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Research Article

New Class of 5-Lipoxygenase Inhibitors: Correlation Between Inhibition of LTB_4 Production and Chemiluminescence of Human Polymorphonuclear Granulocytes

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The effect of several series of compounds on the biosynthesis of leukotriene B_4 in human polymorphonuclear granulocytes (PMNL) was measured after stimulation of the cells by the Ca^{2+} ionophore A23187. Phenylhydrazone derivatives and some pyrrole derivatives strongly inhibited 5-lipoxygenase activity at 10 μM. In contrast, 4-aminopyrrolopyrimidines and 4-oxopyrrolopyrimidines did not exhibit any effect. The compounds examined for inhibition of 5-lipoxygenase were also tested in a chemiluminescence assay for potential effects on the emission of photons as a measure of activated oxygen species generated by the stimulated granulocytes. There was a good correlation between lipoxygenase inhibition and suppression of chemiluminescence; however, some derivatives with no inhibitory activity against lipoxygenase still reduced chemiluminescence, which suggests that an alternative inhibitory mechanism of chemiluminescence must be present. Cyclooxygenase inhibitors such as aspirin did not suppress chemiluminescence of granulocytes. It is, therefore, unlikely that cyclooxygenase is required in the chemiluminescence reaction. In contrast, LTB_4 seems to play an important role in the zymosan-induced chemiluminescence of PMNL.

KEY WORDS: leukotriene B4; 5-lipoxygenase; chemiluminescence; pyrrolopyrimidines; pyrrol derivatives; polymorphonuclear granulocyte (PMNL) activation.

INTRODUCTION

In 1971, Vane found that nonsteroidal antirheumatic drugs such as aspirin inhibit cyclooxygenase, an enzyme that catalyzes the first step of prostaglandin biosynthesis from arachidonic acid (1). A second pathway of arachidonic acid involved in inflammatory and allergic reactions has been found in PMNL[4] (2). In the initial step during the course of this pathway, 5-lipoxygenase catalyzes the oxygenation of arachidonic acid leading to 5-HPETE, which can be further transformed into 5-HETE or the unstable epoxide LTA_4 (3) (Fig. 1). Enzymatic hydrolysis of this epoxide leads to the stable leukotriene B_4 and SRS-A, a mixture of the leukotrienes C_4, D_4, and E_4 (4). The leukotrienes C_4, D_4, and E_4 are potent constrictors of smooth muscles, increase vascular permeability, and seem to play an important role in asthmatic diseases (4). LTB_4 activates PMNL and leads to release of superoxide radicals, chemotaxis, aggregation, and degranulation of PMNL (5–8). Therefore, LTB_4 seems to be a powerful mediator of inflammatory responses, and it is of interest to search for inhibitors of this lipoxygenase pathway and to examine their effect on neutrophil activation.

Measurement of chemiluminescence was used as a parameter of PMNL activation. The generation of activated oxygen in stimulated PMNL ($O_2^{\cdot-}$, $^1O_2$, $^{\cdot}OH$, and H_2O_2) leads to the emission of photons (9–12) and emission of light in a narrow frequency range (13) in the presence of luminol (5-amino-2,3-dihydrophthalazine-1,4-dione). Therefore, various compounds were investigated with respect to their effect on 5-lipoxygenase activity and on chemiluminescence of activated PMNL. The compounds tested here, a new class of pyrrole and pyrrolopyrimidine derivatives and a series of phenylhydrazones, were compared with established cyclooxygenase inhibitors such as aspirin and 5-lipoxygenase inhibitors such as NDGA.

MATERIALS AND METHODS

Materials

All solvents used were either of analytical grade or redistilled before use. Methanol for HPLC was purchased from Merck, Darmstadt/ FRG.

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[4] Abbreviations used: PMNL, polymorphonuclear leukocytes; PGB_2, prostaglandin B_2; LTB_4, leukotriene B_4; LTA_4, leukotriene A_4; 5-HETE, 5-hydroxy-6,8,11,14-eicosatetraenoic acid; 5-HPETE, 5-hydroperoxy-6,8,11,14-eicosatetraenoic acid; NDGA, nordihydroguaiaretic acid; SRS-A, slow-reacting substance of anaphylaxis; PBS, phosphate-buffered saline; 5S,12S-DiHETE, 5S,12S-dihydroxy-6-trans,8-cis,10-trans,14-cis-eicosatetraenoic acid; 6-trans-LTB_4, 5,12-dihydroxy-6,8,10-trans,14-cis-eicosatetraenoic acid; HPLC, high-performance liquid chromatography.

