WF11605, AN ANTAGONIST OF LEUKOTRIENE B_4 PRODUCED BY A FUNGUS

I. PRODUCING STRAIN, FERMENTATION, ISOLATION AND BIOLOGICAL ACTIVITY

EISAKU TSUJII, YASUHISA TSURUMI, SUSUMU MIYATA, KEIKO FUJIE, AKIKO KAWAKAMI, MASANORI OKAMOTO and MASAKUNI OKUHARA

Exploratory Research Laboratories, Fujisawa Pharmaceutical Co., Ltd., 5-2-3 Tokodai, Tsukuba, Ibaraki 300-26, Japan

(Received for publication November 11, 1991)

WF11605, a new antagonist of leukotriene B_4 (LTB_4) was isolated as a product of fungal strain F11605. The molecular formula of WF11605 was determined to be $C_{38}H_{60}O_{11}$. WF11605 inhibited LTB_4-induced chemotaxis of rabbit polymorphonuclear leukocytes (PMNLs) with an IC_{50} value of 1.7×10^{-7} M and blocked ^{3}H-LTB_4 binding to PMNL membranes at 5.6×10^{-6} M (IC_{50}). WF11605 also inhibited LTB_4-induced degranulation of rabbit PMNLs at 3.0×10^{-6} M (IC_{50}). However, WF11605 did not show any inhibitory effect on platelet activating factor (PAF)- and *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP)-induced degranulation at concentrations up to 10^{-4} M. These results suggest that WF11605 is a specific antagonist of LTB_4.

Leukotriene B_4, (5*S*,12*R*)dihydroxy-6,14-*cis*,8,10-*trans*-eicosatetraenoic acid (LTB_4) stimulates the aggregation and degranulation of polymorphonuclear leukocytes (PMNLs), and promotes the chemotaxis and chemokinesis of PMNLs[1]. LTB_4 has been detected in exudates at several sites of inflammation at concentrations sufficient to alter leukocyte functions. This suggests that LTB_4 may serve as a mediator in the cellular phase of the inflammatory response[2]. Therefore, antagonists of LTB_4 action may be useful in treating symptoms associated with inflammatory cell infiltration such as psoriasis[3] and ulcerative colitis[4]. In our screening program for specific LTB_4 antagonists from microbial sources, we discovered WF11605[5] as a fermentation product of fungal strain F11605.

Materials and Methods

Extraction and Analytical Methods

The production of WF11605 in the fermentation was monitored by HPLC using a reverse phase column (Lichrospher RP-18, 250 × 4 mm i.d., Merck). The solvent system was 0.1% H_3PO_4-acetonitrile-methanol (3:4:3), and the detector wavelength was set at 210 nm. The fermentation extracts for HPLC assay were prepared as follows: One ml of acetone was added to 1 ml of fermentation broth, allowed to stand for 1 hour and was centrifuged at 1,500 × *g* for 10 minutes. Ten μl of the supernatant was used for HPLC analysis.

Preparation of Rabbit PMNLs

PMNLs were collected from the peritoneal exudates of male Japanese White rabbits. Sixteen hours after the injection of 0.1% glycogen solution (400 ml/animal, i.p.), peritoneal cells were collected and washed with EAGLE's minimum essential medium (MEM). Over 90% of the cells were judged to be PMNLs after Giemsa staining.

Assay of PMNL Chemotaxis

Chemotactic activity of PMNLs was assayed in modified Boyden chambers using Millipore filters (Millipore Co., U.S.A.) with pores of 0.65 μm diameter. One ml of PMNL suspension (3×10^6 cells/ml) in HANK's balanced solution (HBSS) supplemented with 1% bovine serum albumin (BSA), and several concentrations of WF11605 or vehicle were placed in the upper compartment of the chamber. The lower compartment contained 0.9 ml of LTB_4 solution (3×10^{-11} M, Cayman Chemical Co.,U.S.A.). The chambers were incubated at 37°C in an atmosphere of 95% air and 5% CO_2. After incubation for 5 hours, the filters were removed, fixed in 95% ethanol and stained with hematoxylin. PMNL chemotaxis was quantified microscopically by counting the number of cells that migrated into the filter to a depth of 50 μm. Ten high-power fields (hpf, $\times$400) were examined for each filter and chemotaxis was expressed as cells per 10 hpf.

