BIOCHEMICAL PHARMACOLOGY 75 (2008) 688–697

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/biochempharm

(2R,3R)-2-(3′,4′-dihydroxybenzyl)-3-(3″,4″-dimethoxybenzyl) butyrolactone suppresses fMLP-induced superoxide production by inhibiting fMLP-receptor binding in human neutrophils

Yi-Jia Huang [a], *Ih-Sheng Chen* [b], *Ching-Ping Tseng* [c], *Yuan-Ji Day* [d], *Yin-Chou Lin* [e], *Chang-Hui Liao* [a,*]

[a] *Graduate Institute of Natural Products, College of Medicine, Chang Gung University, Taiwan*
[b] *College of Pharmacy, Kaohsiung Medical University, Kaohsiung, Taiwan*
[c] *Graduate Institute of Medical Biotechnology, College of Medicine, Chang Gung University, Tao-Yuan, Taiwan*
[d] *Department of Anesthesiology, Chang-Gung Memory Hospital, Tao-Yuan, Taiwan*
[e] *Rehabilitation Department, Chang-Gung Memory Hospital, Tao-Yuan, Taiwan*

ARTICLE INFO

Article history:
Received 11 August 2007
Accepted 1 October 2007

Keywords:
Piper philippinum
Lignan
PP-6
Neutrophil
fMLP
Superoxide anion

ABSTRACT

This study investigated the mechanism underlying the inhibiting effect of (2R,3R)-2-(3′,4′-dihydroxybenzyl)-3-(3″,4″-dimethoxybenzyl) butyrolactone (PP-6), a lignan from *Piper philippinum*, on superoxide anion production induced by the chemotactic peptide formly-methionyl-leucyl-phenylalanine (fMLP) in human neutrophils. Human neutrophils were stimulated with fMLP (1 μM), PMA (100 nM) or leukotriene B_4 (LTB_4; 1 μM) and induced superoxide anion release. PP-6 specifically inhibited fMLP-induced superoxide anion production in a concentration-dependent manner with an IC_{50} value of 0.3 ± 0.1 μM. Intracellular signaling caused by fMLP, PMA or LTB_4 were evaluated. PP-6 specifically inhibited fMLP-induced intracellular calcium mobilization and ERK (p42/p44), Akt and p38 phosphorylation. Moreover, PP-6 specifically inhibited fMLP-induced Mac-1 expression without affecting this caused by LTB_4 or PMA. PP-6 did not increase cAMP level in human neutrophils. PP-6 did not inhibit superoxide anion production by NaF (20 mM), a direct activator of G-protein, the target of the inhibitory action of PP-6 appears to be a component of the signal transduction pathway upstream of G-protein. PP-6 inhibited FITC-fMLP binding to neutrophils in a concentration-dependent manner with an IC_{50} of 1.5 ± 0.2 μM. PP-6 did not bring a parallel shift in the concentration response of fMLP-induced superoxide anion. Additionally, the inhibiting effect of PP-6 on fMLP-induced superoxide anion was reversed when PP-6 was washed out. These experimental results suggest that PP-6 exerts non-competitive and reversible antagonistic effect on fMLP receptor.

* *Corresponding author at*: No. 259 Wen-Hwa 1st Road, Kwei-Shan, Tao-Yuan 333, Taiwan, ROC. Tel.: +886 3 2118800x5522; fax: +886 3 2118877.
E-mail address: liaoch@mail.cgu.edu.tw (C.-H. Liao).
0006-2952/$ – see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2007.10.002

1. Introduction

Neutrophils constitute 50–60% of total circulating leukocytes and are the major cellular component of the innate immune response. Neutrophils provide the host with a first line of defense against acute bacterial and fungal diseases, and recurrent severe or unusual infections are often associated with the inherited defects in neutrophil function [1,2]. However, convincing evidence links inappropriate neutrophil-mediated tissue damage to the pathogenesis of conditions such as acute respiratory distress syndrome, ischemia-reperfusion injury and rheumatoid arthritis [3,4]. In response to numerous endogenous and exogenous agents neutrophils undergo chemotaxis toward inflammation sites, both for defense against infectious agents and in various pathologic processes [5,6]. At inflammation sites, they release destructive bioactive compounds from cytoplasmic granules, perform phagocytic functions and produce reactive oxygen species [7]. The release of reactive oxygen species, which are formed in neutrophils by the action of NADPH oxidase, is the so-called respiratory burst and is a crucial bactericidal mechanism [8]. An inappropriately triggered respiratory burst may also cause pathological inflammation, lipid peroxidation and tissue damage.

Chemotactic agents such as *N*-formylated peptides (ex fMLP) and chemokines help to orchestrate these neutrophil functions by initiating various signaling cascades [4]. A clear understanding of the mechanisms of action of neutrophil chemoattractants may elucidate new approaches for selective pharmacological manipulation of neutrophils. Such manipulations may enhance host defense activities and ameliorate conditions such as inflammatory bowel disease and various forms of arthritis in which these neutrophil functions are believed to play pathogenic roles [9]. Studies have demonstrated that small formyl peptide derivatives, acquired as bacterial metabolites or derived from disrupted mitochondria can be potent chemoattractants for phagocytes. Due to its marked ability to activate all physiological functions of neutrophils through cell surface receptors coupled to intracellular effectors through G-protein, fMLP is employed in this study as a model chemoattractant. The interaction of fMLP with its receptor expressed on neutrophils triggers multiple second messengers by activating of PLC [10], PLD and PLA_2 rapidly stimulates PI3K and activates tyrosine phosphorylation [11]. Increased intracellular levels of cAMP [12] and involvement of kinases, such as PKC [13] and MAPKs [14] have also been demonstrated [15]. Activation of these signal transduction pathways is known to trigger various biochemical responses contributing to physiological defense against bacterial infection and cell disruption.

The lignan (2R,3R)-2-(3′,4′-dihydroxybenzyl)-3-(3″,4″-dimethoxybenzyl) butyrolactone (PP-6) is isolated from the stem of *Piper philippinum*. This lignan was the first isolated from its' natural source, although Chen had transferred arctiin to it using human intestinal bacteria [16]. This study investigated the mechanisms of PP-6 on superoxide anion production caused by fMLP and several other chemotactic agents, such as leukotrine B_4 (LTB_4) and PMA in human neutrophils. PP-6 inhibited fMLP-induced superoxide anion release and Mac-1 (CD11b/CD18) expression without affecting these induced by LTB_4 or PMA in human neutrophils. Moreover, PP-6 inhibited intracellular signaling, such as ERK, p38 or Akt phosphorylation, intracellular calcium mobilization, caused by fMLP. The present study revealed that PP-6, using intact human neutrophils, inhibits fMLP binding to its receptor. The inhibiting action of PP-6 was reversible because washing out PP-6 abolished inhibition of superoxide anion production. Furthermore, inhibition by PP-6 of the maximal response to fMLP was not surmountable by at higher concentrations of fMLP. According to these data, PP-6 antagonized fMLP binding to its receptor in a non-competitive and reversible manner.

