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A Sulphonoglycolipid from the Fern *Polypodium decumanum* and its Effect on the Platelet Activating-factor Receptor in Human Neutrophils

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Abstract

The South American fern *Polypodium decumanum*, traditional name calaguala, has documented clinical use in oral treatment of skin disorders, including psoriasis. The inflammatory mediator platelet-activating factor (PAF), has been implicated in the pathogenesis of psoriasis.

A constituent of a calaguala extract has been shown to have inhibitory activity in a PAF-induced exocytosis model in human neutrophils. The compound was identified as the sulphoquinovosyl diacylglycerol 1,2-di-*O*-palmitoyl-3-*O*-(6-sulpho-α-D-quinovopyranosyl)-glycerol by spectroscopic means. When subsequently studied in an in-vitro model for [^{3}H]PAF binding in neutrophils from man the compound caused dose-dependent displacement of [^{3}H]PAF from its receptor with an IC50 value of 2 μM. It is suggested that the compound acts through PAF receptor antagonism in intact human neutrophils.

The South American fern, *Polypodium decumanum* (Polypodiaceae) Willd. with the common name calaguala, has a documented clinical use in the treatment of the skin disorders psoriasis (Padilla et al 1974), atopic dermatitis (Vargas Gonzales & Lopez Gutierrez 1988 and vitiligo (Mohammad 1989). To explain these clinical findings, we have previously presented a study with an in-vivo skin transplantation model which showed a significantly enhanced rejection time for mice receiving the calaguala extract (Tuominen et al 1991). We have also studied the extract's effect in an in-vitro model for biosynthesis of LTB_4, an inflammatory mediator with suggested utility in the clinical symptoms of psoriasis. The inhibitory activity of the extract was found to be caused by polyunsaturated fatty acids (Vasänge-Tuominen et al 1994). Another phospholipid-derived mediator which is implicated in the pathogenesis of psoriasis is platelet-activating factor (PAF) (Koltai et al 1991b). Its involvement in the disease is suggested by the elevated amounts of PAF isolated from psoriatic scales. It is also known to be a potent chemo-attractant and injection of the autocoid induces increased vascular permeability and vasodilatation (Cunningham 1990), which are typical inflammatory signs of psoriasis.

In previous work we have investigated the activity of calaguala extract in two in-vitro models using isolated human neutrophils (Tuominen et al 1992). In the first model, we studied the capacity of the extract to inhibit the biosynthesis of PAF from its biologically inactive precursor, lyso-PAF, and documented dose-dependent inhibition by the extract. In the second model, we examined the effect of the extract in inhibiting the exocytosis induced by PAF and again observed dose-dependent inhibition. The second model was then used to guide the fractionation of the extract and from an acetone supernatant fraction of the extract the nucleoside adenosine was isolated as an active principle.

The objective of the current study was to explain further the PAF-related activity of the extract. This was achieved by isolation of an active constituent using bioactivity-directed fractionation and pharmacological characterization of the identified compound in a model for PAF-induced elastase release and in PAF-receptor binding in human neutrophils.

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Materials and Methods

Plant material

Leaves of *Polypodium decumanum* Willd. (Polypodiaceae) were collected in October 1988 from a cultivation at Ladoge Yogoa, Honduras. The plant was identified by Dr Robert Stolze, Department of Botany, Field Museum of Natural History, Illinois, USA, where a voucher specimen is kept.

Isolation of the active principle

The dried and ground leaves (6·6 kg) were extracted overnight with methanol (puriss; 3 × 35 L) by stirring at room temperature. The extract was filtered, concentrated in-vacuo, and lyophilized (yield 267 g). Part of the methanolic extract (50 g) was then dissolved in water (400 mL) and extracted with chloroform (4 × 600 mL; yield 16 g).

The chloroform extract was adsorbed on silica gel (70–230 mesh; Merck) and eluted through a column packed with silica gel (200 g); the mobile phase was a gradient of chloroform to methanol. Fractions (20 mL) were collected and pooled according to results from comparison by TLC. Ten fractions were obtained and checked for activity in the PAF-induced exocytosis assay. The most active fractions (total weight 1·8 g) were repeatedly chromatographed on silica gel with a gradient of chloroform to methanol and the combined active fractions (1·4 g) were thereafter eluted through a Sephadex LH 20 column (Pharmacia Fine Chemicals, Sweden) with a gradient

of methanol–water (30:70) to 100% methanol as the mobile phase. The penultimate fraction (210 mg) had the highest activity in the assay and was further purified by use of a second Sephadex LH 20 column which was first washed with ethanol and then eluted with methanol. This yielded 10 mg compound 1.