^{3}H-LTB_4 Receptor Assay

PMNLs suspended at a concentration of 5×10^6/ml in EAGLE's MEM were frozen at -20°C and stored. Within 2 weeks, the frozen PMNLs were thawed at room temperature and used as a PMNL membrane suspension.

^{3}H-LTB_4, (5, 6, 8, 9, 11, 12, 14, 15 (*n*)-^{3}H) leukotriene B_4, 20 μCi/ml (Amersham Co., Japan) was diluted 20-fold with 3×10^{-9} M nonradioactive LTB_4 in HBSS containing 10 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid, pH 7.4 (HEPES). The experiment was carried out at 4°C. Fifteen μl of the solution were added to 60 μl HBSS-HEPES. Then, 25 μl of competitor or vehicle and 150 μl of the PMNL membrane suspension were added. After vigorous vortexing, the solution was kept at 4°C for 30 minutes. At the end of incubation, the reaction mixture was immediately filtered through a GF/C glass filter (Whatman Co., U.K.) in order to separate free and bound ^{3}H-LTB_4 using a cell harvester (M-24S, Brandel Co., U.S.A.). The filter papers were washed 5 times with 150 μl of cold HBSS-HEPES, dried and cut off. The cut off papers were placed in a scintillation vial, 3 ml of toluene-based scintillation fluid were added and the radioactivity was counted.

Assay of PMNL Degranulation

PMNL degranulation was quantitated as the amount of β-glucuronidase released from the PMNLs after several stimulations. PMNL suspensions (7×10^6 cells/ml) were incubated with test substance or vehicle at 37°C for 5 minutes in plastic tubes. LTB_4 (7×10^{-8} M), *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP, 7×10^{-10} M, Sigma) or platelet activating factor (PAF, 7×10^{-7} M, Sigma) together with cytochalasin B (10^{-5} M, Sigma) were added and the mixture was incubated for 5 minutes at 37°C. The incubation was terminated by transfer to an ice bath followed by rapid centrifugation. The cell-free supernatants were subsequently assayed for β-glucuronidase activity. β-Glucuronidase activity was measured as follows: The assay mixture consisted of 300 μl of acetate buffer (0.1 M, pH 4.6), 100 μl of sample and 50 μl of 1 mM substrate (4-methylumbelliferyl-β-D-glucuronide, Nakarai Chemical Co., Japan). The reaction was started by addition of the substrate and was terminated after 15 minutes at 37°C by the addition of 2 ml glycine buffer (0.1 M, pH 10.3). Fluorescence properties (excitation; 365 nm, emission; 455 nm) were used to monitor the enzymatic reaction. The results were expressed as percent of total released β-glucuronidase, where "total" means the amount of enzyme released using 0.1% Triton X-100.

Results

Characteristics of Producing Strain

Fungal strain F11605 was isolated from a soil sample collected at Kawamata Town, Fukushima Prefecture, Japan.

Cultural characteristics of strain F11605 on various agar media are summarized in Table 1. The isolate grew rather restrictedly on various agar media, attaining 1.0 to 3.5 cm in diameter after 2 weeks at 25°C, and formed yellowish white to orange white colonies. The vegetative hyphae were septate, straight or flexuous, hyaline, smooth and branched. The hyphal cells were filamentous or cylindrical, and 1 to 5 μm thick.

The strain has produced neither teleomorphic nor anamorphic structures on various media (Table 1),

Table 1. Cultural characteristics[a] of strain F11605.

