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DEVELOPMENT OF A NOVEL METHOD FOR DETERMINATION OF ACETYL-CoA:1-ALKYL-*sn*-GLYCERO-3-PHOSPHOCHOLINE ACETYLTRANSFERASE ACTIVITY AND ITS APPLICATION TO SCREENING FOR ACETYLTRANSFERASE INHIBITORS

INHIBITION BY MAGNOLOL AND HONOKIOL FROM MAGNOLIAE CORTEX

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Abstract—A method was developed for determining the activity of acetyl-CoA:1-alkyl-*sn*-glycero-3-phosphocholine acetyltransferase (EC 2.3.1.67), a key enzyme in the biosynthesis of platelet-activating factor (PAF, 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine). The assay involves measurement of the radioactivity in the trichloroacetic acid (TCA)-precipitated complex of radioactive product and albumin after incubation of 1-alkyl-*sn*-glycero-3-phosphocholine and [^{3}H]acetyl-CoA with rat spleen microsomes or membrane fractions of human polymorphonuclear leukocytes (PMNs). The radioactive product associated with the precipitate was identified as PAF using an ultrahigh-sensitivity TV camera system after extraction and separation by TLC. This TCA method was then used to screen the components of crude preparations that inhibited acetyltransferase activity. Major components from the cortex of Magnoliae (magnolol and honokiol), which have anti-inflammatory and anti-bacterial actions, inhibited the acetyltransferase activity in rat spleen microsomes (IC_{50}, 150 and 150 μM, respectively) and membrane fractions of human PMNs (IC_{50}, 70 and 60 μM, respectively). The inhibitory action of magnolol and honokiol was reversible, and similar to or higher than that of nordihydroguaiaretic acid. PAF production in human PMNs stimulated by the ionophore A23187 was also suppressed dose dependently by magnolol and honokiol. These activities may be relevant to the claimed therapeutic effects of the extract from Magnoliae cortex.

Key words: platelet activating factor; PAF biosynthesis; acetyltransferase; magnold; honokiol

PAF§ [1, 2] has been shown to function as a potent chemical mediator in pathophysiological processes, in particular inflammation and allergy [3, 4]. PAF has a wide spectrum of biological activities, enhancing vascular permeability and inducing hypotension, smooth muscle contraction and edema. In addition, PAF activates numerous types of cells that may be involved in inflammatory and allergic processes, such as neutrophils, basophils, eosinophils, monocytes/macrophages, mast cells and endothelial cells. These cells also synthesize PAF via the remodeling pathway, through acetylation of lyso-PAF generated in response to various stimuli from 1-alkyl-2-acyl-*sn*-glycero-3-phosphocholine by phospholipase A_2 activation. Thus, in addition to the development of specific PAF antagonists, there is considerable interest in the development of new drugs that actively inhibit PAF biosynthesis as potential therapeutic agents for the treatment of PAF-mediated diseases. Conventional assay for the determination of acetyl-CoA:lyso-PAF acetyltransferase activity requires tedious procedures including extraction of PAF with organic solvents and its separation by TLC in order to measure the incorporation of [^{3}H]acetate into lyso-PAF. Accordingly, in the present study, we developed a simple and reproducible method for screening drugs which inhibit acetyltransferase activity.

Magnoliae cortex has been reported to have potent antibacterial action against *Diplococcus*, *Pneumococcus*, *Streptococcus*, *Shigella*, *Bacillus*, *Staphylococcus*, *Salmonella*, *Pseudomonas* and other microorganisms [5], and to inhibit Type I and Type IV allergic reactions [6] through an as yet obscure pharmacological mechanism. This prompted us to test whether extracts obtained from Magnoliae cortex inhibit acetyltransferase activity. Moreover,

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§ Abbreviations: DTT, dithiothreitol; PAF, platelet-activating factor, 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; [^{3}H]acetyl-PAF, 1-alkyl-2-[^{3}H]acetyl-*sn*-glycero-3-phosphocholine; lyso-PAF, 1-alkyl-*sn*-glycero-3-phosphocholine; PMNs, polymorphonuclear leukocytes; TCA, trichloroacetic acid.

we examine the effects of over 50 components isolated from crude preparations, including two major components of Magnoliae cortex extract (magnolol and honokiol), on the acetyltransferase activity in both rat spleen microsomes and membrane fractions of human PMNs, and on PAF biosynthesis in intact human PMNs.

MATERIALS AND METHODS

Materials

1 - Hexadecyl - 2 - acetyl - *sn* - glycero - 3 - phosphocholine (PAF) and 1-hexadecyl-*sn*-glycero-3-phosphocholine (lyso-PAF) were obtained from Bachem Feinchemikalien AG (Bubendorf, Switzerland) and Novabiochem AG (Laüfelfingen, Switzerland), respectively. [^{3}H]Acetyl-CoA (140.6 GBq/mmol), 1 - hexadecyl - 2 - [^{3}H]acetyl - *sn* - glycero - 3 - phosphocholine (370 GBq/mmol, [^{3}H]acetyl-PAF) and EN^{3}HANCER spray were purchased from New England Nuclear Japan (Tokyo). Nordihydroguairetic acid, aspirin, indomethacin, calcium ionophore A23187 and BSA (fraction V, essentially fatty acid free) were products of the Sigma Chemical Co. (St Louis, MO, U.S.A.). Acetyl-CoA, sodium fluoride (NaF), dithiothreitol, baicalein, hydrocortisone, aminopyrine and dipyridamole were purchased from Wako Chemicals (Tokyo, Japan). Magnoliae cortex and other crude drugs were obtained from the Niiya Crude Drug Co. (Shizuoka, Japan). The following components from crude drugs were isolated as described previously: lignans [(−)-arctigenin, arctigenin (B, D and E), lappaol A, matairesinol from *Arctium fructus* [7]], flavonoids [baicalein from *Scutellariae baicalensis* radix, apigenin and AO-1 (galangin) from *Baccharis genisteroides* [8]], sesquiterpenoids [β-eudesmol from *Atractylodis lanceae* rhizoma [9], curdione, K-1 (germacrone), K-9 and K-13 from *Curcuma aromatica* [10]], diterpenoids [AP-1 (andrographolide), AP-2 (14-deoxyandrographolide), AP-5, AP-10 from *Andrographis paniculata* [11] and BG-2 from *Baccharis genisteroides* [8]], saponins (chiyusaponin from Sanguisorbae radix,* LJ-2′ from *Lonicera japonica* [12] and Asp-1 from *Asparagus cochinchinensis**) and others (Ak-9 from seed of *Alpinia katsumadai* [13] and isopimpinellin from *Angelica* sp.*).

Preparation of rat spleen microsomal fractions

Spleens were obtained from male Wistar rats weighing 150–200 g. The tissue was minced in 9 vol. of 0.25 M sucrose solution, 1 mM EDTA, 1 mM DTT and 25 mM NaF, and homogenized by four strokes of a glass–Teflon homogenizer. Homogenization and subsequent fractionation procedures were carried out at 4°. Nuclei and cell debris were removed by centrifugation at 900 *g* for 10 min. Microsomes were pelleted from the 9000 *g* (10 min) supernatant by centrifugation at 105,000 *g* for 75 min. The pellet was suspended in homogenization medium containing no EDTA. The microsomal preparations were stored at −80° and used within 1 month.

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Preparation of human PMNs

PMNs were prepared essentially according to the procedure of Lotner *et al.* [14]. Each 25-mL portion of healthy human donor blood drawn into a 1/10 vol. of 3.8% citrate was mixed with 8.5 mL of 6% Dextran T-500 in 0.15 M NaCl and then allowed to sediment for 30 min. The supernatant plasma was underlayered with 10 mL of Ficoll-Paque and then centrifuged at 500 *g* for 30 min at 25°. PMNs sedimented at the bottom of the tube were resuspended in erythrocyte lysis solution (0.83% NH_4Cl, 0.1 mM EDTA and 0.1% $KHCO_3$) and centrifuged at 275 *g* for 10 min. The sediment was washed twice with HEPES-buffered saline solution (HEPES, 4.2 mM; KCl, 2.6 mM; NaCl, 137 mM; glucose, 5.6 mM; pH 7.4). The pellet was resuspended in HEPES-buffered saline solution containing 1.3 mM $CaCl_2$ and 1 mM $MgCl_2$ (pH 7.4) at a concentration of 1×10^7 or 2.5×10^7 cells/mL. The PMN fraction contained >97% PMNs with >95% viability, as shown by Trypan blue exclusion.