2. Materials and methods

2.1. Materials

(2R,3R)-2-(3′,4′-dihydroxybenzyl)-3-(3″,4″-dimethoxybenzyl)-butyrolactone (PP-6) (Fig. 1) was isolated from *P. philippinum* kindly provided by Dr. Chen Ih-Sheng. *P. philippinum* is woody climber distributed throughout the Philippines and Lanyu and Lutao Islands in Taiwan [16]. The Tau (Yami) aborigines on Lanyu use the older stems in place of pistillate inflorescences of betel for betel quid chewing together with lime and catechu. Briefly, dried stem (4.0 kg) of *P. philippinum* was sliced and extracted with cold MeOH three times. After removal of solvent in vacuum, the extract was partitioned into *n*-hexane soluble fraction (Fr. A, 22 g), $CHCl_3$ soluble fraction (Fr. B, 60 g), *n*-BuOH soluble fraction (Fr. C, 60 g), and H_2O soluble fraction (Fr. D, 120 g). Fr. B (60 g) was chromatographed over silica gel (1.8 kg), eluting with $CHCl_3$ and gradually enriched with acetone to give 12 frs (B-1–B-12). Fr. B-9 (21.5 g, $CHCl_3$–acetone, 80:20) was chromatographed on silica gel (645 g) eluting with $CHCl_3$, gradually increasing the polarity with MeOH to obtain (2R,3R)-2-(3′,4′-dihydroxybenzyl)-3-(3″,4″-dimethoxy-benzyl)-butyrolactone (10.8 mg) [16]. *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP), fura-2 acetoxymethylester (Fura-2/AM)

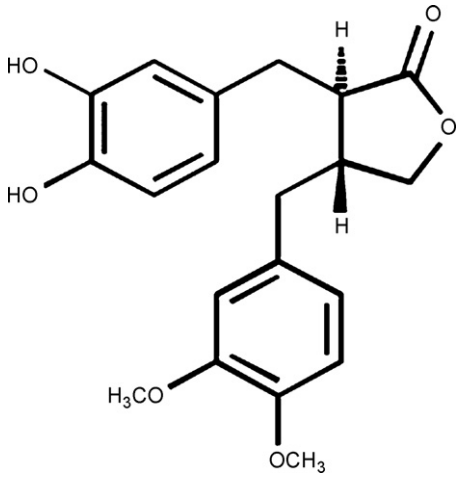

Fig. 1 – Chemical structure of (2R,3R)-2-(3′,4′-dihydroxybenzyl)-3-(3″,4″-dimethoxybenzyl)butyrolactone (PP-6).

and Hank's buffered saline (HBSS) were purchased from Sigma (St. Louis, MO, U.S.A). Hydroethidium and *N*-formyl-norleucyl-leucyl-phenylalanyl-norleucyl-throsyl-lysine-fluorescein (FLPEP) were purchased from Molecular Probe (Eugene, OR, U.S.A). MAC-1 FITC conjugated mouse anti-human monoclonal antibody was purchased from BD PharmMingen Technical (San Diego, CA, U.S.A). Phospho-p44/42 MAP kinase (Thr202/Tyr204) antibody (anti-human) and phospho-p38 MAP kinase (Thr180/Tyr182) antibodies were purchased from Cell Signaling Technology (Danvers, MA, U.S.A).

2.2. Preparation of human neutrophil

Venous blood samples were obtained from healthy volunteers of both gender aged 20–40 years, using syringes containing heparin (final concentration 20 unit/ml). All protocols complied with Chang-Gung Memorial Hospital Ethics Committee guidelines. Neutrophils were isolated from blood samples by Ficoll gradient centrifugation then purified by hypotonic lysis of contamination erythrocytes [17]. Briefly, a blood sample was mixed with an equal volume of 3% dextran solution in a 50 ml-centrifuge tube and incubated in an upright position for 20 min at room temperature to allow for the sedimentation of erythrocytes. The leukocyte-rich upper layer was then collected and centrifuged at 250 × g for 15 min at 4 °C. The pellet was then immediately re-suspended in a volume of phosphate buffer system (PBS) equal to the initial volume of blood. The cell suspension thus obtained was then apportioned into 50-ml centrifuge tubes at 25-ml per tube. Using a pipette, a layer of 10 ml of 1.077 g/ml Ficoll solution was laid beneath the cell suspension. After a final centrifuge (400 × *g* for 40 min at 20 °C), the upper (PBS) and lower (Ficoll) layers were removed, leaving only the granulocyte/erythrocyte pellets. To remove the residual erythrocytes, the pellet was re-suspended in 20 ml cold 0.2% NaCl for 30 s. Twenty millilitres of cold 1.6% NaCl was then added to restore tonicity. Finally, the remaining neutrophils were pelleted, washed twice with ice-cold PBS and re-suspended in an adequate volume of ice-cold Hank's buffered saline (HBSS). The final preparation thus obtained contained more than 95% neutrophils, as estimated by differentially counting 200 Giemsa stained cells under microscope.

2.3. Superoxide anion measurement

Intracellular production of superoxide anion was analyzed by flow cytometer according to the method described by Bassoe et al. [18]. Briefly, neutrophils were incubated at 37 °C for 15 min with 10 μM of hydroethidium. Hydroethidium can be directly oxidized by superoxide anion to ethidium bromide, which fluoresces after intercalating with nucleic acids. After labeling, cells were treated with varying concentrations of PP-6 5 min before stimulation with fMLP (1 μM), LTB_4 (1 μM), PMA (100 nM) or NaF (20 mM). After 15 min stimulation, production of superoxide anion was monitored by FACScan flow cytometer (Becton Dickinson, San Jose, CA, U.S.A) by measuring emission at 590 nm for ethidium bromide. For each sample, 10,000 cells were collected to measure of fluorescence intensity. Data are expressed as mean value of fluorescence intensity (MFI) for each sample as describe above.

2.4. Measurement of MAC-1 up-regulation by flow cytometer

Expression of MAC-1 was analyzed as described by Endeman et al. with some modification [19]. Neutrophils (1×10^6 cells/ml) were incubated with PP-6 (10 μM) or DMSO (0.05%) 5 min before adding fMLP (1 μM), LTB_4 (1 μM) or PMA (100 nM) in the presence of 20 μg/ml of FITC-conjugated anti-MAC-1 antibody or a nonspecific mouse immunoglobulin G (IgG) as a negative control. Cells were fixed with 1% paraformaldehyde 30 min after incubation and analyzed by flow cytometer.