Media	Growth	Surface	Aerial mycelium	Sporulation	Reverse
Malt extract agar	Rather restricted 3.0~3.5 cm[b]	Circular, plane Wrinkly at the center White to pale yellow (4A3)[c]	None	None	Pale yellow (4A3)
Potato glucose agar	Rather restricted 3.0~3.5 cm	Circular, plane Orange white (6A2)	Poor	None	Pale yellow (4A3)
CZAPECK's solution agar	Very restricted 1.0~1.5 cm	Irregular, plane Thin, submerged Colorless	None	None	Colorless
Sabouraud dextrose agar	Rather restricted 3.0~3.5 cm	Circular, plane Wrinkly at the center Light orange (6A4)	Poor	None	Light orange (6A5)
Oatmeal agar	Rather restricted 3.0~3.5 cm[b]	Circular, plane Wrinkly at the center Orange white (6A2) to grayish orange (6B3)	Poor	None	Light orange (6A4) Produced pale orange soluble pigments
Emerson Yp Ss agar	Rather restricted 3.0~3.5 cm	Circular, plane Felty, wrinkly at the margin White, orange white (6A2)	None	None	Pale orange (5A3) Produced pale orange soluble pigments
Corn meal agar	Rather restricted 3.0~3.5 cm	Circular, plane Thin, submerged Colorless to yellowish white (4A2)	None	None	Colorless to yellowish white (4A2)
MY20 agar	Rather restricted 3.0~3.5 cm	Circular, plane Wrinkly White, grayish orange (5B3)	Poor	None	Light yellow (4A4)

[a] Observation after incubation at 25°C for 14 days.
[b] Diameter of colony.
[c] Color descriptions based on the Methuen Handbook of Colour (3rd edition)[8].

nor on a steam-sterilized leaf affixed to a corn meal agar plate. The organism was not induced to sporulate by cultivation in sterile water or on agar media containing some differentiation factors; cyclic AMP or dibutyryl cyclic AMP. Moreover, it has not been observed to form differentiated hyphal structures; sclerotia, bulbils, chlamydospores and clamp connections.

The lack of definitive characteristics suggests that the strain F11605 might be classified as a member of the Agonomycetes (Mycelia sterilia)[6,7]. The fungus was deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan as FERM BP-1730.

Fermentation

A seed medium (100 ml) containing soluble starch 2%, corn starch 1%, glucose 1%, cotton seed meal 1%, dried yeast 1%, Polypepton 0.5%, corn steep liquor 0.5% and $CaCO_3$ 0.2% prepared in tap water (pH was adjusted to 6.0 with 6 N NaOH) was poured into each of three 500-ml Erlenmeyer flasks and sterilized at 121°C for 30 minutes. A loopful of F11605 from a mature slant, grown on potato glucose agar at 25°C for 2 weeks, was used to inoculate each of the flasks. The flasks were shaken on a rotary shaker (220 rpm, 5.1 cm throw) at 25°C for 5 days. The resultant seed culture was used to inoculate (2%

inoculum) 20 liters of sterile fermentation medium which consisted of 3% soluble starch, 1% glucose, 1% wheat germ, 0.5% cotton seed meal and 0.2% $CaCO_3$, pH 7.0 prior sterilization, prepared in tap water into a 30-liter stainless steel jar-fermenter. The fermentation was carried out at 25°C for 5 days employing aeration at 20 liters/minute and agitation at 200 rpm. The concentration of WF11605 in the fermentation broth at 120 hours after inoculation averaged 44.8 μg/ml.

Isolation

An equal volume of acetone was added to 18 liters of culture broth while mixing. The mixture was allowed to stand at room temperature overnight and was filtered. The filtrate was concentrated to 1 liter under reduced pressure. The pH was adjusted to 2.0 with 6 N HCl and the solution was extracted with 2 liters of ethyl acetate. The extract was concentrated and applied on a column (0.8 liter) of silica gel (Silica CC-4, Mallinckrodt). The column was washed with *n*-hexane (1 liter), ethyl acetate (1 liter) and the active substance was eluted from the column with acetone (2 liters). The active fraction was taken to dryness, dissolved in a mixture of chloroform-methanol-acetic acid (200:20:1) and subjected to a column chromatography on silica gel (Kiesel gel 60, Merck, 0.25 liter). The column was developed with the same solvent system. The fractions containing the desired substance were combined and dried to give a powder (150 mg). The powder was dissolved in a small amount of methanol and was allowed to stand overnight at 4°C to yield colorless crystals (150 mg) of WF11605.

Physico-chemical Properties

The physico-chemical properties of WF11605 are summarized in Table 2. WF11605 is slightly soluble in methanol and water, and insoluble in chloroform and ethyl ether. The compound gave positive color reactions using Molisch and $Ce(SO_4)_2$, but was negative to ninhydrin and Dragendorff.