Isolation of membrane fractions of human PMNs

Washed human PMNs prewarmed at 37° for 5 min (1×10^7 cells/mL) were incubated with A23187 (5 μM) at 37° for 5 min and then immediately centrifuged at 400 *g* for 10 min at 4°. The cell pellet was suspended in 0.25 M sucrose solution containing 1 mM DTT and 25 mM NaF (4×10^7 cells/mL), lysed by freeze-thawing three times, sonicated for 1.5 sec 10 times at 4° in a Bioruptor sonicator (UCD-200 TM, COSMO-BIO, Tokyo, Japan) and homogenized by four strokes of a glass–Teflon homogenizer. The PMN membrane fraction was pelleted by centrifugation at 105,000 *g* for 75 min at 4°. The resulting pellet was resuspended in 0.25 M sucrose solution containing 1 mM DTT and 25 mM NaF. The PMN membrane fraction was stored at −80° and used within 1 month.

Assay for acetyl-CoA : lyso-PAF acetyltransferase

TLC method. Unless stated otherwise, microsomal fractions (10–20 μg of protein) or membrane fractions obtained from A23187-stimulated PMNs (30–40 μg of protein) were incubated for 15 min at 37° in a final vol. of 100 μL of 0.1 M Tris–HCl buffer (pH 6.9) containing 1 mM DTT, 25 mM NaF and 0.1% BSA with 4 nmol lyso-PAF and 30 nmol of [^{3}H]acetyl-CoA (0.7 kBq/nmol). The reaction was stopped by addition of 0.5 mL of $CHCl_3$, 1.0 mL of CH_3OH and 0.3 mL of H_2O and then mixed with 0.5 mL of $CHCl_3$ and 0.5 mL of H_2O. The chloroform phase was removed and another 1 mL of $CHCl_3$ was added to the aqueous phase. After vigorous mixing, the chloroform phase was removed and combined with the first fraction. The lipids in the chloroform extract were mixed with 20 nmol of PAF and separated on a silica gel G plate (250 μm; 20 × 20 cm; Uniplate, Analtech, Newark, NJ, U.S.A.) using a solvent system of $CHCl_3$:CH_3OH:H_2O (65:35:6, by vol.). Authentic sphingomyelin, PAF and lysophosphatidylcholine were applied to both sides of the samples on the plate. After development, the plate was exposed to 6-*p*-toluidine-2-naphthalenesulfonic acid and the positions of lysophosphatidylcholine and

sphingomyelin (R_f values, 0.15 and 0.35, respectively) were detected by UV fluorescence. PAF, located on the thin-layer plate between the areas corresponding to sphingomyelin and lysophosphatidylcholine, was scraped off and extracted with 0.1 mL of $CHCl_3$:CH_3OH (1:1, v/v). The radioactivity of the sample was counted in a liquid scintillation counter.

TCA precipitation method. In this study, we improved the following assay system for determination of the enzyme activity. The assay was based on the assumption that BSA does not bind water-soluble compounds (such as acetate and acetyl-CoA), but does bind the radioactive product, [^{3}H]-acetyl-PAF [15]. The reaction was carried out as described in the previous section, and stopped by addition of 20 μL of 7% BSA–saline solution. After addition of 80 μL of 30% trichloroacetic acid (TCA) solution, the reaction mixture was centrifuged at 750 *g* for 2 min to obtain [^{3}H]acetyl-PAF bound to the denatured BSA. The pellet was washed with 400 μL of 7% TCA solution and centrifuged again at 750 *g* for 2 min. The resulting pellet was dissolved in 200 μL of 0.1 M potassium phosphate buffer containing 1% SDS (pH 8.0), mixed with 4 mL of scintillation cocktail (Aquasol-2, Dupont, Boston, MA, U.S.A.), and the radioactivity was determined in an Aloka LSC-3100 liquid scintillation counter. When inhibitory effects of crude drugs and their components on the acetyltransferase activity were investigated, the effects on the binding of 0.1 pmol [^{3}H]acetyl-PAF to BSA were also determined in order to rule out the possibility that the inhibition was due to the detergent effect.

Identification of radioactive products bound to TCA precipitates

After incubation of rat spleen microsomes (10 μg protein) at 37° for 5 or 15 min with or without 40 μM lyso-PAF in 100 μL of reaction mixture consisting of 10 μM [^{3}H]acetyl-CoA (140.6 MBq/nmol), 1 mM DTT, 25 mM NaF, 0.1% BSA and 0.1 M Tris–HCl (pH 6.9), the reaction was stopped by addition of 20 μL of 7% BSA–saline and 80 μL of 30% TCA solution. Products in the TCA precipitate were extracted by the method of Bligh and Dyer [16], and separated on a silica gel G plate using a solvent system of $CHCl_3$:CH_3OH:H_2O (65:35:6, by vol.). After exposure of the plate to EN^{3}HANCER, the radioactivity was determined using an ultrahigh-sensitivity TV camera system (ARGUS-100) [17]. The positions of sphingomyelin, PAF and lysophosphatidylcholine were detected using Dittmer's reagent [18]. For hydrolysis by PAF acetylhydrolase, the PAF fraction, separated on TLC as described above, was re-extracted from silica gel by the method of Bligh and Dyer [16]. The sample dispersed in 10 μL of 0.1% BSA–saline solution was incubated at 37° for 10 min with 90 μL of serum PAF acetylhydrolase in 10 mM potassium phosphate buffer (pH 6.8), which had been partially purified from human serum (sp. act., 5.5 nmol/min/50 μL) as reported previously by Miwa *et al.* [19]. The reaction was stopped by adding 20 μL of 7% BSA–saline solution and 80 μL of 30% TCA solution, and then the mixture was centrifuged at 750 *g* for 2 min to separate the denatured protein. One hundred microliters of the supernatant were mixed with 4 mL of scintillation cocktail, and the radioactivity was determined in a liquid scintillation counter.

Biosynthesis and quantitation of PAF in human PMNs

PMNs in HEPES-buffered saline containing 0.1% BSA, 1.3 mM $CaCl_2$ and 1 mM $MgCl_2$, pH 7.4 (95 μL, 2.4×10^6 cells), prewarmed for 2 min at 37°, were preincubated with various concentrations of magnolol in 5 μL of 50% DMSO for 15 min at 37° and then stimulated with 5 μL of A23187 in DMSO (final concentration, 5×10^{-6} M) for 10 min at 37°. The reaction was stopped by addition of 1.6 mL of C_2H_5OH and 1.1 mL of H_2O. After centrifugation for 10 min at 1000 *g*, the supernatant was evaporated under a stream of N_2 gas. Lipid products were re-extracted by the method of Bligh and Dyer [16] and separated on a silica gel G plate using a solvent system of $CHCl_3$:CH_3OH:H_2O (65:35:6, by vol.). The plate was exposed to 6-*p*-toluidine-2-naphthalenesulfonic acid, and PAF, located on the thin-layer plate between the areas corresponding to sphingomyelin and lysophosphatidylcholine, was scraped off and extracted by the method of Bligh and Dyer [16]. The sample obtained after TLC was used to determine the PAF concentration. The recovery of PAF through the extraction and purification procedures was 67.5 ± 13.6% (eight experiments) as determined by radiotracer studies using 1-hexadecyl-2-[^{3}H]acetyl-*sn*-glycero-3-phosphocholine.