Structure elucidation of compound 1

Mass spectrometry. GC-MS analysis was performed on an HP 5890 gas chromatograph coupled to a HP 5970A quadrupole mass-selective detector operated at an ionization voltage of 70 eV and an electron multiplier voltage of 1800 V. Compound 1 was analysed after a transesterification reaction (Seppänen-Laakso et al 1990) which enables identification of bound fatty acids. For molecular weight determination of compound 1, positive-ion FAB mass spectra was recorded with a Jeol SX 102 instrument with glycerol as matrix.

Carbohydrate analysis. Compound 1 was hydrolysed using trifluoroacetic acid. The hydrolysate was then subjected to reduction then acetylation and the resulting peracetylated compound was compared by gas chromatography with authentic carbohydrates treated in the same manner.

NMR. NMR experiments were performed with a Jeol 270 MHz instrument. A mixture of CH_3OD and $CDCl_3$ was used as solvent and TMS as internal standard.

Pharmacological methods

Cell preparation. Peripheral blood anti-coagulated with heparin was obtained from healthy volunteers at the University Hospital (Uppsala). After sedimentation with 2% Dextran T-500 for 30 min at 20°C the sediments were removed and the neutrophils were separated from the mononuclear cells by centrifugation of the supernatant (Ficoll-Hypaque; 500 *g* for 30 min at 20°C). The pellet was treated with one volume of ice-cold water for 21 s to lyre the remaining erythrocytes. Nine volumes of Mg^{2+}- and Ca^{2+}-free phosphate-buffered saline (PBS) were added to the suspension. After centrifugation (200 *g* for 10 min at 4°C) the leukocytes were suspended in PBS containing Mg^{2+} and Ca^{2+}, and Cytochalasin B (Serva; 5 $\mu g\ mL^{-1}$) at a concentration of 10–30 $\times\ 10^6$ cells mL^{-1}.

For receptor binding experiments the cells were prepared in the same way except that concentrated suspensions of human leukocytes in a CDP-adenine solution was used and in the final step the cells were suspended in the incubation buffer and the cell count was adjusted to $3{\cdot}1 \times 10^6$ cells mL^{-1}.

The viability of the cells was determined to be 97% (tryptan blue dye exclusion test).

PAF-induced exocytosis. The assay was performed as described elsewhere (Tuominen et al 1992). In short, neutrophils (final concentration $1–3 \times 10^6$ cells mL^{-1}) were incubated at 37°C in PBS containing 0·25% bovine serum albumin (BSA) and different concentrations of the inhibitor, *N*-succinyl-L-alanyl-L-alanyl-L-valine-*p*-nitroanilide (Bachem), or the vehicle (maximum 0·1% DMSO) and 10^{-7} M PAF (Bachem) for 10 min. Blank tubes without PAF were run in parallel. The reaction was stopped by addition of 2% citric acid and the samples were, after centrifugation, measured by a UV spectrophotometer at 405 nm.

The absorbance of the corresponding blank tube was subtracted from that of the sample and the inhibition of PAF-induced elastase release was calculated as the relative increase in absorbance compared with that of the vehicle. To exclude the possibility that the inhibitors would influence the elastase release in the absence of PAF, the blank values for tubes containing the inhibitors and tubes containing solely the vehicle were also compared.

The samples were analysed in 4–5 concentrations ranging from 10^{-3} M to 10^{-7} M (because of the small amount of the isolated compound available, the highest dose tested for compound 1 was 2×10^{-5} M). They were dissolved in 10% dimethylsulphoxide (compound 1) or cyclodextrin (BN 52021) and then diluted with the buffer. The final concentration of dimethylsulphoxide or cyclodextrin never exceeded 0·1%.