Biological Activity

Effect of WF11605 on LTB_4-induced Chemotaxis in Rabbit PMNLs

LTB_4 (3×10^{-11} M) showed chemotactic activity vs. rabbit PMNLs. The mean migrating cell number was 28.3 ± 2.4 cells/10 hpf (n=3) in vehicle treated cells. WF11605 significantly inhibited migration using concentrations ranging from 10^{-7} to 10^{-5} M (Fig. 1). The IC_{50} value was 1.7×10^{-7} M.

Effect of WF11605 on ^{3}H-LTB_4 Binding to PMNL Membranes

Specific binding (defined as total binding minus non-specific binding with 0.1 μm, nonlabeled LTB_4) was about 60% of the total binding. As shown in Table 3, WF11605 blocked ^{3}H-LTB_4 binding to PMNL membranes with an IC_{50} value of 5.6×10^{-6} M.

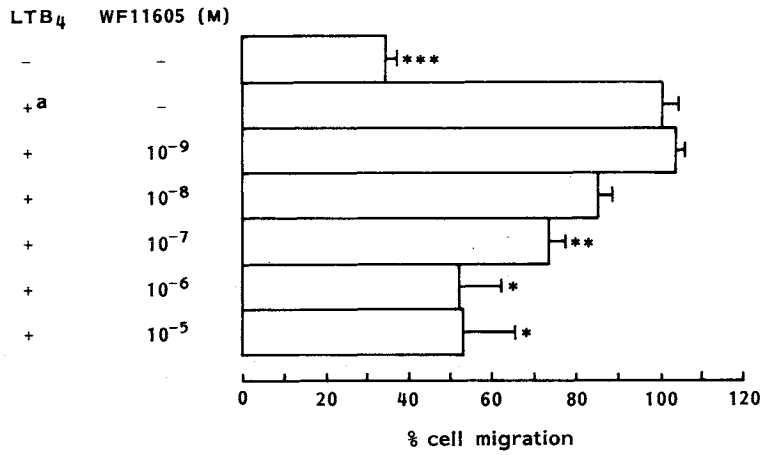
Effect of WF11605 on PMNL Degranulation

LTB_4, PAF and FMLP induced β-gluculonidase release from cytochalasin B-treated rabbit PMNLs. The percent of the β-gluculonidase released by LTB_4 (7×10^{-8} M), PAF (7×10^{-7} M) and FMLP

Table 2. Physico-chemical properties of WF11605.

Melting point:	291°C
Molecular formula:	$C_{38}H_{60}O_{11}$
Elemental analysis:	
Found:	C 64.69, H 8.49
Calcd for $C_{38}H_{60}O_{11}\cdot H_2O$:	C 64.20, H 8.79
Molecular weight (FAB-MS *m/z*):	715 (M+Na)
Specific rotation $[\alpha]_D^{23}$:	−46° (*c* 0.5, MeOH)
Ultraviolet absorption spectrum:	
Acidic and neutral:	225 (sh), ε: 6,900
Basic:	End absorption in MeOH
Thin layer chromatography (Silica gel plate):	
$CHCl_3$-MeOH-AcOH (200:40:1)	Rf 0.56
BuOH-AcOH-H_2O (4:1:2)	Rf 0.83

Fig. 1. Inhibition of rabbit PMNL chemotaxis by WF11605.



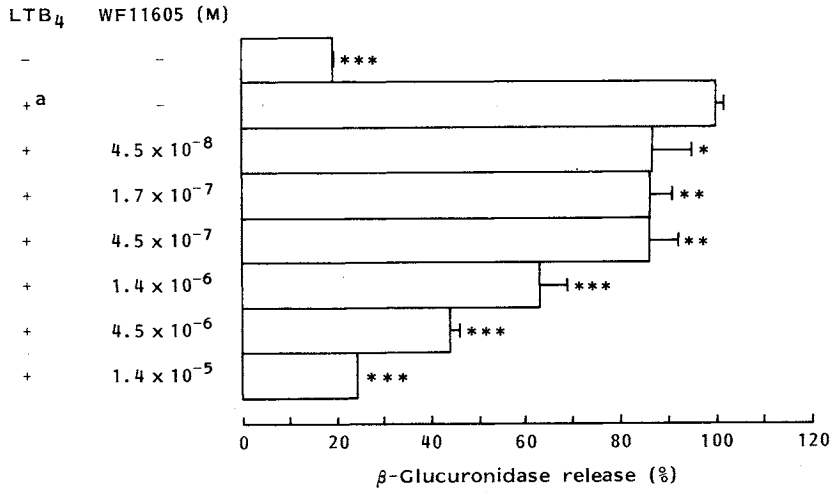
Results are expressed as percent of migrating cell number (mean ± SE, n = 3).
*$P<0.05$, **$P<0.01$, ***$P<0.001$.
[a] PMNL migration was induced by 3×10^{-11} M LTB_4.