PAF was quantified by bioassay using rabbit platelet aggregation [20]. Washed rabbit platelets were obtained by a modification of the procedure described by Pinckard *et al.* [21]. Each 45-mL portion of rabbit blood was withdrawn into 5 mL of 41 mM citric acid–75 mM trisodium citrate–130 mM glucose solution and centrifuged at 170 *g* for 10 min. The supernatant platelet-rich plasma was centrifuged at 400 *g* for 10 min. The platelet pellet was gently suspended in Tris–HCl-buffered saline, pH 7.2 (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 5 mM glucose and 45 mM HCl) and centrifuged at 400 *g* for 10 min. The resulting pellet was resuspended in Tyrode–gelatin buffer (137 mM NaCl, 2.6 mM KCl, 1 mM $MgCl_2 \cdot 6H_2O$, 110 mM glucose, 12 mM $NaHCO_3$, 0.25% gelatin; pH 7.2) at a concentration of 1.25×10^9 cells/mL. In the platelet aggregation assay, 10 μL of the sample dispersed in 0.1% BSA–saline solution were added to washed rabbit platelets in Tyrode–gelatin buffer containing 1 mM $CaCl_2$ (pH 7.2) (1×10^8 cells, 400 μL), and changes in light transmittance were monitored by an aggregometer (Nikko Hematracer PAT-2A, Niko Bioscience, Tokyo, Japan). PAF was quantified by comparison of the increase in maximal light transmission through platelet suspensions stimulated by experimental samples with that through a suspension stimulated by a known quantity of 1-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine.

Analytical method

Protein content was determined using BCA protein assay reagent (Pierce, Rockford, IL, U.S.A.).

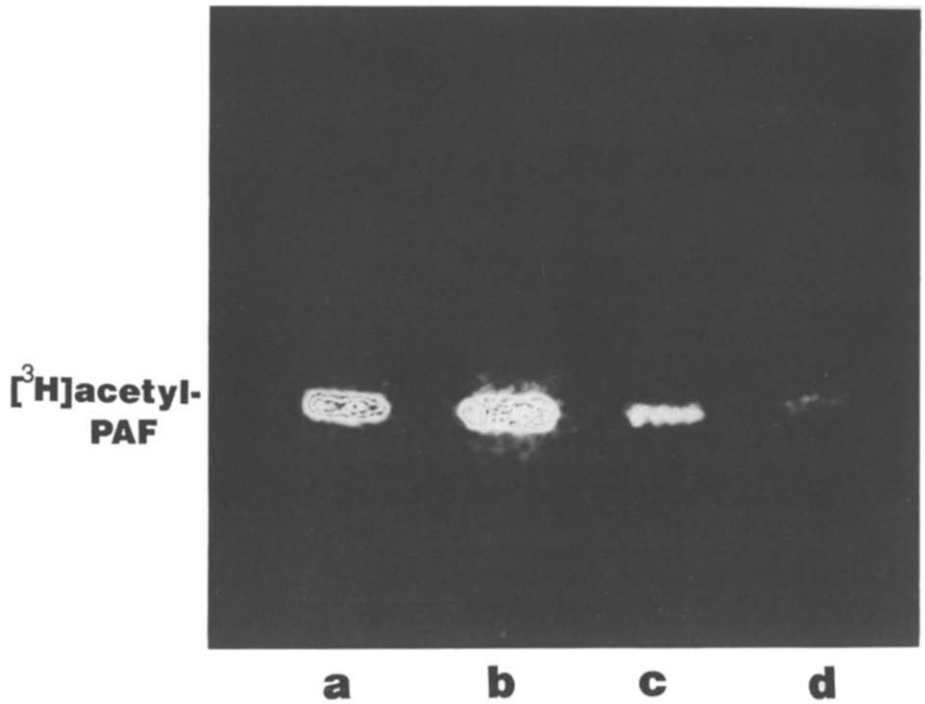


Fig. 1. Identification of radioactive products bound to the TCA precipitate. Radioactive products were detected as described in Materials and Methods. (a) Authentic [^{3}H]acetyl-PAF; (b) and (c) radioactive products obtained after 15 min and 5 min incubation of rat spleen microsomes (10 μg protein) with 10 μM [^{3}H]acetyl-CoA (140.6 MBq) and 40 μM lyso-PAF; (d) radioactive product obtained after 15 min incubation of rat spleen microsomes (10 μg protein) with 10 μM [^{3}H]acetyl-CoA (140.6 MBq) without 40 μM lyso-PAF. The results from one of three experiments are shown.

RESULTS

Development of a simple and reproducible method for measuring acetyl-CoA : lyso-PAF acetyltransferase activity

We have developed a procedure involving radioactivity to measure the product from [^{3}H]acetyl-PAF and albumin by TCA for the routine assay of acetyl-CoA : lyso-PAF acetyltransferase activity. [^{3}H]acetyl-CoA (100, 200, 300 or 400 μM) suspended in 120 μL of 1% BSA–saline solution was mixed with 80 μL of 30% TCA solution and the mixture centrifuged for 5 min at 750 g; 0.36–0.46% of the radioactivity was recovered in the precipitate after treatment with 400 μL of 30% TCA solution. In contrast, in the case of [^{3}H]acetyl-PAF (100 μM), 99.0% of the radioactivity was recovered in the precipitate. This indicates that it is possible to separate substrate ([^{3}H]acetyl-CoA) and reaction product ([^{3}H]acetyl-PAF) upon precipitation of the complex of [^{3}H]acetyl-PAF and albumin by TCA. Accordingly, after incubation of rat spleen microsomes and human PMN membrane fraction with [^{3}H]acetyl-CoA and lyso-PAF, the reaction was stopped by adding BSA and TCA, and the mixture was centrifuged to separate TCA-soluble materials. The main product of the reaction of [^{3}H]acetyl-CoA and lyso-PAF with rat spleen microsomes (Fig. 1) and human PMN membrane fraction (data not shown) was [^{3}H]acetyl-PAF, which had a mobility identical to that of authentic PAF and whose radioactivity was abolished after hydrolysis with serum PAF acetylhydrolase. Formation of a small amount of [^{3}H]acetyl-PAF was observed in the absence of added lyso-PAF, suggesting that either endogenous lyso-PAF or lysophosphatidylcholine may be used as an acetyl acceptor (Figs 1 and 3B). The values of acetyltransferase activity measured by our TCA precipitation method were compatible with those for [^{3}H]acetyl-PAF obtained by the conventional TLC method (Fig. 2). The correlation coefficient between the results obtained by the TLC method and the TCA precipitation method was 0.996, indicating that this method provides a reliable and accurate means of determining acetyl-CoA : lyso-PAF acetyltransferase activity by measuring the radioactivity recovered in the TCA precipitate.

Figure 3 shows the kinetics of PAF synthesis in relation to time, rat spleen microsome protein concentration, acetyl-CoA concentration and lyso-PAF concentration. Linearity for up to 15 min of incubation and 20 μg of rat spleen microsome protein was observed. The apparent K_m value for rat spleen microsomes was 36 μM lyso-PAF in the presence of 300 μM acetyl-CoA. However, since lyso-PAF at concentrations higher than 50 μM suppressed PAF synthesis, a routine enzyme assay was performed at an optimal concentration of lyso-PAF of 40–50 μM. As the apparent K_m and V_{max} values for rat spleen

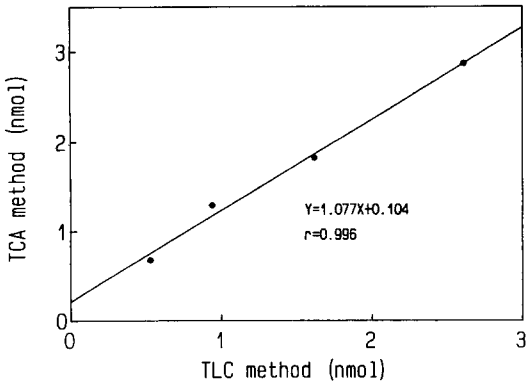


Fig. 2. Correlation of acetyl-CoA:lyso-PAF acetyltransferase activities obtained from radioactivities of the acetylated product, [^{3}H]acetyl-PAF, of lyso-PAF in the TCA precipitate and by the conventional TLC method. Rat spleen microsomes (10–30 μg protein) were incubated with 300 μM [^{3}H]acetyl-CoA and 40 μM lyso-PAF in 100 μL of 0.1 M Tris–HCl buffer (pH 6.9) containing 1 mM DTT, 25 mM NaF and 0.1% BSA at 37° for 15 min. Acetyltransferase activity was measured by the TLC method and the TCA precipitation method as described in Materials and Methods. The results are expressed as means ± SD of one representative experiment, in triplicate.