In-vitro [^{3}H]PAF binding assay. [^{3}H]PAF (C18; 1-*O*-[^{3}H]octadecyl-2-acetyl-sn-3-phosphocholine; Amersham, UK) receptor binding was studied in human neutrophils ($2{\cdot}5 \times 10^6$ cells mL^{-1}) by use of a modification of methods previously described by Marquis et al (1988) and O'Flaherty et al (1986). Incubations were performed for 120 min at 20°C in a total volume of 1·0 mL containing 0·6 mM Na_2HPO_4, 0·6 mM NaH_2PO4, 25 mM Tris HCl, 130 mM NaCl, 5·5 mM KCl, 1·4 mM $CaCl_2$, 0·7 mM $MgCl_2$, 10 mM glucose, 0·5% BSA, 0·35–0·40 nM [^{3}H]PAF and the inhibitors, which were added before the PAF. Final pH was adjusted to 7·0 at the temperature used and the incubation was initiated by the addition of the cells, final concentration $2{\cdot}5 \times 10^6\ mL^{-1}$. Non-specific binding was determined with 1 μM C16-PAF (Bachem, Switzerland). This method is characterized by a K_d value of 0·09 nM and a B_{max} of 2880 sites/cell. Non-specific interactions are considerable in the assay but the level of specific binding can be determined to about 55% of the total binding by using antagonists, such as BN 50730, with no structural similarity to the agonist.

The incubation contents were rapidly (< 15 s) filtered under vacuum over Whatman GF/C glass-fibre filters using a 12-well Skatron cell harvester (Skatron A/S, Norway) and washed with 3×2 mL incubation buffer containing 0·1% BSA. Filters were also pre-soaked with incubation buffer containing 0·1% BSA. After filtration the filters were dried in an oven at 70°C for 30 min and equilibrated for 2 h in the scintillation vials before counting in a Packard scintillation counter at an efficiency of 55%.

The competition-binding assays were performed in triplicate at several concentrations ($10^{-11} - 10^{-3}$ M) of compound 1 and the receptor antagonists BN 50730 and BN 52021 (kindly provided by Dr P. Braquet, Institut Henri Beufour). Compound 1 and BN 50730 (10^{-2} M of each) were dissolved in 10% dimethylsulphoxide, BN 52021 in cyclodextrin (1 mg mL^{-1}) and then further diluted with the incubation buffer.

Results

Identification of compound 1

Mass spectrometry. GC-MS analysis after transesterification was performed for the analysis of the bound fatty acids. A single peak was observed in the GC-chromatogram; the mass spectrum of the peak had major signals at m/z 270 (M^+), 239, 227, 213, 199, 185, 157, 143, 129, 97, 87, 74, 59, 43 and was

thus identified as the methyl ester of palmitic acid. The FAB MS of compound 1 had a molecular ion $[M+1]^+$ at m/z = 817 corresponding to a molecular formula of $C_{42}H_{77}O_{12}SNa$ for the compound.

Carbohydrate analysis. Analysis based on comparison of GC retention times indicated the presence of glucose.

NMR. The ^1H NMR and ^{13}C NMR shift values for compound 1 are shown in Table 1. The values could be arranged in three groups (acyl, glycerol and glucosyl) and were then matched with reported values for the sulphoquinovosyl diacylglycerol (Amarquaye et al 1994) isolated from the tropical American tree *Byrsonima crassifolia*. Compound 1 was thereby identified as 1,2-di-*O*-palmitoyl-3-*O*-(6-sulpho-α-D-quinovopyranosyl)-glycerol (Fig. 1).

Pharmacological studies

PAF induced exocytosis. The dose–response curves for compound 1 and for the PAF receptor antagonist ginkgolide BN 52021 (Braquet 1987) are shown in Fig. 2. The IC50 values were determined to be 10 μM and 80 μM, respectively.

[^{3}H]PAF binding assay. The displacement curves of the ginkgolide BN 52021, the hetrapazine-derived PAF receptor antagonist BN 50730 (Braquet & Esanu 1991) and compound 1 are presented in Fig. 3. The IC50 values were determined to be 2 μM for compound 1, 25 μM for BN 52021 and 0·04 μM for BN 50730. At lower concentrations (below $10^{-5.5}$ M) compound 1 caused an increase in the total binding of [^{3}H]PAF in several experiments. This phenomenon, which for the time being has not been explained, results in the relatively large s.e.m. values for the curve.

Table 1. Proton and carbon chemical shifts of 1,2-di-*O*-palmitoyl-3-*O*-(6-sulpho-α-D-quinovopyranosyl)-glycerol (compound 1).

