Platelet Activating Factor (PAF) Antagonists Contained in Medicinal Plants: Lignans and Sesquiterpenes

Satoshi IWAKAMI, Jin-Bin WU, Yutaka EBIZUKA and Ushio SANKAWA*

Faculty of Pharmaceutical Sciences, The University of Tokyo, 7–3–1 Hongo, Bunkyo-ku, Tokyo 113, Japan. *Received November 20, 1991*

Hot aqueous extracts of medicinal plants were tested for their inhibitory effect on the binding of platelet activating factor (PAF) to rabbit platelets. The extracts of *Forsythia suspensa* VAHL. (Oleaceae), *Arctium lappa* L. (Compositae) and *Centipeda minima* (L.) A. BRAUN *et* ASCHERS (Compositae) showed significant activities. Since the main constituents of *F. suspensa* and *A. lappa* are lignans, 30 lignans were tested for their inhibitory effects on PAF binding to platelets and 9 lignans were found active. Four sesquiterpenes were isolated as active compounds from *C. minima*. In particular 6-*O*-angeloylplenolin and 6-*O*-senecioylplenolin are the most potent and specific PAF antagonists found in this study.

Keywords *Centipeda minima*; platelet activating factor (PAF); antagonist; lignan; sesquiterpene; 6-*O*-angeloylplenolin; 6-*O*-senecioylplenolin

Platelet activating factor (PAF) is a compound released from activated basophils to induce platelet aggregation.[1] Extensive studies on PAF have clarified that this compound acts as a chemical mediator in a wide range of physiological phenomena not limited to anaphylaxis response. The search for PAF antagonists has been the subject of studies to find new lead compounds for drug development and PAF analogues were synthesized to find PAF antagonists or agonists. CV-3988 is the first PAF antagonist found among synthetic PAF analogues,[2] and following this, several PAF analogues possessing antagonist activity were synthesized.[3] Shen *et al.* first reported the isolation of a naturally occurring PAF antagonist, kadsurenone, from *Piper futokadsura* SIEB. *et* ZUCC. (Piperaceae), which has been used in Chinese traditional medicine for anti-allergy purposes including in asthma.[4a] Kadsurenone is a neolignan with potent PAF antagonist activity and has been proved to be active against *in vivo* experimental allergy. A synthetic compound, L-652,731, which has been designed from the structure of kadsurenone, is a highly active PAF antagonist.[4b] A sesquiterpene, L-652,469, has been isolated as a PAF antagonist from *Tussilago farfara* L. which is a medicinal plant used in Chinese medicine.[5] Ginkgolides, diterpenes of *Ginkgo biloba* L., were also found to have PAF antagonist activity.[6] More recently coumarins contained in *Peucedanum praeruptorum* DUUN were identified as PAF antagonists.[7]

In our previous studies on anti-allergic compounds contained in medicinal plants, we surveyed literature on medicinal plants used for anti-allergy purposes in traditional oriental medicine, and followed this by screening studies using *in vivo* passive cutaneous anaphylaxis (PCA) tests and *in vitro* inhibition tests on induced histamine release from rat mast cells.[8] Flavonoids, sesquiterpenes and lignans were isolated and identified as active compounds from medicinal plants which were reported to be active for allergic diseases at the clinical level.[8,9] We have introduced PAF binding assay to test the inhibitory effects of the extracts of medicinal plants.[10] The search for PAF antagonists is usually carried out by measuring the inhibitory effect on platelet aggregation induced by PAF. The binding assay has a disadvantage in that antagonist and agonist cannot be distinguished, however we dared to use the binding assay from the point of its convenience and efficiency to evaluate biological activities of extracts and fractions in isolation and separation processes. In a previous communication we briefly reported the identification of lignans and sesquiterpenes as PAF antagonists.[10] This paper reports the details of the identification of PAF antagonist from medicinal plants used in traditional medicines.

The hot aqueous extracts of medicinal plants prepared and used in our previous screening studies were used to find active medicinal plants in PAF binding assay.[8,11] Of the extracts tested the fruits of *Forsythia suspensa* VAHL. (Oleaceae), the seeds of *Arctium lappa* L. (Compositae) and the herbs of *Centipeda minima* (L.) A. BRAUN *et* ASCHERS

