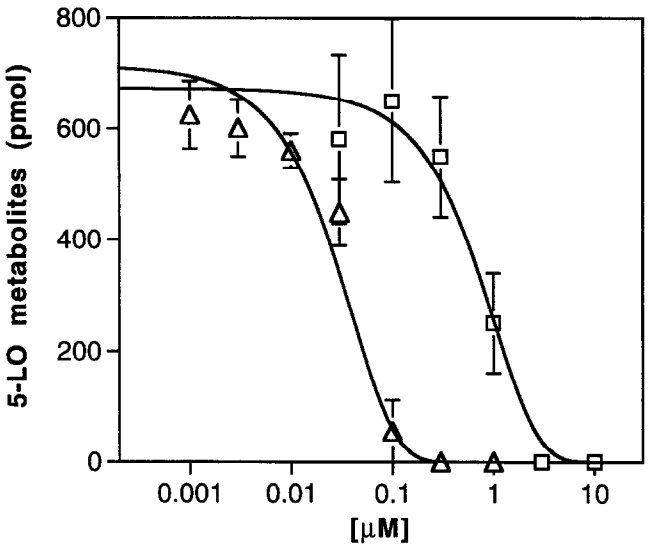

FIG. 2. Production of 5-lipoxygenase metabolites by human polymorphonuclear leukocytes challenged with the Ca^{++}-ionophore A23187. Human polymorphonuclear leukocytes (10^6 cells) were challenged with Ca^{++}-ionophore A23187 (1 μM, 10 min at 37°C) in the presence of increasing concentrations of MK-886 (△) or BAY-X1005 (□). 5-lipoxygenase metabolites (5-HETE and LTA_4-derived metabolites) were quantited by reverse-phase HPLC. Control PMNL synthesized 639 ± 43 pmol of 5-LO metabolites. Values are expressed as means ± SD of 4 different PMNL preparations.

with published data, MK-886, BAY-X1005 (Fig. 2) and zileuton were able to concentration-dependently inhibit the synthesis of 5-LO metabolites. The IC_{50} values for the 3 compounds tested were 0.044 μM, 0.85 μM, and 1.5 μM, respectively.

The aminoacid sequence of LTC_4-S obtained from molecular cloning in myelocytic leukemia cell lines showed no identity with other glutathione S-transferases, but did demonstrate significant identity with the integral membrane protein FLAP. Identity reached 44% for the N-terminus of the protein, and the three transmembrane sequences of the two proteins were basically overlapping [3–4]. According to this observation, compound MK-886, a FLAP-binding molecule known to efficiently inhibit leukotriene synthesis in intact cells through interaction with FLAP, was found to inhibit THP-1 LTC_4-S as well as COS-expressed LTC_4-S [3–4].

We report that LTC_4-S in intact platelets is inhibited by two different, structurally unrelated leukotriene synthesis inhibitors, known to bind with high affinity to a specific site on FLAP [5–6]. We provide pharmacological evidence that LTC_4-S in intact platelets is not different from myelocytic leukemia cell line and COS-expressed LTC_4-S. This is in agreement with the reported cross-reactivity of platelet LTC_4-S with a polyclonal antibody raised against human lung LTC_4-S [13]. Although no direct evidence of a potential interaction of MK-886 or BAY X1005 with the platelet LTC_4-S has been provided, inhibition of recombinant LTC_4-S by MK-886 has recently been reported [14]. Because a total methanol extract of the platelet incubation was obtained, the effect of Bay X1005 and MK-886 on the export step of LTC4 into the supernatant could be ruled out.

Strict conservation of relative potencies for the two inhibitory activities (MK-886 was 19.3- and 19.6-fold more potent than Bay X1005 for 5-LO and LTC_4-S inhibition, respectively) suggests that the observed homology of FLAP and LTC_4-S preserves functionally similar binding sites for inhibitors. This observation may represent an important lead in the development of new potent LTC_4-S inhibitors.

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