2.5. Intracellular calcium measurement

The method of Pollock and Rink, with some modification, was used for intracellular calcium measurement [20]. Briefly, neutrophils (1×10^6 cells/ml) were incubated with fura-2/AM (2 μM) at 37 °C for 30 min then centrifuged at 200 × *g*. The resultant pellet was washed with HBSS. After centrifugation, neutrophils were re-suspended in HBSS containing calcium (1 mM). Neutrophils (1×10^6 cells/ml) were treated with PP-6 (10 μM) 5 min before challenge with fMLP (1 μM) or LTB_4 (1 μM). Fluorescence (excitation 340 nm and 380 nm; emission 500 nm) was measured by Hitachi fluorescence spectrophotometer (model F4500; Tokyo, Japan) at 37 °C. At the end of the experiment, the cells were treated with triton X-100 (0.1%) and EGTA (10 mM) to obtain the maximal and minimal fluorescence, respectively. Intracellular calcium was calculated as described for fura-2 using the calcium-dye dissociation constant 224 nM [20].

2.6. Western blotting analysis

To analyze affect of ERK phosphorylation, neutrophils (2×10^6 cells/ml) were incubated with or without PP-6 (10 μM) for 5 min at 37 °C and then stimulated with fMLP (1 μM), LTB_4 (1 μM) or PMA (100 nM). The reaction was terminated by placing the cells on ice and subjected them to immediate centrifugation. The pellet was re-suspending in 1× Laemmli sample buffer. After boiling for 10 min, the proteins were stored in −70 °C for immunoblotting assay. The sample was electrophoresed in 8–10% SDS-polyacrylamide gels and transferred electrophoretically to nitrocellulose. Blots were stained with Ponceau-S (0.2% Ponceau-S 3% TCA, and 3% sulfosalicylic acid) to visualize Mr markers then destained with deionized water followed by TBST (10 mM Tris–HCl (pH 7.5), 100 mM NaCl, and 0.1% Tween-20). Blots were blocked for 1 h in 5% nonfat milk in TBST. Antibodies were diluted in PBS (pH 7.3) containing 3% bovine serum albumin (BSA) and 0.02% sodium azide. Blots were incubated with the appropriate antibody (Phospho-p44/42 MAP kinase or Phospho-p38; 1/1000) for 2 h at 25 °C then thoroughly washed (three times, 10 min each) with TBST. Next, blots were incubated for 1 h with an appropriate horseradish peroxidase-conjugated secondary antibody (1/5000) in 5% nonfat milk in TBST, washed thoroughly and examined by enhanced chemiluminescence.

2.7. Flow cytometer analysis of fMLP receptor

Expression of fMLP receptor on the surface of neutrophils was monitored with a fluorescent analogue of fMLP, *N*-formyl-nor-

leucyl-leucyl-phenylalanyl-norleucyl-throsyl-lysine-fluorescein (FLPEP) [21]. Briefly, 3×10^5 neutrophils were treated with various concentrations of PP-6 or DMSO (0.5%) for 5 min. Neutrophils were labeled 30 min at 4 °C with FLPEP (2 nM). Alternatively, nonspecific binding was determined with cells incubated with over amount of fMLP (10 μM) before FLPEP was added. Finally, cells were washed in PBS and re-suspended in PBS before flow cytometer analysis with a FACScan flow cytometer (Becton Dickinson, San Jose, CA). Acquisition was performed at 10,000 events per sample.

2.8. Cyclic nucleotides analysis

Intracellular cyclic AMP (cAMP) was measured as described in a previous study [22]. Briefly, human neutrophils were incubated with DMSO (0.5%; resting), Forskolin (10 μM), IBMX (50 μM) or PP-6 (10 μM) for 5 min. The reactions were terminated by adding EDTA (10 mM) and then followed by boiling for 2 min. The cAMP content was determined by enzyme immunoassay.

3. Results

3.1. PP-6 specific inhibits fMLP-induced superoxide anion production in human neutrophils

Stimulation of human neutrophils with fMLP (1 μM), PMA (100 nM) or LTB_4 (1 μM) induced production of superoxide anion. PP-6 attenuated fMLP-induced superoxide anion in a dose-dependent manner. The IC_{50} value for the inhibiting action of PP-6 was 0.3 ± 0.1 μM (Fig. 2A). Treatment of the cells with a high concentration of PP-6 (10 μM) did not affect the PMA- or LTB_4-induced superoxide anion (Fig. 2B and C).

3.2. PP-6 inhibits fMLP-induced intracellular calcium mobilization and ERK phosphorylation

Intracellular calcium mobilization in human neutrophils was induced by fMLP (1 μM) or LTB_4 (100 nM) (Fig. 3A; (a and b)). PP-6 (10 μM) prevented intracellular calcium mobilization induced by fMLP (Fig. 3A; (a)). However, PP-6 did not affect intracellular calcium mobilization induced by LTB_4 (Fig. 3A; (b)). In another series of experiments, fMLP (1 μM), PMA (100 nM) or LTB_4 (1 μM) induced ERK and p-38 phosphorylation (Fig. 3B). The PP-6 (10 μM) inhibited fMLP-induced ERK (Fig. 3B; (a)) and p38 (Fig. 3B; (b)) phosphorylation. However, PP-6 (10 μM) did not affect ERK and p38 phosphorylation caused by PMA and LTB_4. The ERK phosphorylation caused by fMLP as a positive control and inhibited by PD98059 (Fig. 3B; (b)).

3.3. PP-6 does not inhibit NaF-stimulated superoxide anion production in human neutrophils

A G-protein activator, NaF (20 mM), induced moderate superoxide anion production by human neutrophils. Treatment with wortmannin (1 μM), a PI3 kinase inhibitor, significantly inhibited NaF-induced production of superoxide anion, whereas PP-6 (10 μM) did not suppress the NaF-induced production of superoxide anion on human neutrophils