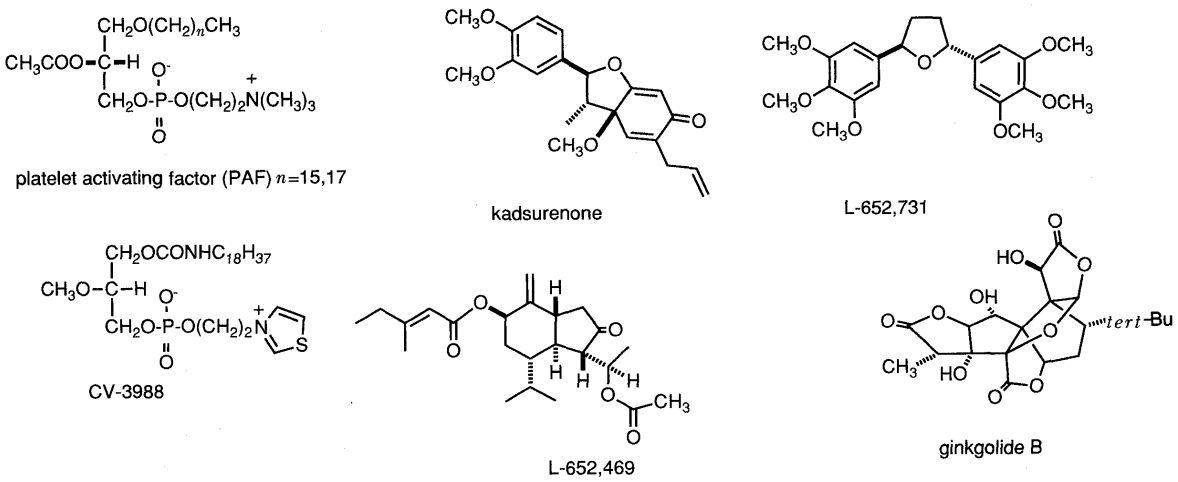
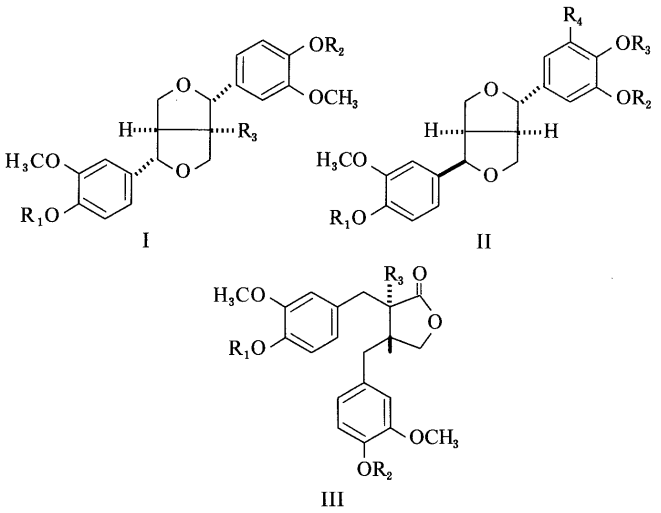


Fig. 1. Structures of PAF and PAF Antagonists

(Compositae) showed significant inhibitory effects on PAF binding to platelets. The inhibition test of medicinal plant extracts on PAF binding to platelets gave significant results only when the extracts contained compounds possessing very strong PAF receptor binding activity, since the addition of extracts tended to disturb the binding of radioactive PAF to the receptor and many cases showed an increase of binding compared to control experiments without extracts. This probably resulted from the increase of non-specific binding of radioactive PAF to platelet cell membrane, which was caused by the presence of plant extract. The extract of seeds of *A. lappa* inhibited PAF binding 74% at 200 μg/ml concentration. *F. suspensa* and *C. minima* extracts showed 89 and 63% inhibitions at a concentration of 100 μg/ml, respectively. The main constituents of *F. suspensa* and *A. lappa* are lignans,[12,13] therefore we tested the PAF receptor binding activities of 30 lignans of tetrahydrofuran, bistetrahydrofuran and butanolide types including those contained in these two plants. PAF receptor binding activities of bistetrahydrofuran and butanolide type lignans are summarized in Table I, in which the lignans of enough high activities to give IC_{50} values are listed. Tetrahydrofuran type lignans tested gave no positive results (data not shown). The presence of at least one 3,4-dimethoxyphenyl group is essential for high activities in the bistetrahydrofuran and butanolide type lignans (Table I). Bistetrahydrofuran type lignans (**1**, **6**) also inhibited *in vitro* platelet aggregation induced by PAF, indicating that these lignans act as PAF antagonists. The IC_{50} values of pinoresinol (**1**), fargesin (**6**) and CV-3988 for platelet aggregation induced by PAF were 31, 38 and 9.8 μM, respectively.