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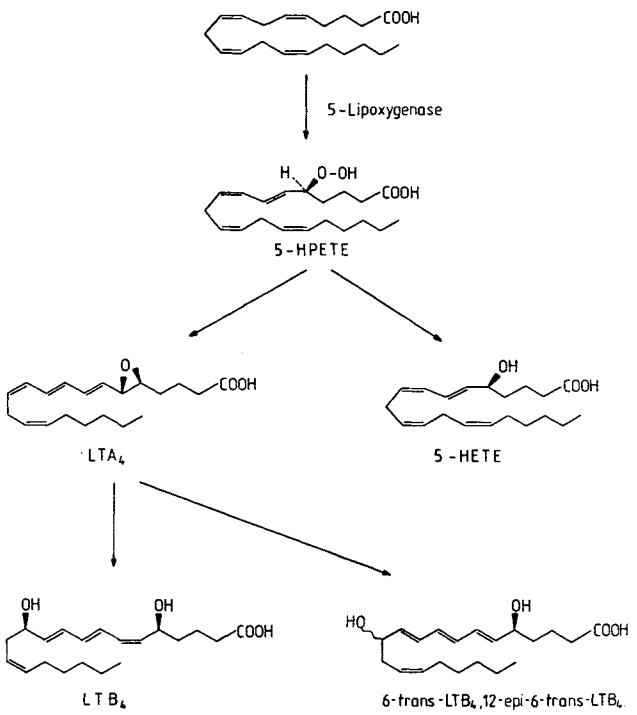


Fig. 1. Transformation of arachidonic acid in human PMNL.

Ca^{2+} ionophore A23187 (free acid), arachidonic acid, and prostaglandin B_2 were obtained from Sigma, St. Louis, Mo. PBS-Dulbecco containing Ca^{2+}, Mg^{2+} as used in chemiluminescence was obtained from Biochrom KG, Berlin, FRG. PM 16 from Serva served as incubation buffer in the lipoxygenase assay. Luminol was purchased from Lumac, Basel, Switzerland. Percoll was obtained from Pharmacia, Freiburg, FRG. HPLC standards were either generous gifts from Dr. C. O. Meese (Dr. Margarete Fischer Bosch Institute, Stuttgart, FRG) or obtained from Paesel GmbH, Frankfurt, FRG.

Opsonized zymosan was prepared as previously described (12).

Methods

Isolation of Polymorphonuclear Granulocytes (PMNL). Suspensions of human PMNL were prepared according to the method of Hjorth *et al.* (14): 8000 mg NaCl, 200 mg KCl, 1150 mg $Na_2HPO_4 \times 6H_2O$, 200 mg KH_2PO_4, and 100 mg $CaCl_2 \times 2H_2O$ were dissolved in 1000 ml percoll to obtain a solution which is referred to as 100% percoll. Seventy and 55% percoll solutions were prepared using an isotonic NaCl solution to give refractive indices of 1,3474 and 1,3448, respectively. Four milliliters of EDTA blood was pipetted carefully into a polystyrol tube containing 4 ml 70% percoll, overlayered with 4 ml 55% percoll. After centrifugation at 350*g* for 20 min, the PMNL fractions were collected and washed with 0.9% saline. This was followed by lysis of the erythrocytes with lysis medium (14). Finally, the PMNL were washed again with saline and resuspended in the final incubation buffer.