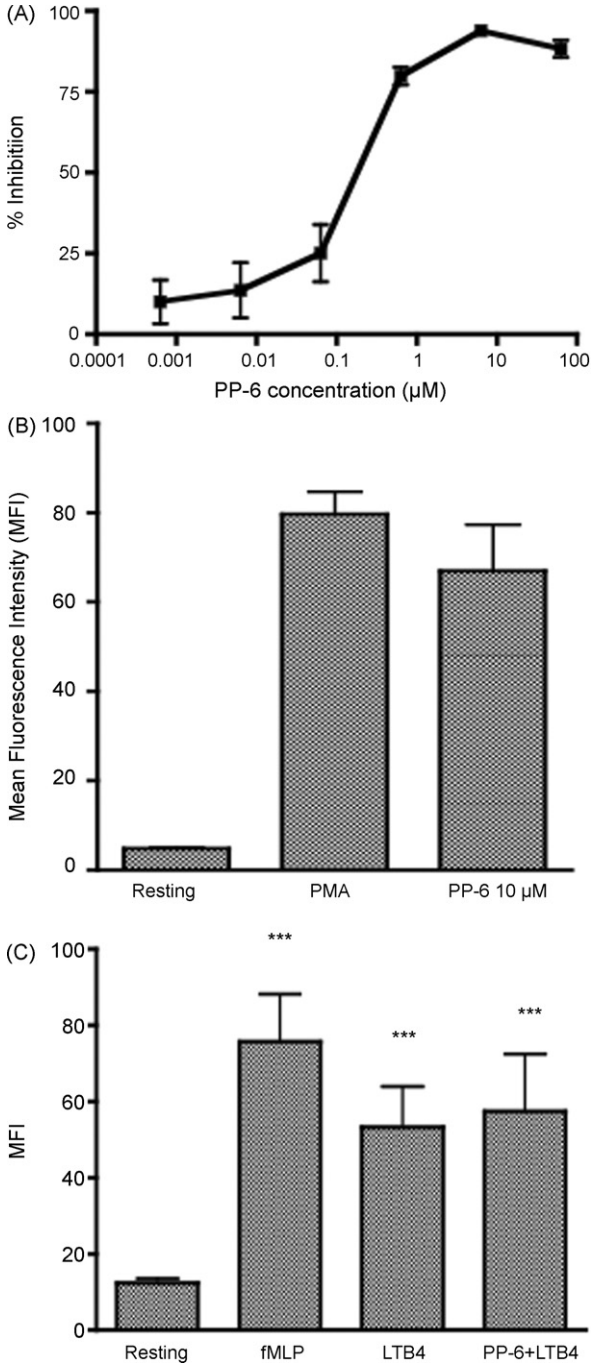

Fig. 2 – Specific effect of PP-6 on fMLP-induced superoxide anion production in human neutrophils. (A) Concentration response of PP-6 on fMLP (1 μM) induced superoxide anion production. The IC_{50} value for PP-6 inhibition of fMLP-induced free radical is 0.3 ± 0.1 μM. (B) PMA (100 nM) induced significant superoxide anion production. PP-6 (10 μM) did not affect superoxide anion production by PMA. (C) Leukotriene B_4 (LTB_4 1 μM) induced superoxide anion production by a Gi-protein couple receptor. PP-6 did not affect the superoxide anion production caused by LTB_4. fMLP (1 μM) as a positive control. ***P < 0.001 as compared with resting state.

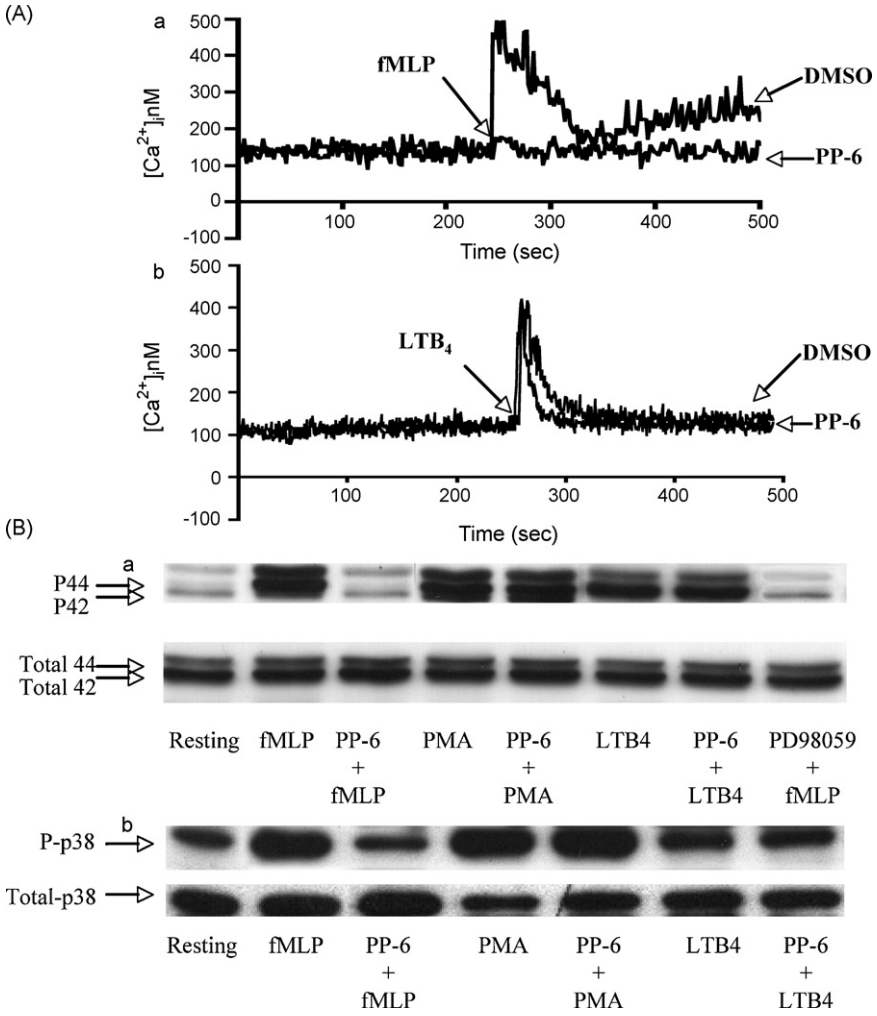

Fig. 3 – PP-6 specific affects fMLP-induced-intracellular signaling. (A) fura-2/AM loaded neutrophils were incubated with DMSO or PP-6 (10 μM) as illustrated for 5 min then fMLP (1 μM; (a)) or LTB_4 (100 nM; (b)) was then added to trigger intracellular calcium mobilization. (B) Human neutrophils were incubated with DMSO or PP-6 (10 μM) for 5 min before adding fMLP (1 μM), PMA (100 nM) or LTB_4 (1 μM) was added, respectively. Reactions were stopped with sample buffer then applied to 10% gel. P42/44 (a) and p38 (b) were detected with Ab.

(Fig. 4). This experimental result indicated that the inhibition of fMLP-induced superoxide anion production by PP-6 is due to its inhibition of a component of the signal transduction pathway upstream of G-protein.

3.4. *PP-6 does not increase cAMP in human neutrophils*

Human neutrophils were treated with PP-6 (10 μM) for varying time periods in the presence or absence of IBMX (50 μM). Treatment with PP-6 did not increase cAMP level in the presence or absence of IBMX (Table 1). Forskolin or IBMX alone was positive control for cAMP levels (Table 1).

3.5. *PP-6 inhibits FITC-fMLP-receptor binding in human neutrophils*

Whole cell of human neutrophils were studied to elucidate the effect of PP-6 on the interaction between fMLP and its receptor. Binding of FLPEP to neutrophils was inhibited by fMLP in a concentration-dependent manner (Fig. 5A). Moreover, FLPEP binding to neutrophils was inhibited by PP-6 in a concentration-dependent manner (Fig. 5B and C). The IC_{50} value for the inhibiting action of PP-6 was 1.5 ± 0.2 μM (Fig. 5C).