TABLE I. Inhibitory Effects of Lignans on PAF Binding to Platelets



		R_1	R_2	R_3	R_4	IC_{50} for PAF binding (μM)
Ia	(1)	Me	Me	H		1.2
Ib	(2)	Me	Me	OAc		0.67
Ic	(3)	Me	Me	OH		2.8
IIa	(4)	Me	Me	H	H	1.6
IIb	(5)	Me	Me	Me	H	0.91
IIc	(6)	Me	$-CH_2-$		H	0.66
IId	(7)	Me	Me	Me	MeO	0.42
IIIa	(8)	H	Me	H		2.9
IIIb	(9)	Me	Me	H		0.56

Ia: (+) pinoresinol dimethyl ether; Ib: (+)-1-acetoxypinoresinol dimethyl ether; Ic: (+) isogmelinol; IIa: phillygenin; IIb: (+) epipinoresinol dimethyl ether; IIc: fargesin; IId: isomagnolin; IIIa: arctigenin; IIIb: arctigenin methyl ether.

Next we investigated PAF antagonists in *C. minima*. Since preliminary small scale fractionation and bioassay testing indicated that active compounds were soluble in hexane, hexane soluble fraction corresponding to 390 g of *C. minima* herbs was repeatedly fractionated by column chromatographies using silica gel, LH-20, Lobar RP-18 and ODS 80TM (Tosoh). During the course of separation three known triterpenes[14,15] and one known thymol derivative[16] were isolated, however they were inactive in the PAF binding assay. Active compounds were finally obtained by preparative high performance liquid chromatography (HPLC) separation and were identified as plenolin type sesquiterpenes, 6-*O*-angeloylplenolin (**10**),[17] 6-*O*-senecioylplenolin (**11**),[9a] microhelenin B (**12**)[18] and arnicolide B (**13**)[19] (Fig. 2). The sesquiterpenes with unsaturated ester groups are more active than those with saturated ester groups, indicating that the double bonds of ester groups highly contribute to the activity. IC_{50} values of 6-*O*-angeloylplenolin (**10**) and CV-3988 for platelet aggregation induced by PAF are 3.1 and 6.7 μM, respectively. This indicates that 6-*O*-angeloylplenolin (**10**) is a PAF antagonist as potent as CV-3988. Specificity of PAF antagonist activity was demonstrated in the inhibition experiments of platelet

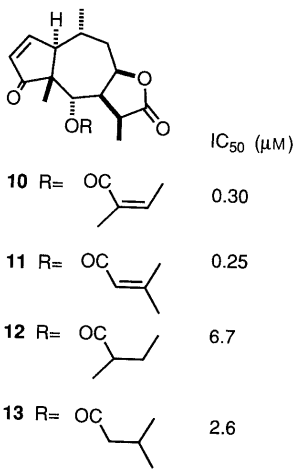


Fig. 2. Structures of Sesquiterpenes Isolated from *Centipeda minima* and Their IC_{50} Values for PAF Receptor Binding

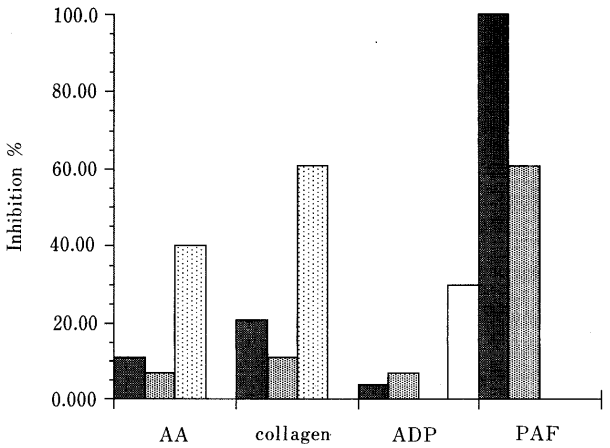


Fig. 3. Inhibition of Platelet Aggregation Induced by PAF by CV-3988 and 6-*O*-Angeloylplenolin (**10**)

The concentrations of AA, collagen, ADP and PAF are 60, 10, 1.7 and 0.08 μg/ml, respectively. The concentrations of inhibitors, 6-*O*-angeloylplenolin (**10**), CV-3988, indomethacin and adenosine are 10, 10, 2 and 5 μM, respectively. ■, 6-*O*-angeloylplenolin; ▨, CV-3988; ⊡, indomethacin; □, adenosine.

aggregation induced by arachidonic acid (AA), collagen, adenosine diphosphate (ADP) and PAF. As it appears in Fig. 3, 6-*O*-angeloylplenolin (**10**) and CV-3988 specifically inhibit platelet aggregation induced by PAF. A sesquiterpene, L-652,469, has been reported to have PAF antagonist activity,[5] however its IC_{50} value was one order less than that of CV-3988. It is worth noting that plenolin type sesquiterpenes contained in *C. minima* possess inhibitory effects on histamine release from rat mast cells as well as on PAF binding. The results so far obtained in this study provide further examples of PAF antagonists contained in medicinal plants.