	^1H shift (ppm)	^{13}C shift (ppm)
Acyl groups*		
C(n)	0·9	14·3
C(n-1)		23·4
C(n-2)		32·7
C, methylene	1·25	20·9, 30·2, 30·5
C3	1·6	25·7
C2	2·3	34·8, 35·0
C1		173·7, 173·7
Glycerol group†		
sn-1a	4·5	64·0
sn-1b	4·2	64·0
sn-2	5·3	71·3
sn-3a	4·1	66·7
sn-3b	3·6	66·7
Glucosyl group†		
C1	4·5	99·0
C2	3·4	73·0
C3	3·6	74·6
C4	3·1	74·6
C5	4·0	69·4
C6a	3·3	54·0
C6b	3·0	54·0

*(n) refers to the carbon at the methyl end of the acyl group, C1 refers to the carbonyl carbon. † The positions of the carbon atoms are shown in Fig. 1.

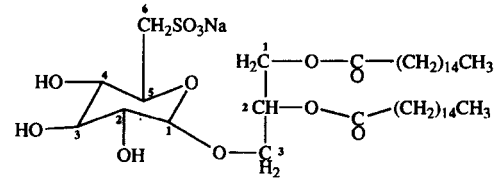

FIG. 1. The chemical structure of 1,2-di-*O*-palmitoyl-3-*O*-(6-sulpho-α-D-quinovopyranosyl)-glycerol (compound 1). The index figures in the glycerol and glucosyl groups refer to the carbon numbers in Table 1.

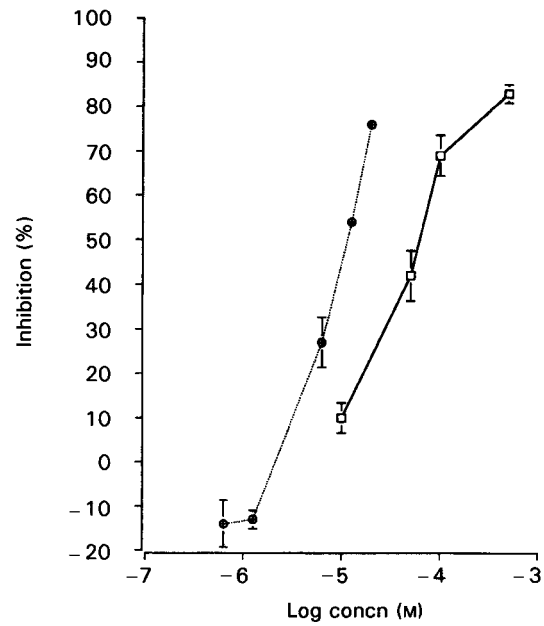

FIG. 2. Inhibition of PAF-induced elastase release in human neutrophils by 1,2-di-*O*-palmitoyl-3-*O*-(6-sulpho-α-D-quinovopyranosyl)-glycerol (compound 1) and BN 52021. Each point represents the mean ± s.e.m. of 4–8 experiments, all performed in duplicate. □, BN52021; ○, compound 1.

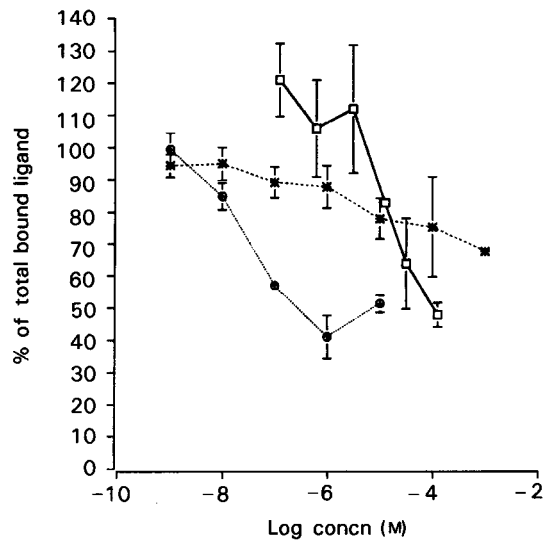

FIG. 3. Displacement curves of 1,2-di-*O*-palmitoyl-3-*O*-(6-sulpho-α-D-quinovopyranosyl)-glycerol (compound 1), BN 52021 and BN 50730 on [^{3}H]PAF bound to its receptor in human neutrophils. The figure shows the decrease in d min^{-1} measured as percentage of total bound ligand. The level of non-specific binding was determined to be approximately 45% of total bound ligand. Each point represents the mean ± s.e.m. of 2–4 experiments, all performed in triplicate. □, Compound 1; ○, BN50730; *, BN52021

Discussion

Platelet-activating factor (PAF; 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is a phospholipid-derived mediator of allergy and inflammation which is generated by various cell types, including platelets, leukocytes and endothelial cells. In-vitro, PAF exhibits a diversity of effects, including aggregation and degranulation of leukocytes and inhibition of lymphocyte proliferation. In-vivo effects include hypotension, acute renal failure and increase in vascular permeability (Koltai et al 1991a). PAF is implicated to be associated with a number of pathological conditions, such as shock, airway hypersensitivity, inflammation, psoriasis, arthritis and graft rejection (Braquet & Esanu 1991).