Lipoxygenase Assay. Human PMNL ($1.0-1.4 \times 10^7$ cells/ml), suspended in 1 ml PM 16 Serva buffer salt solution, supplemented with 1 m*M* Ca^{2+}, 0.1% glucose, and 0.1% human albumin, were preincubated with the test compounds for 5 min. In the control experiments, the test compounds were omitted. The reaction was started with 2.5 μl of Ca^{2+} ionophore solution (1 mg/ml in methanol) or with a mixture of the ionophore solution and 10 μmol of arachidonic acid. After 6 min, the reaction was stopped with 1 ml methanol, the tubes were chilled on ice and acidified with 1 *N* HCl, and finally, PGB_2 was added as internal standard. After centrifugation, the eicosanoids were extracted from the supernatant liquid as previously described (15). Extraction of eicosanoids was done with a modified Baker 10 extraction system, using Baker C_{18} disposable columns. The columns were conditioned with 2 ml methanol and 2 ml PBS buffer, pH 3.0. The samples were then put over the columns and washed with water and with 25% methanol. Finally, the leukotrienes were eluted with 100% methanol, and the extract was evaporated to dryness with a stream of nitrogen at 37°C on a water bath and resuspended in 100 μl methanol. Using 20 μl of this solution, HPLC was performed on a 5 C_{18} nucleosil column (Macherey Nagel, Dueren, FRG), 4 × 250 mm, with methanol/water/acetic acid (70/30/0.01) as the mobile phase (2,16–19). Absorbance was monitored at 280 nm with a Waters 480 detector. The ε values of Ref. 2 were used for calculation of the eicosanoid amounts. In order to detect the leukotrienes and HETEs simultaneously in a single run, the compounds were first eluted for 16 min with methanol/water/acetic acid (70/30/0.01), followed by methanol/water/acetic acid (80/20/0.01). This was achieved using two Waters pumps (Models 510 and M-45), connected with a Waters automated gradient controller 680. Ultraviolet spectra were recorded in methanol/water/acetic acid (70/30/0.01) with a Beckmann DU-50 spectrophotometer connected on-line with the HPLC equipment.

Chemiluminescence Assay. The test system according to Kato *et al.* (20) was performed as follows: 100 μl of PMNL suspension (0.3×10^6 cells/ml) was pipetted into 500 μl of PBS buffer containing 0.1% glucose, 0.1% human albumin, and the test compound. Cells were preincubated for 10 min in the presence of luminol (50 μl of a 0.2 m*M* alkaline solution) before the reaction was started with 20 μl of zymosan. Measurements were performed on a six-channel Biolumat LB 9505 (Berthold, Wildbad, FRG) and terminated after 70 min. The integrals over this period were used for calculations.

RESULTS

Various pyrrole derivatives, pyrrolopyrimidines (21–23), and phenylhydrazones were investigated with respect to their effect on lipoxygenase activity and chemiluminescence of PMNL. All compounds were tested at a concentration of 10^{-5} *M*. Figure 2A shows the typical HPLC profile of the arachidonic acid metabolites produced by human PMNL. Peaks I–VII were identified as those corresponding to 20-OH- and 20-COOH-LTB_4 (not separated), PGB_2, 5S,12R-6-trans-LTB_4, 5S,12S-6-trans-LTB_4, LTB_4, 5S,12S-DiHETE (also referred to as the "double lipoxygenase product"), and 5-HETE. Ultraviolet spectra of LTB_4 and 5-HETE are illustrated in Fig. 3. LTB_4 as well as peaks, I, III, IV, and VI showed the typical triene structure, with an UV maximum at 270 nm. The peaks were further identified by their relative retention times with respect to PGB_2