3.6. *PP-6 does not shift the dose–response curve for fMLP-induced superoxide anion production to the right*

To confirm the mode of PP-6 action, the effect of PP-6 on the concentration–response curve for fMLP-induced superoxide anion production was examined. The fMLP was found to induce superoxide anion production in a concentration-dependent manner (Fig. 6). PP-6 (0.1, 1 or 10 μM) did not cause parallel shift in the fMLP concentration–response curve to the right. Additionally, the inhibition by PP-6 of the maximal response to fMLP was not surmountable at higher concentration of fMLP (Fig. 6). This experimental result indicated that the

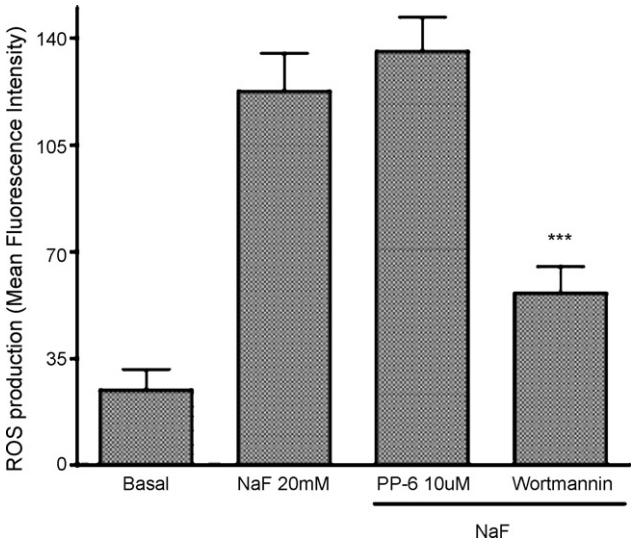

Fig. 4 – Effect of PP-6 on NaF-induced superoxide anion production in human neutrophils. Neutrophils were pretreated with wortmannin (1 μM) or PP-6 (10 μM) at 37 °C for 5 min and stimulated with NaF (20 mM). Superoxide anion production was measured by peak value of mean fluorescence intensity (MFI). Data are means ± S.E.M., $n = 5$. ***$P < 0.001$ as compared with NaF alone.

inhibiting effect of PP-6 is a non-competitive antagonistic action [23,24].

3.7. *Washing out of PP-6 abolishes the inhibition of fMLP-induced superoxide anion production*

To examine the reversibility of the inhibiting effect of PP-6, neutrophils were treated with PP-6 (10 μM) for 5 min, and PP-6 was washed out before stimulation with fMLP. Fig. 7 shows that the fMLP-induced superoxide anion production recovered after PP-6 had been washed out. This result indicates that the inhibiting properties of PP-6 are reversible.

Table 1 – PP-6 did not increase cAMP level in human neutrophil

	cAMP (pg/ml)
Resting	2.5 ± 0.5
Forskolin (10 μM)	18.3 ± 0.2***
IBMX (50 μM)	12.5 ± 0.3***
Forskolin + IBMX	35.7 ± 0.6***
PP-6 10 μM	2.8 ± 0.2
PP-6 + IBMX	11.5 ± 0.5***
PP-6 + Forskolin	20.3 ± 0.8***

Human neutrophils were incubated with DMSO (0.5%), Forskolin (10 μM), IBMX (50 μM), Forskolin + IBMX or PP-6 (10 μM) for 5 min. The reactions were terminated by adding of EDTA (10 mM) and then followed by boiling 2 min. The cAMP content was determined by enzyme immunoassay. Values are presented as means ± S.E.M. ($n = 6$).
*** $P < 0.001$ as compared with resting.

3.8. *Specific effect of PP-6 on fMLP-induced MAC-1 expression*

To confirm the specific inhibiting effect of PP-6 on fMLP-activated human neutrophils, MAC-1 expression on human neutrophils was evaluated. MAC-1 is the main adhesion receptor and plays an important role in migration of neutrophils during the process of inflammation. MAC-1 is expressed by various inflammatory receptors which initiates a signal transduction cascade [25]. Stimulation of human neutrophils with fMLP (1 μM), PMA (100 nM) or LTB_4 (100 nM) for 15 min significantly induced MAC-1 expression (Fig. 8A–C). Pretreatment neutrophils with PP-6 (10 μM) attenuated fMLP increased MAC-1 expression; moreover, PP-6 did not affect the MAC-1 expression induced by LTB_4. However, PP-6 had an insignificantly effect on MAC-1 expression induced by PMA (100 nM) (Fig. 8B and C).

4. Discussion

This study investigated the inhibiting mechanisms of PP-6, a natural product from *P. philippinum*, on superoxide anion production in human neutrophils. Several chemoattractants, such as fMLP, LTB_4 and PMA were using for triggering superoxide anion production. According to our data, PP-6 specific inhibited fMLP-induced superoxide anion release, therefore, we evaluated the mechanism of PP-6 on fMLP. The cell viability was not affected by high concentration of PP-6 in present study.

Following fMLP binding, fMLP receptor undergoes a conformational change enabling interaction pertussis toxin (PTX)-sensitive G proteins of Gi-family in human neutrophils. Downstream of the heterotrimeric G protein, a number of signaling systems are activated in human neutrophils [26]; activation of PLC_β results in hydrolysis of phosphatidylinositol 4,5-bisphosphate, generating diacylglycerol (DAG) which activates PKC isoforms and inositol-1,4,5-trisphosphate (IP_3) which release Ca^{2+} from intracellular stores. Activation of the enzyme $PI3K_\gamma$ isoform converts [Ptlns(4,5)P2] into inositol-3,4,5-trisphosphate; this regulates localization and possible cross-linking/stabilization of actin filaments. Other intracellular effectors coupled to fMLP receptor signaling cascade include PLA_2, PLD, MAPKs and phosphorylation mediated by tyrosine kinase [26]. Activation of these signal transduction pathways is known to provoke various biochemical responses contributing to physiological defense against bacterial infection and cell disruption.