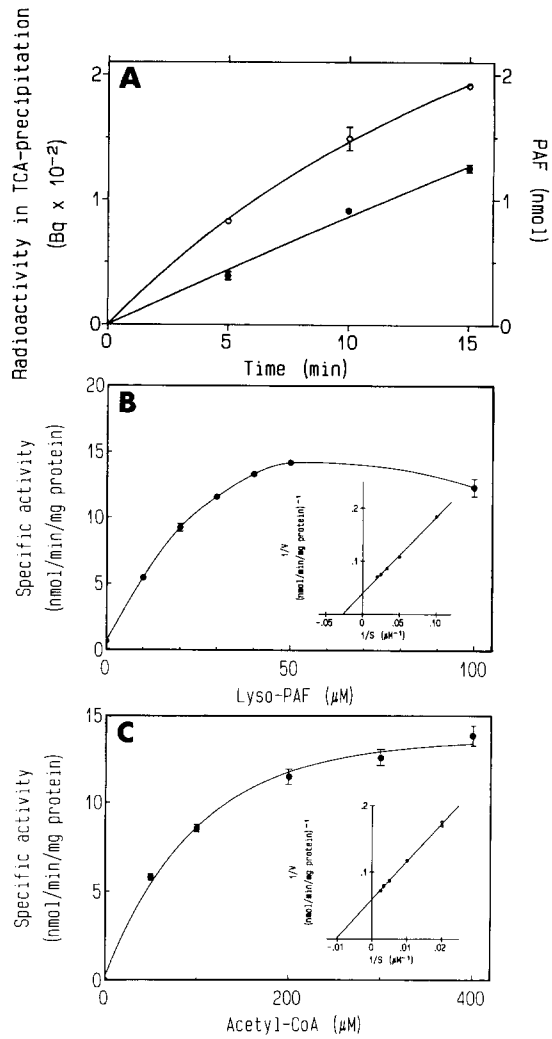


Fig. 3. Effects of incubation time and concentration of rat spleen microsome protein and substrate on acetyl-CoA:lyso-PAF acetyltransferase activity. (A) Rat spleen microsomes [(●) 10 μg, (○) 20 μg protein] were incubated with 300 μM [^{3}H]acetyl-CoA and 40 μM lyso-PAF in 100 μL of 0.1 M Tris–HCl buffer (pH 6.9) containing 1 mM DTT, 25 mM NaF and 0.1% BSA for the desired periods at 37°. (B) and (C) Rat spleen microsomes (10 μg protein) were incubated with 300 μM [^{3}H]acetyl-CoA and various concentrations of lyso-PAF (B), or with 40 μM lyso-PAF and various concentrations of [^{3}H]acetyl-CoA (C) in 100 μL of 0.1 M Tris–HCl buffer (pH 6.9) containing 1 mM DTT, 25 mM NaF and 0.1% BSA at 37° for 15 min. The acetyltransferase activity was measured by our TCA precipitation method, as described in Materials and Methods. Inset shows double reciprocal plots of acetyltransferase activity for various concentrations of substrates. The results are expressed as means ± SD of one representative experiment, in triplicate.

microsomes in the presence of 40 μM lyso-PAF were 100 μM acetyl-CoA and 16.9 nmol PAF/min/mg protein, respectively, the routine enzyme assay was done using 300 μM acetyl-CoA, unless otherwise stated. In subsequent experiments, we analysed the optimal conditions for the acetyltransferase assay. Since acetyltransferase has been suggested to be activated by phosphorylation of the enzyme and inactivated by its dephosphorylation, the effect of the protein phosphatase inhibitor NaF on the acetyltransferase activity in rat spleen microsomes was investigated. Sodium fluoride at a concentration of 25 mM enhanced the enzyme activity 1.2-fold, but it was inhibitory above 25 mM. Therefore, we used 25 mM NaF for the enzyme preparation and enzyme assay. Moreover, use of 0.1% BSA resulted in a 1.4-fold reaction yield and this concentration was regard as optimal.

Inhibition of acetyl-CoA:lyso-PAF acetyltransferase by magnolol and honokiol

Pre-incubation of the membrane fraction of human PMNs (30 μg protein) for 15 min with a crude methanol extract of Magnoliae cortex (barks of *Magnolia obovata*) caused dose-dependent inhibition of acetyl-CoA:lyso-PAF acetyltransferase activity (IC_{50}, 47 μg/mL). To isolate and identify the active components, the methanol extract (13 g) of Magnoliae cortex (100 g) was fractionated with water–ethyl acetate (1:1, v/v). The ethyl acetate-soluble fraction (7 g) had higher inhibitory activity (IC_{50}, 39 μg/mL) than the water-soluble fraction (5.5 g, IC_{50}, 346.7 μg/mL). The ethyl acetate extract was evaporated to dryness *in vacuo* and the residue was applied to a silica gel column (3 × 40 cm). The column was eluted stepwise with hexane, hexane-ethyl acetate 4:1 (v/v), 3:1 (v/v), 2:1 (v/v) and 1:1 (v/v). The fractions eluted with hexane-ethyl acetate 1:1 (v/v) were pooled and evaporated to dryness *in vacuo*. The residue (1.5 g) was further chromatographed using a preparative HPLC system (880,

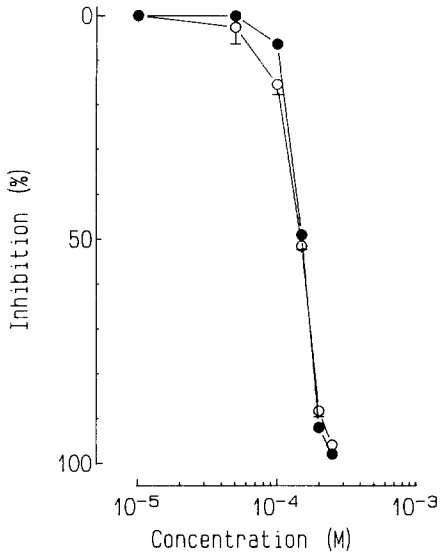


Fig. 4. Effects of magnolol and honokiol on acetyl-CoA:lyso-PAF acetyltransferase activity in rat spleen microsomes. Acetyltransferase activity was measured by the TCA precipitation method as described in Materials and Methods. Rat spleen microsomes (10 μg protein) were incubated with various concentrations of magnolol (●) and honokiol (○) dissolved in DMSO (final concentration, 5%) in 100 μL of 0.1 M Tris–HCl buffer (pH 6.9) containing 300 μM [^{3}H]acetyl-CoA, 40 μM lyso-PAF, 1 mM DTT, 25 mM NaF and 0.1% BSA at 37° for 15 min. The results are expressed as means ± SD of one of three experiments, in triplicate, that showed similar results.