Table 3. Inhibition of ^{3}H-LTB_4 binding to rabbit PMNL membrane by WF11605.

WF11605 (M)	Unlabeled LTB_4 (M)	n	^{3}H-LTB_4 binding[a] Means ± SEM (dpm)	Inhibition of specific binding (%)
—	—	3	1,156 ± 36.3	0
—	10^{-7}	3	425 ± 23.7***	100
10^{-7}	—	3	1,122 ± 87.8	4.7
10^{-6}	—	3	963 ± 27.8*	26.4
10^{-5}	—	3	721 ± 57.2**	59.5

[a] 4.8×10^{-10} M ^{3}H-LTB_4 was used.
*$P<0.05$, **$P<0.01$, ***$P<0.001$.

Fig. 2. Effect of WF11605 on LTB_4-induced degranulation in rabbit PMNLs.



Results are expressed as percent of β-glucuronidase release (mean ± SE).
*$P<0.05$, **$P<0.01$, ***$P<0.001$.
[a] PMNLs degranulation was induced by 7×10^{-8} M LTB_4.

(7×10^{-10} M) was 29.5±0.52% (n=11), 17.7±0.77% (n=4) and 18.3±3.1% (n=4), respectively. WF11605 significantly decreased the LTB_4-induced PMNL degranulation in a dose-dependent manner from 10^{-7} to 10^{-5} M (Fig. 2) without showing any agonistic activity. The IC_{50} value was 1.8×10^{-6} M. In contrast, WF11605 did not show any inhibitory effect on the degranulation induced by PAF and FMLP at concentrations up to 10^{-4} M.

Acute Toxity

The 50% lethal dose of WF11605 by intraperitoneal injection to male *dd*Y mice (6 weeks old) was more than 1 g/kg.

Discussion

Recently, considerable attention has been focused on the possibility that LTB_4 is an endogenous mediator of various inflammatory responses. An antagonist of the LTB_4 receptor could be potentially useful for modulation of the inflammatory process.

WF11605, a new antagonist of LTB_4 receptor having no agonistic activity could be a useful drug in several inflammatory diseases, although further examination of anti-LTB_4 activity in an animal model is needed to make this determination. The *in vivo* activity of WF11605 is currently under study, and the results will be published later.

References

1) Ford-Hutchinson, A. W.; M. A. Bray, M. V. Doig, M. E. Shipley & M. J. H. Smith: Leukotriene B, a potent chemokinetic and aggregating substance released from polymorphonuclear leukocytes. Nature 286: 264~265, 1980
2) Simmons, P. M.; J. A. Salmon & S. Moncada: The release of leukotriene B_4 during experimental inflammation. Biochem. Pharmacol. 32: 1353~1359, 1983
3) Grabbe, J. & B. M. Czarnetzki: Identification of chemotactic lipoxygenase products of arachidonate metabolism in psoriatic skin. J. Invest. Dermatol. 82: 477~479, 1984
4) Sharon, P. & W. F. Stenson: Enhanced synthesis of leukotriene B_4 by colonic mucosa in inflammatory bowel disease. Gastroenterology 86: 453~460, 1984
5) Okamoto, M.; E. Tsujii, T. Kaizu, H. Hatanaka, M. Okuhara, K. Sawada & H. Tanaka (Fujisawa Pharm.): WF11605 and its derivatives. Jpn. Kokai 252298 ('89), Oct. 6, 1989
6) Onions, A. H. S.; D. Allsopp & H. O. W. Eggins: Smith's Introduction to Industrial Mycology. p. 108, John Wiley & Sons, 1981
7) Barron, G. L. (*Ed.*): The Genera of Hyphomycetes from Soil. Williams & Wilkins Co., 1968
8) Kornerup, A. & J. H. Wanscher: Methuen Handbook of Colour. 3rd Ed., Methuen, London, 1988