Experimental

The samples of lignans were kind gifts from Prof. S. Nishibe of Higashi Nippon Gakuen University, Prof. T. Ichihara of Hokkaido University, Prof. K. Mori of the University of Tokyo and Dr. K. Tomioka of the University of Tokyo. The sesquiterpenes were from our collection used in our previous studies.[9a,10]

^1H- and ^{13}C-nuclear magnetic resonance (NMR) spectra were measured on a JEOL JNM GX-400, mass spectra (MS) on JEOL JMS-DX300, infrared (IR) spectra on a JASCO model 701G and ultraviolet (UV) spectra on a Hitachi spectrophotometer model 100-60. Melting points were determined on a Yanagimoto micro-melting point apparatus or in a silicon bath with a glass capillary and were uncorrected.

Assay Methods PAF binding assay was performed as described in literature with some modification.[2] Sample solutions for PAF binding assay were prepared by using polyvinylpyrrolidone (PVP, average molecular weight 40000) as a cosolubilizing agent as described in previous papers.[8,9d] Rabbit platelet rich plasma in Tris–Tyrode buffer (pH 7.3) (NaCl, 8 g; KCl, 0.194 g; glucose, 1 g; Tris 1.21 g; BSA, 2.5 g in 1 l) was adjusted to contain 2.22×10^8 platelets/ml. [^3H] PAF (15 μl, 0.6 nM final concentration, 3.3×10^4 dpm) and a sample solution (10 μl) was mixed in an Eppendorf tube (1.5 ml) and then platelet solution (225 μl, 5×10^7 cells) was added to the tube. The reaction mixture was left at r.t. for 20 min and then filtered through a glass filter sheet, Whatman GF/C, to collect platelets. The glass filter sheet was washed with Tris–Tyrode buffer several times to remove free [^3H] PAF, dried under a heating lamp and submitted for radioactive counting. Obtained radioactivity represents total binding value. The reaction mixture of the control experiment to measure non-specific binding contained 120 nM non-labelled PAF together with radioactive PAF. The value of specific binding was calculated by reducing the value of non-specific binding from that of total binding. Platelet aggregation induced by PAF was measured according to literatures,[20] in which PAF was added to a reaction mixture at a concentration of 5.0×10^{-8} M instead of ADP. Aggregation reactions between 0 to 10 min were measured continuously by an aggregometer (Payton, 600B).

A Preliminary Extraction and Fractionation of *Centipeda minima* Herbs *C. minima* herbs (100 g), collected in Taiwan, were extracted with 70% aqueous acetone. After removing acetone from the extract, the aqueous solution was extracted with hexane and then with EtOAc. The hexane extract obtained upon evaporating solvent inhibited PAF binding in platelets almost 100% at a higher concentration than 10 μg/ml, while the inhibition of the EtOAc soluble fraction was 23% at 100 μg/ml.

A Large Scale Extraction and Separation of PAF Antagonist from *C. minima* Herbs *C. minima* herbs (1.1 kg) were extracted with 70% aqueous acetone and fractionated as described in the preliminary extraction and fractionation to give hexane extract (57 g). A part of hexane extract (20 g) was chromatographed over silica gel (1.5 kg) and eluted with hexane–acetone to give 143 fractions. Two known triterpenes, taraxasteryl palmitate and taraxasteryl acetate,[14] were isolated from fr. 51—55, however they showed no PAF antagonist activity. Fraction 95—112 that were eluted from the column with hexane–acetone (10 : 1) showed strong PAF antagonist activity (100% inhibition at 4 μg/ml) and were further separated by a LH-20 column ($CHCl_3$–MeOH, 1 : 2) to remove chlorophyls. The most active fractions, fr. 66—83 (100% inhibition at 4 μg/ml), were further fractionated by a silica gel column (hexane–AcOEt, 3 : 1) to afford active fractions, fr. 42—58, which inhibit PAF binding 100% at a concentration 4 μg/ml. The first silica gel chromatography also afforded two known compounds, lup-20(29)-ene-3β, 16β-diol[15] and 9,10-diisobutyryloxy-8-hydroxythymol,[16] however they were not active. The active fractions of the second silica gel column chromatography, fr. 42—58, contained many compounds and were further separated by a Lobar RP-18 column (65% MeOH) to give active fractions, fr. 18—24, which were finally separated by an ODS 80TM column (H_2O–MeOH) to afford four known sesquiterpenes. 6-*O*-Angeloylplenolin,[17] 6-*O*-senecioylplenolin,[9a] microhelenin B[18] and arnicolide B[19] were identified by the comparison of their NMR, MS and IR spectral data with those of authentic samples or with those reported.

Acknowledgments The authors thank the Ministry of Education, Science and Culture Japan for scientific grants. The authors also thank Prof. S. Nishibe of Higashi Nippon Gakuen University, Prof. T. Ichihara of Hokkaido University, Prof. K. Mori of the University of Tokyo and Dr. K. Tomioka of the University of Tokyo for their kind supply of samples of lignans.

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