Table 1. Effects of magnolol, honokiol and nordihydroguaiaretic acid on acetyl-CoA:lyso-PAF acetyltransferase activity in rat spleen microsomes and human PMN membrane fraction

Compound	IC_{50} (μM)	
	Spleen microsomes	PMN membrane fraction
Magnoliae cortex		
honokiol	150	60
magnolol	150	70
Xylem of *Larrea divaricata*		
Nordihydroguaiaretic acid	290	60

The acetyltransferase activity was measured by the TCA precipitation method as described in Materials and Methods. Rat spleen microsomes (10 μg protein) or PMN membrane fraction (30 μg protein) were incubated with various concentrations of compounds dissolved in DMSO (final concentration, 5%) in 100 μL of 0.1 M Tris–HCl buffer (pH 6.9) containing 300 μM [^{3}H]acetyl-CoA (0.7 kBq/nmol), 40 μM lyso-PAF, 1 mM DTT, 25 mM NaF and 0.1% BSA at 37° for 15 min.

JASCO, Tokyo) equipped with an octadecyl silica HPLC column (2.5 × 30 cm) using a solvent system of H_2O–acetonitrile (3:7, v/v) at 7.0 mL/min to give honokiol (150 mg) and magnolol (500 mg). On a silica gel G plate developed by hexane-ethyl acetate 2:1 (v/v), the fractions of magnolol and honokiol gave single spots and had an R_f value of 0.55. On an octadecyl silica HPLC column (5 × 300 mm) using a solvent system of H_2O–acetonitrile in a gradient mode at 1.0 mL/min, honokiol and magnolol were eluted as a single peak at 9.5 and 12.2 min, respectively, and the purities were greater than 98%. Both components yielded an ion of mass 267 due to $[MH]^+$ of dimer of phenylpropanoide and were identified as magnolol and honokiol by ^{1}H- and ^{13}C-NMR spectra, respectively. Magnolol and honokiol had inhibitory effects on the acetyl transferase activity in rat spleen microsomes (IC_{50}, 39.6 (150 μM) and 39.6 (150 μM) μg/mL, respectively) and the membrane fraction of human PMNs (IC_{50}, 18.5 (70 μM) and 15.8 (60 μM) μg/mL, respectively) (Fig. 4 and Table 1). When in the presence of magnolol and honokiol instead of [^{3}H]acetyl-CoA under the same conditions for the PAF biosynthesis assay, we investigated the binding activity of BSA to 0.1 pmol [^{3}H]acetyl-PAF by the TCA precipitation method, the amounts of [^{3}H]acetyl-PAF bound to BSA in the presence of magnolol and honokiol at concentrations of 50, 100, 200 and 300 μM were the same as in the absence of magnolol and honokiol. These observations ruled out the possibility that magnolol and honokiol prevent the binding of BSA to [^{3}H]acetyl-PAF to cause the decrease in the radioactivity in the BSA precipitate denatured by TCA. In addition, when we investigated the effect of magnolol and honokiol at a concentration of 150 μM (IC_{50} obtained by the TCA precipitation method) on the acetyl-CoA:lyso-PAF acetyltransferase activity in rat spleen microsomes by the conventional TLC method, the amount of [^{3}H]-acetyl-PAF produced decreased to 50% of that in the absence of magnolol and honokiol. These results indicate that the effects of magnolol and honokiol were reproduced using the TLC method.

The cyclooxygenase and 5-lipoxygenase inhibitor nordihydroguaiaretic acid has been shown to be a potent inhibitor of acetyltransferase [22]. In our assay, the IC_{50} values of nordihydroguaiaretic acid against the acetyltransferase activity in rat spleen microsomes and the membrane fraction of human PMNs were 290 and 60 μM, respectively, which were larger than or similar to those of magnolol and honokiol (Table 1). Aspirin and indomethacin were weak inhibitors of the acetyltransferase activity in rat spleen microsomes (IC_{50}, 3000 and 360 μM, respectively). Other anti-inflammatory drugs, hydrocortisone and aminopyrine, and the acyltransferase inhibitor dipyridamole had no inhibitory activity.

In order to investigate whether the inhibitory action of magnolol and honokiol is reversible, the human PMN membrane fraction was incubated with 143 μM magnolol, honokiol or vehicle added to the reaction mixture with [^{3}H]acetyl-CoA and lyso-PAF. In the absence of BSA, the inhibition of acetyltransferase activity by magnolol and honokiol was 74.9% and 73.7%, respectively, whereas addition of 0.2% BSA decreased the extent of inhibition to

Table 2. Effect of BSA on inhibition of acetyltransferase activity by magnolol and honokiol

Pre-incubation	Addition	Acetyltransferase activity (nmol/min/mg protein)	Relative activity (%)
Vehicle	Vehicle	0.61 ± 0.18	100
100 μM magnolol	Vehicle	0.15 ± 0.03	25.1 ± 4.8
100 μM honokiol	Vehicle	0.16 ± 0.08	26.3 ± 6.7
Vehicle	0.2% BSA	0.99 ± 0.01	100
100 μM magnolol	0.2% BSA	0.88 ± 0.01	82.3 ± 0.6
100 μM honokiol	0.2% BSA	0.57 ± 0.04	56.6 ± 3.3

Human PMN membrane fraction (30 μg protein) in 60 μL of 0.1 M Tris–HCl buffer (pH 6.9) containing 1 mM DTT and 25 mM NaF was pre-incubated with 10 μL of 1 mM magnolol or honokiol dissolved in 50% DMSO or vehicle at 37° for 15 min, and then 20 μL of 1% BSA dissolved in 0.1 M Tris–HCl buffer (pH 6.9) containing 1 mM DTT and 25 mM NaF or vehicle, and 10 μL of 3 mM [^{3}H]acetyl-CoA (2 kBq) and 400 μM lyso-PAF dissolved in 0.1 M Tris–HCl buffer (pH 6.9) containing 1 mM DTT and 25 mM NaF were added to the reaction mixture, which was subsequently incubated for 15 min. The acetyltransferase activity was measured as described in Materials and Methods.

The results are expressed as means ± SD of one of three experiments in triplicate, that showed similar results.

17.7% and 43.4%, respectively (Table 2). This result indicated that the inhibitory action was reversible and did not result from denaturation of the enzyme protein.

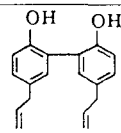
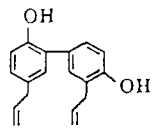
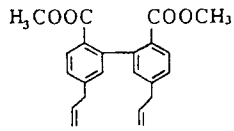
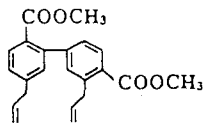
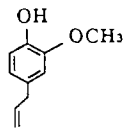
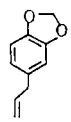
Magnolol and honokiol both have a phenylpropanoid dimer structure. Therefore we compared the inhibitory actions of compounds related to phenylpropanoids on the acetyltransferase activity in the human PMN membrane fraction. At a concentration of 100 μM, diacetylmagnolol and diacetylhonokiol had more marked inhibitory actions (18.8% and 20.5% inhibition, respectively) than magnolol and honokiol (6.3% and 15.4% inhibition, respectively). However, since the solubility of diacetylhonokiol is low, we were unable to obtain the precise IC_{50} values. Monophenylpropanoids such as eugenol, safrole and *o*-allylphenol had no appreciable activity under these experimental conditions, indicating that the phenylpropanoid dimer form but not the hydroxy residue of magnolol and honokiol may be essential for the inhibitory action (Table 3).

We also investigated the effect of magnolol and honokiol on PAF biosynthesis in intact human PMNs. Pre-incubation of human PMNs with magnolol and honokiol for 15 min resulted in dose-dependent inhibition of PAF biosynthesis by A23187-stimulated human PMNs (Fig. 5A). The IC_{50} of magnolol and honokiol was 70 and 50 μM, respectively. In order to determine whether the inhibition of PAF biosynthesis in human PMNs by magnolol and honokiol was dependent on the pre-incubation time with magnolol and honokiol, the influence of addition time of magnolol and honokiol was studied. A 15-min incubation with 100 μM magnolol and 70 μM honokiol before stimulation of human PMNs caused 77.6% and 85.1% inhibition, respectively, of the PAF biosynthesis in A23187-stimulated PMNs. However, the addition of magnolol and honokiol 3 min before stimulation of human PMNs decreased the magnitude of inhibition (25.9% and 30.5% inhibition, respectively) as shown in Fig. 5B. When magnolol or honokiol was added to human PMNs immediately after stimulation of human PMNs, PAF biosynthesis occurred to the extent of 10.3% and 13.1% of control, respectively. After pre-incubation of human PMNs with 150 μM magnolol for 15 min, their viability was almost 100% as estimated by Trypan blue exclusion. This observation confirmed that the inhibition of PAF biosynthesis in human PMNs by magnolol was not the result of a cytotoxic action. In addition, magnolol at a concentration of 200 μM did not affect 0.6 μM A23187-stimulated rabbit platelet aggregation and did not cause hemolysis of red cells, indicating that magnolol does not show cytotoxicity toward these cells.