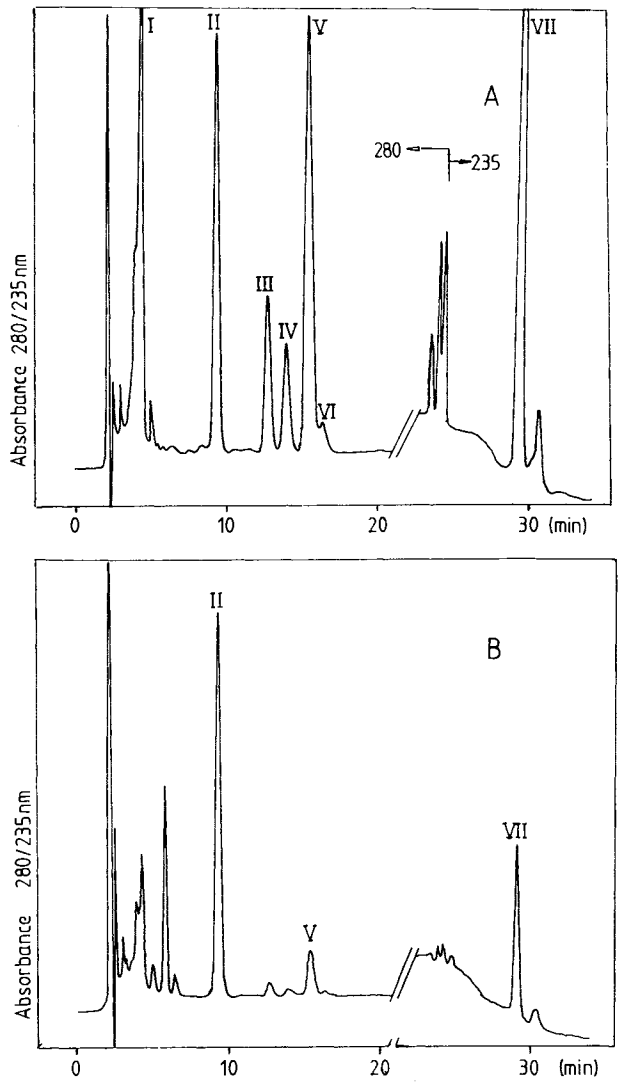


Fig. 2. (A) A HPLC chromatogram of the arachidonic acid metabolites isolated from a suspension of human PMNL after stimulation with the Ca^{2+} ionophore A23187. (B) A chromatogram of cells preincubated with compound **26** (5 μM) for 5 min before stimulation.

(internal standard) (17,19) and coelution with the synthetic standards 20-OH-LTB_4, LTB_4, 5S,12S-DiHETE, and 5-HETE. Using the methanol/water system as the mobile phase, LTB_4 was well separated from its 6-trans isomers. In contrast, the separation of LTB_4 from 5S,12S-DiHETE was more difficult. While these two compounds could be separated under our standard conditions, they failed to separate when the solvent polarity was reduced and when the injection volumes or the injected quantities of these arachidonic acid metabolites were increased. The cells produced only trace amounts of 5S,12S-DiHETE and no 12- and 15-HETEs (Fig. 2A). When Ca^{2+} or the ionophore A23187 was omitted, no lipoxygenase products could be detected. In the control experiments the amount of LTB_4 produced by the PMNL varied between 122 and 172 ng/10^7 cells. However, the percentage inhibition was not affected by these variable amounts of LTB_4 produced by the PMNL of different donors.

We investigated the effects of a series of phenylhydrazones on 5-lipoxygenase and chemiluminescence (Table I). The phenylhydrazone derivatives **4** and **5** were chosen because they are structurally related to NDGA. All the phenylhydrazone derivatives except cyclohexanone phenylhydrazone had a strong inhibitory effect on lipoxygenase activity and chemiluminescence (97 and 77%, respectively). Cyclohexanone phenylhydrazone, which failed to inhibit 5-lipoxygenase at a concentration of 10^{-5} *M*, showed a significantly lower inhibition of chemiluminescence.

The 4-aminopyrrolopyrimidines (Table IV), which possess antiphlogistic activity *in vivo* (27), were also tested in both assays in order to determine their mode of action and to investigate a possible correlation between lipoxygenase and chemiluminescence inhibition of human PMNL. None of the 4-aminopyrrolopyrimidines had any effect on LTB_4 production. However, the 4-aminopyrrolopyrimidines inhibited chemiluminescence ranging from 40 to 60%, except compounds **10**, **12**, and **13** (Table II), which had no significant effect. The activity of these compounds varied with the substituents R^1, R^2, and R^3. Oxygenated groups in R^1 and R^2 as well as replacement of the phenyl ring by a furanyl results in a loss of inhibitory activity. Even at concentrations of 5×10^{-5} *M*, LTB_4 production was not affected by most of the compounds. The 4-oxopyrrolopyrimidines, which showed no antiphlogistic activity *in vivo* (22,27), inhibited neither LTB_4 synthesis nor chemiluminescence in a significant manner (Table III).