Superoxide anion production in human neutrophils is elicited by fMLP, LTB_4 or PMA by activating NADPH oxidase. PP-6 does not affect the enzyme activity of NADPH oxidase nor the assemble activity of NADPH oxidase in a cell free system (data not shown). Moreover, PP-6 does not scavenge superoxide anion in a xanthine/xanthine oxidase system (data not shown). This finding suggests that PP-6 may affect intracellular signaling for activating NADPH oxidase. PP-6 specific inhibited superoxide anion production when human neutrophils were stimulated by fMLP; however, PP-6 did not inhibit PMA-induced superoxide anion production. In addition, PP-6 did not affect the ERK, p38 or Akt phosphorylation caused by

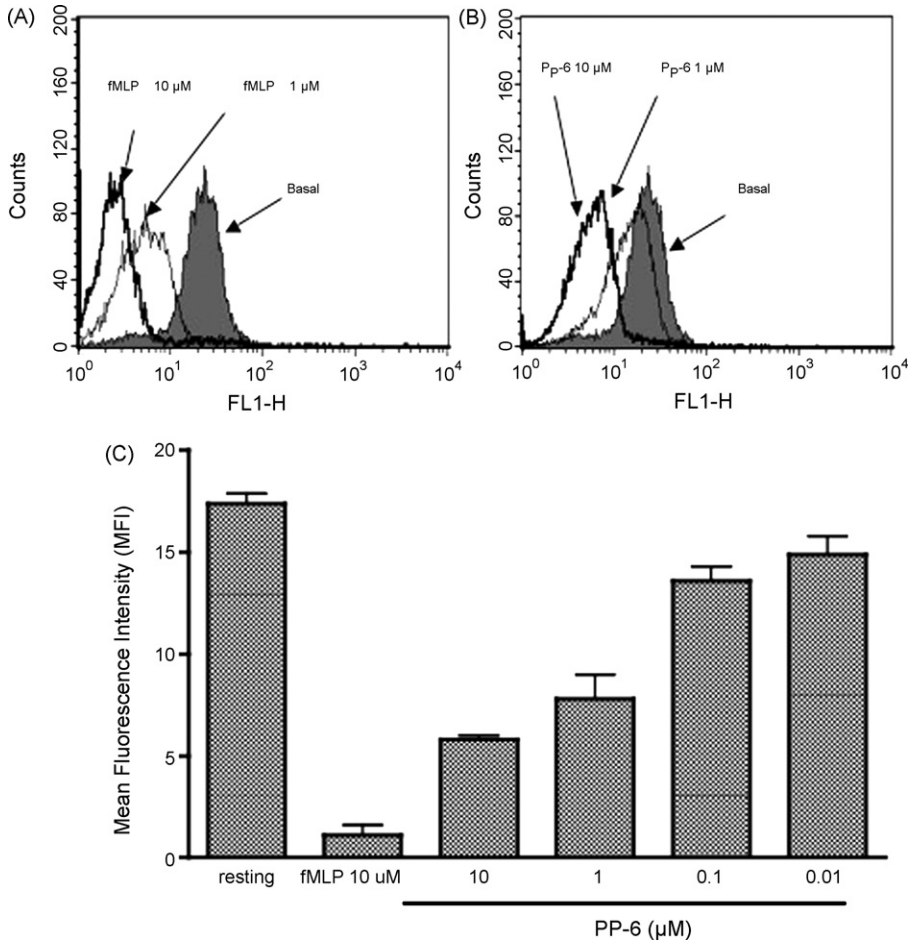

Fig. 5 – Inhibitory effect of PP-6 on FLPEP binding to membrane receptor. (A) Human neutrophils were incubated at different concentrations of fMLP (1 or 10 μM) for 5 min before adding FITC-conjugated fMLP for an additional 20 min. Binding to FITC-fMLP was determined by flow cytometer. (B) Human neutrophils were incubated at various concentrations of PP-6 (1 or 10 μM) 5 min before adding FITC-conjugated fMLP for an additional 20 min. Samples were analyzed by flow cytometer. (C) Concentration response of PP-6 to inhibition of FLPEP binding on human neutrophil. fMLP (10 μM) served as a positive control.

PMA. These data indicate that PP-6 does not affect the PKC activity. Furthermore, inflammatory mediator-LTB_4 induced superoxide anion production in human neutrophils through LTB_4 receptor. Two membranes receptors for LTB_4 have been identified in human neutrophils; BLT1 and BLT2 [27]. Both receptors are members of the heptohelical, G protein-coupled receptor family. In this study, PP-6 did not affect superoxide anion production by LTB_4, suggesting an inhibiting effect of PP-6 specifically on fMLP.

To clarify the specific effect of PP-6 on fMLP-induced neutrophil activation, intracellular signals induced by fMLP, LTB_4 or PMA were evaluated. Intracellular calcium mobilization, ERK- and p38-phosphorylation were all induced by fMLP or LTB_4 through fMLP receptor or LTB_4 receptor, respectively. The role of calcium as a primary or secondary messenger in neutrophil activation induced by fMLP or LTB_4 has been extensively studied [28,29]. PP-6 inhibited the intracellular calcium mobilization induced by fMLP; however, PP-6 does not affect the intracellular calcium mobilization induced by LTB_4. Moreover, PP-6 inhibits ERK and p38 phosphorylation induced by fMLP without affecting these proteins (ERK and p38) phosphorylation caused by PMA or LTB_4. In another series of studies, fMLP, PMA and LTB_4 increased MAC-1 expression in human neutrophil. PP-6 specific inhibited fMLP-induced MAC-1 expression. Moreover, PP-6 did not suppress NaF-induced superoxide anion production. This result indicated that the inhibition of fMLP-induced superoxide anion production by PP-6 is due to its inhibition of a component of the signal transduction pathway upstream of G-protein. The specific effects of PP-6 on fMLP-induced free radical production, intracellular calcium mobilization, ERK and P38 phosphorylation and MAC-1 expression, suggest PP-6 may be a fMLP receptor antagonist.

To evaluate the hypothesis that PP-6 is a fMLP receptor antagonist on human neutrophil, FITC-conjugated fMLP (FLPEP) was used in this study. The fMLP inhibiting the binding of FLPEP to the human neutrophil served a positive control. These data confirm the specific binding effect of FLPEP on fMLP receptor on human neutrophil. PP-6 inhibited FLPEP binding to human neutrophil in a concentration-dependent

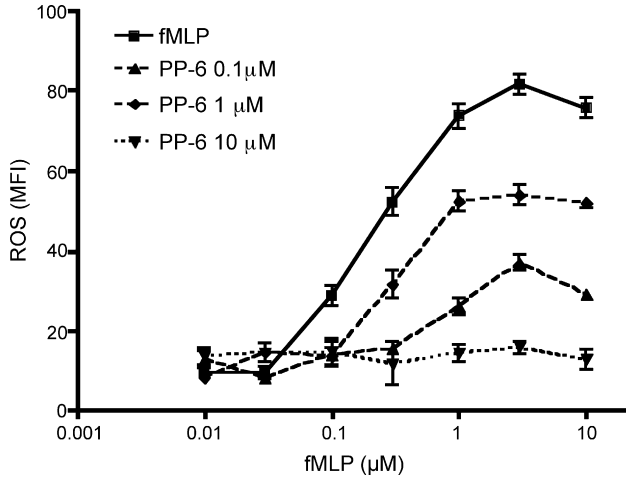

Fig. 6 – Effect of PP-6 on the dose–response curve for fMLP-induced superoxide anion production. Neutrophils were pretreated with vehicle or various concentration of PP-6 (0.1, 1 or 10 μM) at 37 °C for 5 min and stimulated with fMLP at the indicated concentrations (0.01–10 μM). Superoxide anion production was measured from peak value of MFI. Data are shown as means ± S.E.M., $n = 5$.