Screening of inhibitors of acetyl-CoA:lyso-PAF acetyltransferase among components isolated from crude preparations

We screened over 50 components isolated from crude extracts to find inhibitors of acetyl-CoA:lyso-PAF acetyltransferase. Figure 6 shows a comparison of the inhibitory actions of components found to be active. These were: lignans (magnolol and honokiol from Magnoliae cortex and nordihydroguaiaretic acid by xylem of *Larrea divaricata*), flavonoids (baicalein, apigenin and galangin), sesquiterpenoids [β-eudesmol, curdione, K-1 (germacrone), K-9 and K-13], diterpenoids (AP-1, AP-2, AP-5, AP-10 and BG-2), saponins (chiyusaponin, LJ-2′ Asp-1) and others (Ak-9 and isopimpinellin). Magnolol and honokiol at a concentration of 200 μM suppressed the acetyltransferase activity in rat spleen microsomes to 8.0% and 11.7% of the control, respectively, whereas other tested lignans at a concentration of 500 μM, matairesinol, (−)-arctigenin, lappaol A, arctignan B, arctignan D and arctignan E from *Arctium fructus*, had no inhibitory action. Baicalein had almost the same inhibitory action as magnolol and honokiol (IC_{50}, 148 μM). However, the inhibitory actions of the other flavonoids, apigenin and galangin, were not as strong as those of magnolol

Table 3. Effects of phenylpropanoids on acetyltransferase activity in rat spleen microsomes

		Inhibition* (%)	IC_{50} (μM)
Magnolol		6.3 ± 0.4	150
Honokiol		15.4 ± 2.4	150
Diacetylmagnolol		18.8 ± 0.5	ND
Diacetylhonokiol		20.4 ± 0.6	ND
Eugenol		0	>1000
Safrole		0	>1000
o-Allylphenol		0	>1000

Rat spleen microsomes (10 μg protein) were incubated with 300 μM [^{3}H]acetyl-CoA and 40 μM lyso-PAF in 100 μL of 0.1 M Tris–HCl buffer (pH 6.9) containing 1 mM DTT, 25 mM NaF and 0.1% BSA in the presence of various concentrations of phenylpropanoids dissolved in DMSO (final concentration, 5%) at 37° for 15 min. The acetyltransferase activity was measured by the TCA precipitation method as described in Materials and Methods.

The results are expressed as means ± SD of one representative experiment, in triplicate. ND, not determined.

* Percentage inhibition by 100 μM phenylpropanoid.

and honokiol. Chiyusaponin and LJ-2′ were relatively strong inhibitors under our experimental conditions. The effects of other tested components were not as marked as those of magnolol and honokiol.

DISCUSSION

PAF is biosynthesized in specific enzymatic reactions, which include acetyl-CoA : lyso-PAF acetyltransferase (remodeling pathway) and CDP-choline : 1-alkyl-2-acetyl-glycerol cholinephosphotransferase (*de novo* pathway). Inhibitors of acetyltransferase, which is an important regulatory enzyme in the biosynthesis of PAF by the remodeling pathway, are expected to be of therapeutic interest. In the present study, we screened acetyltransferase inhibitors from crude drugs and their components. Since the conventional method for assay of acetyltransferase activity, which consists of PAF extraction and its purification by TLC, requires troublesome procedures, and it is not easy to measure the enzyme activities in many samples at one time, it is necessary to establish a more convenient method. PAF binds to albumin to form a complex, whereas

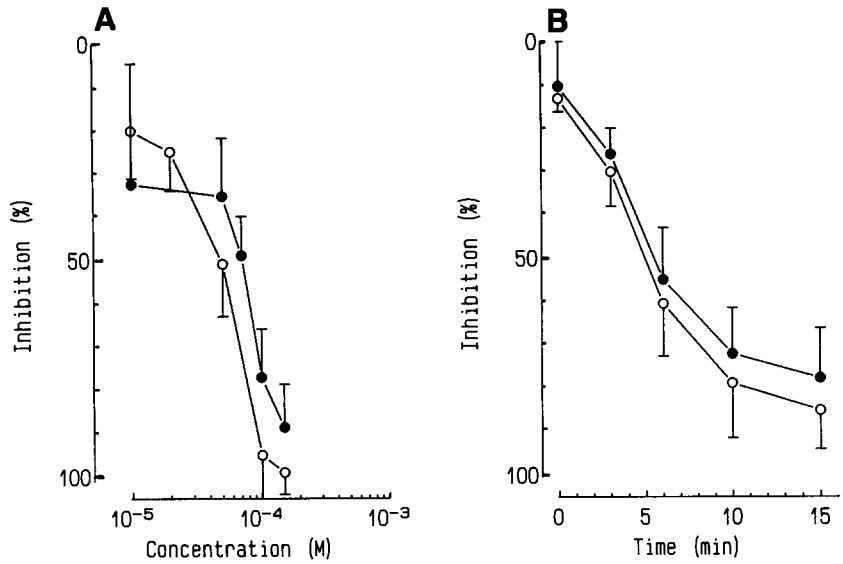


Fig. 5. Dose (A)- and preincubation time (B)-dependent effects of magnolol and honokiol on PAF biosynthesis in human PMNs stimulated with A23187. Stimulation of magnolol (●)- or honokiol (○)-pretreated PMNs with A23187 and determination of the amount of PAF produced are described in Materials and Methods. Pre-incubation time-dependent effects were measured using 100 μM magnolol (●) and 70 μM honokiol (○). The results are expressed as means ± SD of one of three experiments, in triplicate, that showed similar results.

acetyl-CoA does not bind to albumin. We utilized these properties to develop the assay method, which was based on the separation of [^{3}H]acetyl-PAF in the precipitate formed from [^{3}H]acetyl-CoA in the supernatant by addition of BSA followed by TCA. The radioactivity in the precipitate was that of [^{3}H]-acetyl-PAF (Fig. 1), and this TCA precipitation method was found to be reliable compared with the TLC method (Fig. 2). Under the optimum experimental conditions (Fig. 3), BSA at a concentration of 1–2 mg/mL increased the yield of the enzyme reaction, probably because of binding of BSA to the reaction product, PAF, and thus removal of it from the enzyme. With rat spleen microsomes and the membrane fraction of stimulated human PMNs (which have been shown to possess high acetyltransferase activity [23, 24]) as enzyme sources, the acetylhydrolase activity was less than about 1/9 of the acetyltransferase activity under our experimental conditions (data not shown). Therefore, our new method was applicable for screening of inhibitors present in crude plant drugs and their components.