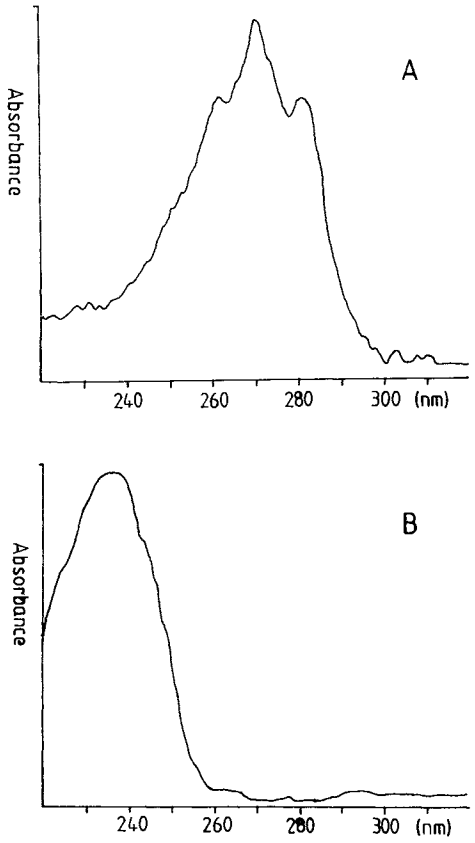
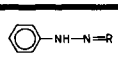
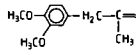
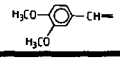


Fig. 3. Ultraviolet spectra of LTB_4 (A) and 5-HETE (B) recorded as described under "Methods."

Table I. Effect of Phenylhydrazones on LTB_4 Synthesis and Chemiluminescence (Values Are Expressed as Percentage Inhibition of Control)



	R =	LTB_4 synthesis	Chemiluminescence (%)
1		13	32.5
2		94	79
3		95	77
4		100	76
5		100	78

In contrast to the bicyclic compounds, some of the pyrrole derivatives (Table IV) inhibited LTB_4 synthesis. Compounds **24**, **25**, and **26** inhibited leukotriene biosynthesis in the range of 92 to 94% at 10^{-5} *M*. Their I_{50} values were 5.2, 3.6, and 1.8 μM, respectively. Figure 4 shows the dose–response profile of compounds **25** and **26**. Compounds **23** and **27**, which differ only in the substituent R, were less effective (20 and 32%; I_{50} values of 29 and 19 μM, respectively). Compounds **28**, **29**, and **30**, which are structurally more distinct from compounds **23–27**, were inactive, even at concentrations of 5×10^{-5} *M*.

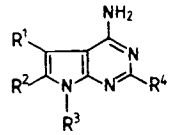
In order to evaluate the mode of action of compounds **24**, **25**, and **26**, the cells were incubated with additional arachidonic acid (10 μM). The compounds reduced 5-HETE, LTB_4, and 6-trans-LTB_4 release from the PMNL to the same extent.

The pyrroles that showed a high activity in the lipoxygenase assay exhibited at least a 50% inhibition of chemiluminescence. The effects of the pyrroles and BW 755C on the lipoxygenase reaction and chemiluminescence were in the same range at a concentration of 10^{-5} *M* (Tables IV and V). In contrast to these strong lipoxygenase inhibitors, the other pyrrole derivatives showed an inhibition of chemiluminescence only by 45% or less, with compounds **29** and **30** being the least active in the lipoxygenase as well as in the chemiluminescence assay. When compounds **23** and **24** were tested with the Biolumat in the same run, compound **24**, representing a stronger lipoxygenase inhibitor, was always approximately 10% more effective than compound **23**. Cyclooxygenase inhibitors showed no significant effects in both assays, while the lipoxygenase inhibitors NDGA and BW 755C inhibited the LTB_4 production and the chemiluminescence of PMNL (Table V).

DISCUSSION

In this study, the effect of several series of compounds on LTB_4 release from PMNL and a possible relationship between inhibition of lipoxygenase and inhibition of chemiluminescence were investigated.