manner, which confirms that PP-6 is a fMLP receptor antagonist. To study the typical action of PP-6, the effect of PP-6 on dose–response curve of fMLP-induced superoxide anion production was examined. PP-6 exhibited no parallel

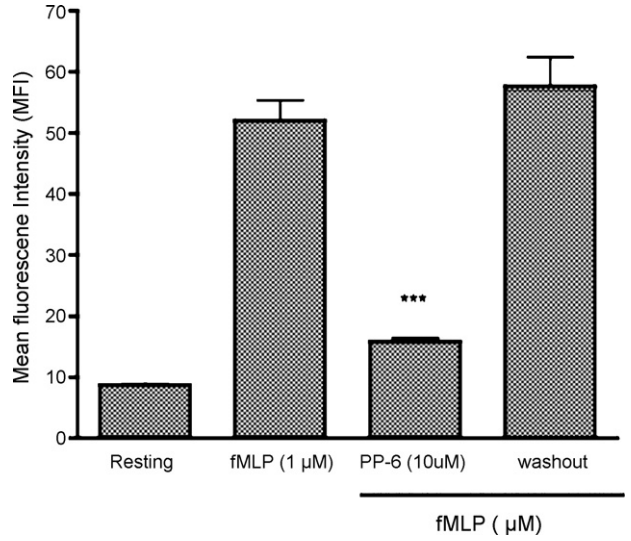

Fig. 7 – Washing out of PP-6 abolishes the inhibition of fMLP-induced superoxide anion production. Neutrophils were pretreated with PP-6 (10 μM) for 5 min, and then PP-6 was washed out with HBSS before stimulation with fMLP (1 μM). Control cells were pretreated, washed and re-suspended with HBSS. The peak value of MFI was regarded as indicative of the level of superoxide anion production and is expressed as percentage of control. Data are shown as means ± S.E.M., $n = 5$. ***$P < 0.001$ as compared with control (fMLP).

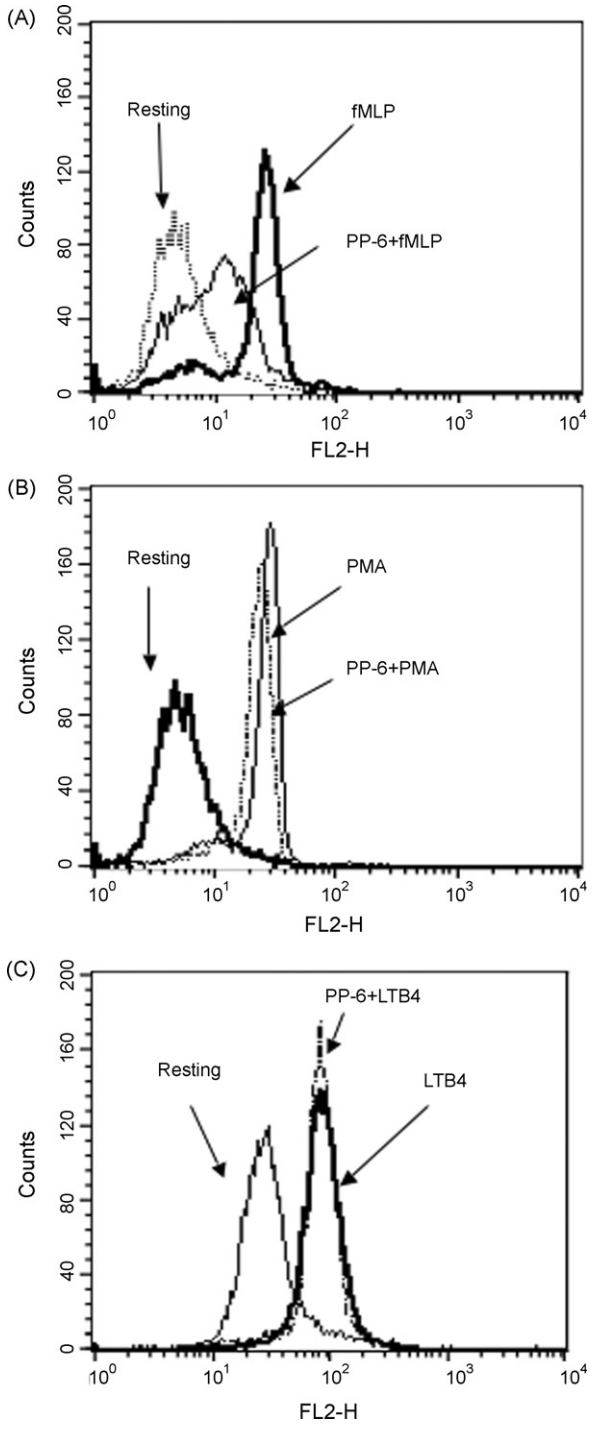

Fig. 8 – Specific effect of PP-6 on fMLP-induced MAC-1 expression. Human neutrophils (1×10^6/ml) were pretreated with DMSO (0.05%; resting and control) or PP-6 (10 μM) at 37 °C 5 min before fMLP (1 μM) (A); PMA (100 nM) (B); and LTB_4 (1 μM). Expression MAC-1 was evaluated by flow cytometer as discussed in Section 2 ($n = 5$).

shift in the fMLP concentration–response curve to the right. Additionally, inhibition by PP-6 of the maximal response to fMLP was not surmountable by at higher concentrations of fMLP. According to previous study, a non-competitive

antagonist prevents the agonist, at any concentration, from producing a maximum effect on a given receptor [23,24,30]. Therefore, the result of this experiment shows that the inhibiting effect of PP-6 is a non-competitive antagonistic action. Furthermore, the inhibiting action of PP-6 was reversible because washing out PP-6 abolished inhibition of superoxide anion production. According to these data, PP-6 is a non-competitive antagonist of fMLP-receptor, and this antagonistic action is a reversible effect.

In the case of *H. pylori* infection, lots of neutrophils are known to infiltrate into the gastric mucosa and injure the host tissue. Mooney et al. [31] reported a substance that reacted with fMLP antibody was produced by *H. pylori* and it activated neutrophils. PP-6 has been shown to inhibit the binding of fMLP to fMLP-receptor in this study. Considering the results obtained in the present study, it seems that PP-6 may inhibit the binding of *H. pylori*-derived fMLP to fMLP-receptor and suppress the activation of neutrophil activation probably. However, it needs more studies to prove it.