There are several reports of substances that inhibit PAF synthesis in intact cells. Calmodulin antagonists (trifluoperazine and *N*-6-aminohexyl-5-chloro-1-naphthalene sulfonamide) [25], antiflammins which interfere with the activation of phospholipase A_2 [26], and nitroprusside and 3-morpholinosydnonimine, which stimulate the production of cGMP [27], have been shown to inhibit PAF biosynthesis in human PMNs, rat peritoneal macrophages and human endothelial cells. The quinoline-based compounds PF-5901 and Wy-50295 have been shown to exert an inhibitory effect on PAF synthesis in rat peritoneal mast cells in addition to both inhibitory effects on leukotriene synthesis and antagonistic actions on the leukotriene D_4 receptor [28]. Furthermore, ketotifen [29, 30] and the sesquiterpene lactone scandenolide isolated from *Mikania cordata* [31], which are used as anti-allergic and anti-inflammatory drugs, have been reported to suppress PAF production in mouse bone marrow-derived mast cells, rat peritoneal leukocytes and human PMNs. However, it has been shown that a cyclooxygenase and 5-lipoxygenase inhibitor (nordihydroguaiaretic acid) and anti-inflammatory drugs such as diflunisal and benoxaprofen are relatively potent inhibitors of acetyltransferase [22]. Several crude drugs including Magnoliae cortex have been established as anti-inflammatory and anti-allergic drugs from experience accumulated over a long period, but the basis of most of their pharmacological actions remains to be clarified. Therefore, inhibitors of PAF synthesis, i.e. acetyltransferase inhibitors, were investigated in over 50 components isolated from crude drugs possessing anti-inflammatory and anti-allergic actions, and their inhibitory actions were compared with that of nordihydroguaiaretic acid.

The IC_{50} value of nordihydroguaiaretic acid under our experimental conditions, using the membrane fraction of human PMNs (60 μM), was 10 times higher than that reported by White and Faison (6 μM) [22], who used rat pleural PMN lysate as an enzyme source. This difference in IC_{50} values may have been due to the fact that (1) the enzyme sources of the acetyltransferases were different, (2) we measured the enzyme activity in the presence of BSA, which suppressed the inhibition by magnolol and honokiol as shown in Table 2, and (3)

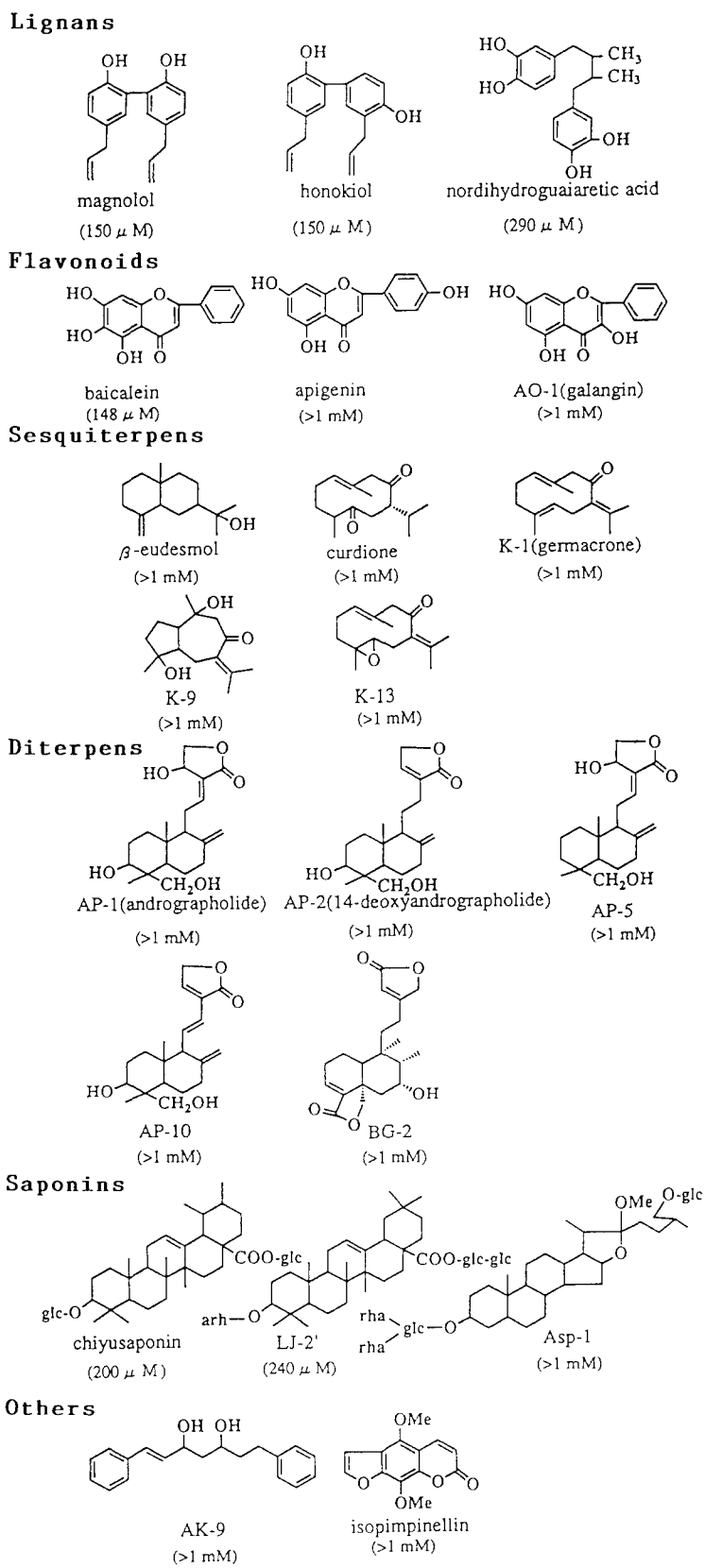


Fig. 6. Chemical structures of compounds isolated from crude drugs and their inhibitory effect on acetyl-CoA : lyso-PAF acetyltransferase activity. Rat spleen microsomes (10 μg protein) were incubated with 300 μM [^{3}H]acetyl-CoA and 40 μM lyso-PAF in 100 μL of 0.1 M Tris–HCl buffer (pH 6.9) containing 1 mM DTT, 25 mM NaF and 0.1% BSA in the presence of various concentrations of compounds dissolved in DMSO (final concentration, 5%) at 37° for 15 min. The acetyltransferase activity was measured by the TCA precipitation method as described in Materials and Methods. IC_{50} values are shown in parentheses.

the concentration of acetyl-CoA in the present experiment (300 μM) was higher than that in the previous one (2.3 μM). Under our experimental conditions, Magnoliae cortex extract, which has been reported to inhibit picryl chloride-induced contact dermatitis in mice [6], produced significant inhibition of PAF synthesis by the human PMN membrane fraction. We subsequently purified effective components from Magnoliae cortex, and observed that two of the major ones, magnolol and honokiol, were active in inhibiting PAF synthesis not only in rat spleen microsomes and the human PMN membrane fraction (Table 1) but also in intact human PMNs (Fig. 5). The inhibition of PAF synthesis by magnolol and honokiol was stronger or similar to that shown by nordihydroguaiaretic acid, and may be of biological relevance for the anti-inflammatory and anti-allergic effect of Magnoliae cortex.

Although it still remains to be clarified whether or not the inhibition of PAF synthesis by magnolol and honokiol is due to direct effects on the acetyltransferase enzyme, the inhibitory action was not due to protein denaturation or toxicity since the enzyme activity was recovered by addition of BSA (Table 2) and these compounds did not affect the viability of human PMNs under the experimental conditions we employed. With regard to the mode of action of magnolol and honokiol, it is interesting that the phenylpropanoid dimer structure appeared necessary for the inhibition of PAF synthesis. The inhibition of PAF synthesis by the saponins chiyusaponin and LJ-2′, although similar to that induced by magnolol and honokiol, may be due to detergent action on membranes, since an inhibitory effect of some detergents on acyl-CoA : lysolecithin acyltransferase in rat tissue microsomes has been reported [32]. The inhibition of PAF synthesis by baicalein is of particular interest, because the effective concentration of this compound was similar to those of magnolol and honokiol, and because the anti-allergic drug amlexanox was developed from this compound. Accordingly, it may be possible to develop new inhibitors of PAF synthesis based on magnolol and honokiol. Finally, the biochemical activity of magnolol and honokiol described in this study, i.e. an inhibitory action toward PAF biosynthesis, may explain at least some of the beneficial pharmacological effects of Magnoliae cortex.