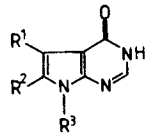
Table II. Effect of 4-Aminopyrrolopyrimidines on LTB_4 Synthesis and Chemiluminescence [Values Are Expressed as Percentage Inhibition of Control (Mean ± SE of Two or Three Independent Experiments)]



	R^1	R^2	R^3	R^4	LTB_4 synthesis	Chemiluminescence (%)
6	CH_3	CH_3		H	nI[a]	41 ± 1
7	CH_3	CH_3		H	nI	44 ± 1
8	CH_3	CH_3		H	nI	41 ± 3
9	CH_3	CH_3		H	nI	37 ± 4
10	CH_3	CH_3		H	nI	16 ± 2
11[b]	CH_3	CH_3		H	nI	28 ± 3
12	CH_3	CH_2OH		H	nI	16 ± 9
13	COOH	COOH		H	nI	15 ± 7
14	H	H		H	nI	52 ± 8
15	CH_3	CH_3		NH_2	nI	64 ± 3

[a] No inhibition.
[b] Employed as salicylate salt.

Table III. Effect of 4-Oxopyrrolopyrimidines on LTB_4 Synthesis and Chemiluminescence [Values Are Expressed as Percentage Inhibition of Control (Mean ± SE of Two or Three Independent Experiments)]



Nr	R^1	R^2	R^3	LTB_4 synthesis/Chemiluminescence (%)	
16	CH_3	CH_3		nI[a]	14 ± 2
17	CH_3	CHO		nI	5 ± 13
18	CHO	CH_3		nI	11 ± 9
19	CH_3	NO_2		nI	15 ± 4
20	CN	CH_3		nI	28 ± 5
21				nI	12 ± 5
22				nI	11 ± 2

[a] No inhibition.

Both assay systems address different aspects of the mode of action of the test compounds. The lipoxygenase assay is a specific system to test drugs for their effect on lipoxygenase. On the other hand, chemiluminescence is a relatively nonspecific measurement of cell parameters that indicate cell activation and release of oxygen radicals. Compounds that show an effect in this system can interfere with several steps involved in the activation of PMNL. As a relatively nonspecific antioxidant, NDGA inhibits leukotriene biosynthesis and exhibits a very strong inhibitory action on chemiluminescence (Table V) as expected.

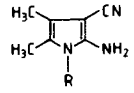
Since LTB_4 leads to activation and superoxide release of PMNL (8) and is produced during the activation of such cells with zymosan (24), inhibition of LTB_4 production should result in an inhibitory effect on chemiluminescence. Indeed, all pyrroles that inhibit LTB_4 production cause at least 50% inhibition of chemiluminescence. The structurally related pyrroles with an I_{50} of more than 10 μM (compounds 23 and 27) still showed up to 45% inhibition at 10 μM (Table IV). This may be due to different assay conditions, e.g., cell number or the manner of cell stimulation, which may lead to a variable effect of these compounds on leukotriene production in the leukotriene and chemiluminescence assay. Since the active compounds reduced LTB_4, 6-trans-LTB_4, and 5-HETE release in the same range in the presence of arachidonic acid, one can conclude that the compounds act via 5-lipoxygenase inhibition, not affecting the succeeding enzymatic steps in the cascade.

Phenylhydrazone derivatives have previously been shown to have inhibitory effects on soybean lipoxygenase (25) and 12-lipoxygenase (26). Like the pyrroles, all the phenylhydrazones listed in Table I except cyclohexanone phenylhydrazone showed a strong inhibitory effect in both assays.

Among the 4-amino-pyrrolopyrimidines, compounds **6**, **8**, and **9**, which are known to possess antiphlogistic activity *in vivo* (27), showed no inhibition of LTB_4 synthesis. Since inhibition of cyclooxygenase had no significant influence on chemiluminescence of PMNL, one can conclude that these compounds interfere with other cellular mechanisms. Compounds showing an effect in the chemiluminescence assay can interfere with several enzymatic steps involved in the activation of PMNL, e.g., the activated oxygen-producing systems such as NADPH-oxidase (28) responsible for the chemiluminescence of PMNL or reactions induced by the generated leukotrienes, thereby causing the inhibition of up to 60% (e.g., compounds **14**, **15**, and **28**) without affecting leukotriene biosynthesis. This hypothesis also accounts for the fact that inhibitors of LTB_4 production show different ranges of inhibition in the chemiluminescence assay (approximately 50% for pyrrole derivatives, 70% for phenylhydrazones, and 95% for NDGA). However, an additional nonspecific reduction mechanism of highly active compounds such as NDGA cannot be ruled out. In the case of the pyrrolopyrimidines and pyrroles, this kind of mechanism is very unlikely, since it would not explain the structural requirements of these compounds for their inhibitory activity in the chemiluminescence and lipoxygenase assay.