Acknowledgements

This work was supported in part by National Science Council Grant NSC-95-2320-B-182-053, and a Chang Gung Memorial Hospital Grant.

REFERENCES

[1] Malech HL, Gallin JI. Current concepts: immunology. Neutrophils in human diseases. N Engl J Med 1987;317: 687–94.
[2] Kobayashi SD, Voyich JM, DeLeo FR. Regulation of the neutrophil-mediated inflammatory response to infection. Microbes Infect 2003;5:1337–44.
[3] Hernandez LA, Grisham MB, Twohig B, Arfors KE, Harlan JM, Granger DN. Role of neutrophils in ischemia-reperfusion-induced microvascular injury. Am J Physiol 1987;253:H699–703.
[4] Witko-Sarsat V, Rieu P, Descamps-Latscha B, Lesavre P, Halbwachs-Mecarelli L. Neutrophils: molecules, functions and pathophysiological aspects. Lab Invest 2000;80: 617–53.
[5] Berkow RL, Wang D, Larrick JW, Dodson RW, Howard TH. Enhancement of neutrophil superoxide production by preincubation with recombinant human tumor necrosis factor. J Immunol 1987;139:3783–91.
[6] Downey GP, Fukushima T, Fialkow L, Waddell TK. Intracellular signaling in neutrophil priming and activation. Semin Cell Biol 1995;6:345–56.
[7] Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci USA 1990;87: 1620–4.
[8] Hampton MB, Kettle AJ, Winterbourn CC. Inside the neutrophil phagosome: oxidants, myeloperoxidase, and bacterial killing. Blood 1998;92:3007–17.
[9] Parkos CA, Colgan SP, Madara JL. Interactions of neutrophils with epithelial cells: lessons from the intestine. J Am Soc Nephrol 1994;5:138–52.
[10] Ferretti ME, Nalli M, Biondi C, Colamussi ML, Pavan B, Traniello S, et al. Modulation of neutrophil phospholipase C activity and cyclic AMP levels by fMLP-OMe analogues. Cell Signal 2001;13:233–40.
[11] Okada T, Sakuma L, Fukui Y, Hazeki O, Ui M. Blockage of chemotactic peptide-induced stimulation of neutrophils by wortmannin as a result of selective inhibition of phosphatidylinositol 3-kinase. J Biol Chem 1994;269:3563–7.
[12] Fabbri E, Spisani S, Biondi C, Barbin L, Colamussi ML, Cariani A, et al. Two for-Met-Leu-Phe-OMe analogues trigger selective neutrophil responses. A differential effect on cytosolic free Ca^{2+}. Biochim Biophys Acta 1997;1359: 233–40.
[13] Dekker LV, Leitges M, Altschuler G, Mistry N, McDermott A, Roes J, et al. Protein kinase C-beta contributes to NADPH oxidase activation in neutrophils. Biochem J 2000;347(Pt 1):285–9.
[14] Selvatici R, Falzarano S, Traniello S, Pagani Zecchini G, Spisani S. Formylpeptides trigger selective molecular pathways that are required in the physiological functions of human neutrophils. Cell Signal 2003;15: 377–83.
[15] Selvatici R, Falzarano S, Mollica A, Spisani S. Signal transduction pathways triggered by selective formylpeptide analogues in human neutrophils. Eur J Pharmacol 2006;534:1–11.
[16] Chen YC, Liao CH, Chen IS. Lignans, an amide and anti-platelet activities from *Piper philippinum*. Phytochemistry 2007;68:2101–11.
[17] Liao CH, Hsiech YJ, Lin YC. Celecoxib simulates respiratory burst through pertussis toxin-sensitive G-protein, a possible signal for beta 2-integrin expression on human neutrophils. Eur J Pharmacol 2004;484:29–39.
[18] Bassoe CF, Li N, Ragheb K, Lawler G, Sturgis J, Robinson JP. Investigations of phagosomes, mitochondria, and acidic granules in human neutrophils using fluorescent probes. Cytometry 2003;51:21–9.
[19] Endemann G, Feng Y, Bryant CM, Hamilton GS, Perumattam J, Mewshaw RE, et al. Novel anti-inflammatory compounds prevent CD11b/CD18, alpha M beta 2 (Mac-1)-dependent neutrophil adhesion without blocking activation-induced changes in Mac-1. J Pharmacol Exp Ther 1996;276:5–12.
[20] Pollock WK, Rink TJ. Thrombin and ionomycin can raise platelet cytosolic Ca^{2+} to micromolar levels by discharge of internal Ca^{2+} stores: studies using fura-2. Biochem Biophys Res Commun 1986;139:308–14.
[21] Thivierge M, Parent JL, Stankova J, Rola-Pleszczynski M. Modulation of formyl peptide receptor expression by IL-10 in human monocytes and neutrophils. J Immunol 1999;162:3590–5.
[22] Wang JP, Chang LC, Raung SL, Hsu MF, Huang LJ, Kuo SC. Inhibition of superoxide anion generation by YC-1 in rat neutrophils through cyclic GMP-dependent and -independent mechanisms. Biochem Pharmacol 2002;63:577–85.
[23] Foreman JC, Johansen T. Textbook of receptor pharmacology. Salem: CRC Press, Inc.; 1996.
[24] Kenakin TP, Bond RA, Bonner TI. Definition of pharmacological receptors. Pharmacol Rev 1992;44:351–62.
[25] Harris ES, McIntyre TM, Prescott SM, Zimmerman GA. The leukocyte integrins. J Biol Chem 2000;275:23409–12.
[26] Niggli V. Signaling to migration in neutrophils: importance of localized pathways. Int J Biochem Cell Biol 2003;35: 1619–38.
[27] Serezani CH, Aronoff DM, Jancar S, Peters-Golden M. Leukotriene B4 mediates p47phox phosphorylation and membrane translocation in polyunsaturated fatty acid-stimulated neutrophils. J Leukoc Biol 2005;78:976–84.
[28] Tintinger GR, Theron AJ, Potjo M, Anderson R. Reactive oxidants regulate membrane repolarization and store-

operated uptake of calcium by formyl peptide-activated human neutrophils. Free Radic Biol Med 2007;42:1851–7.
[29] Sitrin RG, Emery SL, Sassanella TM, Blackwood RA, Petty HR. Selective localization of recognition complexes for leukotriene B4 and formyl-Met-Leu-Phe within lipid raft microdomains of human polymorphonuclear neutrophils. J Immunol 2006;177:8177–84.
[30] Hardman JG, Limbird LE, Molinoff PB, Ruddon RW, Alfred GG. Goodman and Gilman's The pharmacological basis of therapeutics, 9th ed., The McGraw-Hill Companies; 1996
[31] Mooney C, Keenan J, Munster D, Wilson I, Allardyce R, Bagshaw P, et al. Neutrophil activation by *Helicobacter pylori*. Gut 1991;32:853–7.