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REFERENCES

1. Hanahan DJ, Platelet activating factor: a biologically active phosphoglyceride. *Annu Rev Biochem* **55**: 483–509, 1986.
2. Prescott SM, Zimmerman GA and McIntyre TM, Platelet-activating factor. *J Biol Chem* **265**: 17381–17384, 1990.
3. Meade CJ, Heuer H and Kempe R, Biochemical pharmacology of platelet-activating factor (and PAF antagonists) in relation to clinical and experimental thrombocytopenia. *Biochem Pharmacol* **41**: 657–668, 1991.
4. Braquet P, Touqui L, Shen TY and Vargaftig BB, Perspectives in platelet-activating factor research. *Pharmacol Rev* **39**: 97–145, 1987.
5. Namba T, Tsunezuka M and Hattori M, Dental caries prevention by traditional chinese medicines. *J Med Plant Res* **44**: 100–106, 1982.
6. Koda A, Nishiyori T, Nagai H, Matsuura N and Tsuchiya H, Anti-allergic actions of crude drugs and blended chinese traditional medicines. Effect on Type I and Type IV allergic reactions. *Folia Pharmacol Jpn* **80**: 31–41, 1982.
7. Umehara K, Sugawa A, Kuroyanagi M, Ueno A and Taki T, Studies on differentiation-inducers from *Arctium fructus*. *Chem Pharm Bull*, in press.
8. Kuroyanagi M, Fujita K, Kazaoka M, Matsumoto S, Ueno A, Fukushima S and Katsuoka M, Studies on the constituents of *Baccharis genistelloides*. *Chem Pharm Bull* **33**: 5075–5078, 1985.
9. Kuroyanagi M, Ebihara T, Tsukamoto K, Fukushima S, Ishizeki C and Satake M, Screening of antibacterial constituents of crude drugs and plants. In: *J Pharmacobio-Dyn* **8**, *Proceedings of the 5th Symposium on the Development and Application of Naturally Occurring Drug Materials, Hiroshima, Japan, 30–31 July 1984* (Chief Ed. Y. Kasuya), p. S-62. Pharmaceutical Society of Japan, Tokyo, 1985.
10. Kuroyanagi M, Ueno A, Ujiie K and Sato S, Structures of sesquiterpenes from *Curcuma aromatica* Salisb. *Chem Pharm Bull* **35**: 53–59, 1987.
11. Matsuda T, Kuroyanagi M, Sugiyama S and Ueno A, Studies on the cell differentiation inducers of *Andrographis paniculata*. In: *Proceedings of the 33rd Symposium on the Chemistry of Natural Products, Osaka, Japan, 3–5 October 1991* (Ed. Hayashi Y), pp. 433–439. Osaka City University, Osaka, 1991.
12. Kawai H, Kuroyanagi M, Umehara K, Ueno A and Satake M, Studies on the Saponins of *Lonicera japonica* Thunb. *Chem Pharm Bull* **36**: 4769–4775, 1988.
13. Kuroyanagi M, Noro T, Fukushima S, Aiyama R, Ikuta A, Itokawa H and Morita M, Studies on the constituents of the seeds of *Alpinia katsumadai* Hayata. *Chem Pharm Bull* **31**: 1544–1550, 1983.
14. Lotner GZ, Lynch JM, Betz SJ and Henson PM, Human neutrophil-derived platelet activating factor. *J Immunol* **124**: 676–684, 1980.
15. Miwa M, Miyake T, Yamanaka T, Sugatani J, Suzuki Y, Sakata S, Araki Y and Matsumoto M, Characterization of serum platelet-activating factor (PAF) acetylhydrolase: correlation between deficiency of serum PAF acetylhydrolase and respiratory symptoms in asthmatic children. *J Clin Invest* **82**: 1983–1991, 1988.
16. Bligh EG and Dyer W, A rapid quantitative method of total lipid extraction and purification. *Can J Biochem Physiol* **37**: 911–919, 1959.
17. Miwa M, Matsumoto M, Tezuka M, Okada S, Ohsuka S and Fujiwake H, Quantitative fluorographic detection of ^{3}H and ^{14}C on two-dimensional thin-layer chromatographic sheets by an ultra-high-sensitivity TV camera system. *Anal Biochem* **152**: 391–395, 1986.
18. Dittmer JC and Lester RL, A simple, specific spray for the detection of phospholipids on thin-layer chromatograms. *J Lipid Res* **5**: 126–127, 1964.
19. Miwa M, Sugatani J, Ikemura T, Okamoto Y, Ino M, Saito K, Suzuki Y and Matsumoto M, Release of newly synthesized platelet-activating factor (PAF) from human polymorphonuclear leukocytes under *in vivo* conditions: contribution of PAF-releasing factor. *J Immunol* **148**: 872–880, 1992.
20. Miwa M, Hill C, Kumar R, Sugatani J, Olson MS and Hanahan DJ, Occurrence of an endogenous inhibitor of platelet-activating factor in rat liver. *J Biol Chem* **262**: 527–530, 1987.

21. Pinkard RN, Farr RS and Hanahan DJ, Physicochemical and functional identity of rabbit platelet-activating factor (PAF) released *in vivo* during IgE anaphylaxis with PAF released *in vitro* from IgE sensitized basophils. *J Immunol* **123**: 1847–1857, 1979.
22. White HL and Faison LD, Inhibition of lyso-PAF:acetyl-CoA acetyltransferase by salicylates and other compounds. *Prostaglandins* **35**: 939–944, 1988.
23. Wykle RL, Malone B and Snyder F, Enzymatic synthesis of 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine, a hypotensive and platelet-aggregating lipid. *J Biol Chem* **255**: 10256–10260, 1980.
24. Mueller HW, O'Flaherty JT and Wykle RL, Biosynthesis of platelet activating factor in rabbit polymorphonuclear neutrophils. *J Biol Chem* **258**: 6213–6218, 1983.
25. Billah MM and Siegel MI, Calmodulin antagonists inhibit formation of platelet-activating factor in stimulated human neutrophils. *Biochem Biophys Res Commun* **118**: 629–635, 1984.
26. Tetta C, Camussi G, Bussolino F, Herrick-Davis K and Baglioni C, Inhibition of the synthesis of platelet-activating factor by anti-inflammatory peptides (antiflammins) without methionine. *J Pharmacol Exp Ther* **257**: 616–620, 1991.
27. Heller R, Bussolino F, Ghigo D, Garbarino G, Pescarmona G, Till U and Bosia A, Nitrovasodilators inhibit thrombin-induced platelet-activating factor synthesis in human endothelial cells. *Biochem Pharmacol* **44**: 223–229, 1992.
28. Hogaboam CM, Donigi-Gale D, Shoupe TS, Bissonnette EY, Befus AD and Wallace JL, Platelet-activating factor synthesis by peritoneal mast cells and its inhibition by two quinoline-based compounds. *Br J Pharmacol* **105**: 87–92, 1992.
29. Joly F, Bessou G, Benveniste J and Ninio E, Ketotifen inhibits PAF-acether biosynthesis and β-hexosaminidase release in mouse mast cells stimulated with antigen. *Eur J Pharmacol* **144**: 133–139, 1987.
30. Nakamura T, Kuriyama M, Ishihara K, Matsumura Y and Miyamoto T, Platelet-activating factor (PAF) in allergic diseases: Inhibitory effects of anti-allergic drugs, ketotifen and three kampo medicines on PAF production. *Lipids* **26**: 1297–1300, 1991.
31. Ysrael MC and Croft KD, Inhibition of leukotriene and platelet-activating factor synthesis in leukocytes by the sesquiterpene lactone scandenolide. *Planta Med* **56**: 268–270, 1990.
32. Shier WT, Inhibition of acyl coenzyme A:lysolecithin acyltransferases by local anesthetics, detergents and inhibitors of cyclic nucleotide phosphodiesterases. *Biochem Biophys Res Commun* **75**: 186–193, 1977.