In contrast to the 4-aminopyrrolopyrimidines with antiphlogistic activity, their 4-oxopyrrolopyrimidine analogues showed no effect *in vivo* (22,27) and in our test systems (Table III). Apparently the 4-amino group is essential for an inhibitory activity of pyrrolopyrimidines in the chemiluminescence test and for an antiphlogistic effect *in vivo*, as has

Table IV. Effect of Pyrrole Derivatives on LTB_4 Synthesis and Chemiluminescence [Values Are Expressed as Percentage Inhibition of Control (Mean ± SE of Two or Three Independent Experiments)]



		LTB_4 synthesis/Chemiluminescence (%)	
23		20 ± 2	44 ± 3
24		92 ± 0	55 ± 3
25		94 ± 5	53 ± 1
26		94 ± 5	52 ± 2
27		32 ± 7	45 ± 3
28		nI[a]	42 ± 2
29		nI	14 ± 2
30		nI	24 ± 6

[a] No inhibition.

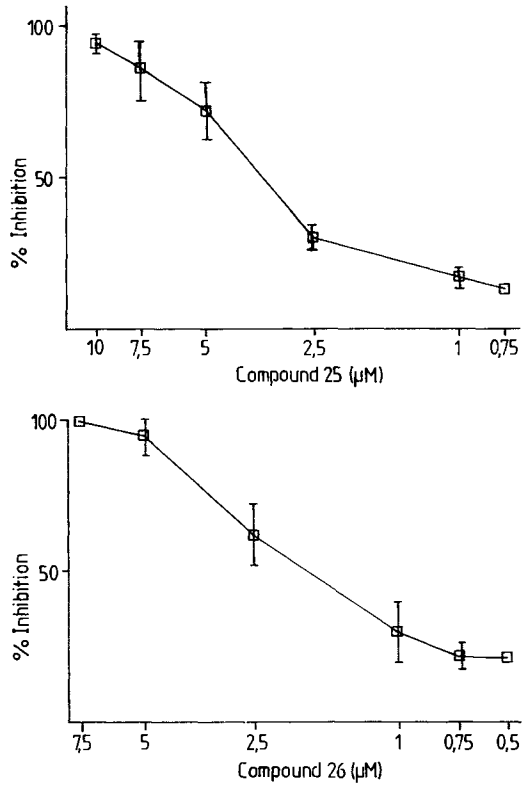


Fig. 4. Effects of compounds **25** and **26** on the formation of LTB_4 in human PMNL. Values are the means ± SE of two separate experiments.

Table V. Effect of Various Compounds on LTB_4 Synthesis and Chemiluminescence (Values Are Expressed as Percentage Inhibition of Control)

Compound	LTB_4 synthesis/Chemiluminescence (%)	
BW 755C	87	53
NDGA	100	95
Fenbufen	nI[a]	20
Indomethacin	nI	3
Diclofenac	nI	26
Acetylsalicylic acid	nI	15

[a] No inhibition.

been previously found (22,27), but a general correlation between these two parameters needs to be investigated further.

In conclusion, it can be stated that inhibition of leukotriene biosynthesis seems to inhibit activation of PMNL, as measured by the chemiluminescence assay. Our data are in agreement with the finding that LTB_4 is able to activate PMNL (8). Since such an activation occurs in inflammation and rheumatic disease, it is of interest to search for lipoxygenase inhibitors and investigate their effect on stimulated cells. Even though the pyrrole derivatives may prove to have toxic properties, they are useful tools to elucidate the pathological and physiological role of leukotrienes.

Flavonoids (29), gossypol (30), and AA 860 (31) have previously been reported to inhibit 5-lipoxygenase. We present here a new group of compounds that inhibit leukotriene biosynthesis; studies with other pyrrole derivatives at lower concentrations are in progress.

ACKNOWLEDGMENTS

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