Calaguala which is a methanolic extract of the fern *Polypodium decumanum*, has been reported to be clinically efficient in oral treatment of psoriasis and related skin disorders (Del Pino Gamboa et al 1982; Jimenez et al 1987). The mechanism of action and the constituents responsible for the action in the extract have, however, not been fully identified. We have previously studied calaguala in connection with mediators that are considered significant in inflammatory disorders, including PAF. One of the pharmacological models used is the PAF-induced release of the proteolytic enzyme elastase in human neutrophils, on which calaguala had dose-related inhibitory activity (Tuominen et al 1992). The present fractionation of calaguala with the aim of identifying components of the plant responsible for the activity resulted in the isolation of the sulphoquinovosyl diacylglycerol 1,2-di-*O*-palmitoyl-3-*O*-(6-sulpho-α-D-quinovopyranosyl)-glycerol. The IC50 of the compound in the exocytosis model is 10 μM which is comparable with that of the well-known PAF-receptor antagonist, the ginkgolide BN 52021 (80 μM).

Sulphoquinovosyl diacylglycerols, with the sulphonyl-substituted glucose group and a variety of fatty acyl chain compositions, are one of the four major components of polar lipids in chloroplast membranes of all photosynthetic organisms, with the exception of some bacteria (Benning et al 1993). They are structural components of the thylakoid membrane and it is suggested that they are involved with the electron-transport chain in photosynthesis and in the construction of chloroplasts (Morimoto et al 1993). Although they have not yet been intensively studied pharmacologically, they have been found to have some biological effects, such as HIV-1 inhibitory activity in man (Gustafson et al 1989) and anti-tumour promoting activity (Shirahashi et al 1993). They have not to our knowledge previously been studied in relation to PAF.

The effects of PAF are mediated by a G protein-coupled receptor in the plasma membrane of the target cell (Chao & Ohlson 1993). Binding to the receptor results in functional responses, including exocytosis of lysosomal enzymes, like elastase, and these responses have been shown to be sensitive to inhibition by receptor antagonists (Dewald & Baggiolini 1987; Marquis et al 1989). The exocytosis model used in the experiments with calaguala is thus correlated with receptor antagonism but the action is not exclusive. To elucidate whether a compound acts through interference with the receptor, a receptor-binding assay using labelled agonist or antagonist must be used. Previously, several laboratories have defined the PAF receptor in human polymorphonuclear neutrophils (O'Flaherty et al 1986; Marquis et al 1988).

1,2-Di-*O*-palmitoyl-3-*O*-(6-sulpho-α-D-quinovopyranosyl)-glycerol was consequently studied in a PAF receptor-binding model using [^{3}H]PAF as the radioligand. The IC50 value for the compound was determined to be 2 μM which is in the same range as its IC50 value in the exocytosis model (10 μM).

The results suggest that the effect the sulphoquinovosyl diacylglycerol exerts on neutrophils is inhibition of binding of PAF to its receptors. This action could be mediated by the compound through its structural similarity to PAF, which is also an acylated glycerol derivative with phosphocholine as the polar head group (Braquet et al 1987). The chemical and physical properties of both compounds should, however, be taken into consideration when interpreting the results. PAF and sulphoquinovosyl diacylglycerols are amphipathic molecules with detergent properties. PAF has been reported to act as a general membrane perturbant and, at micromolar concentrations, to alter the behaviour of biological membranes and membrane proteins. It is suggested that some of the properties of PAF at concentrations above its critical micellar concentration (3 μM) might be because of these unspecific detergent-like actions (Sawyer & Andersen 1989). This could also apply to the sulphoquinovosyl diacylglycerols.

The inclusion of BSA in the incubation medium of the binding assay, on the other hand, counteracts the non-specific effects of PAF (Clay et al 1990) and the same is also likely to be true of the sulphoquinovosyl diacylglycerol. The confirmation of the IC50 value of the compound in the model for PAF-induced elastase release which is a functional response of neutrophils to PAF binding to its receptor further supports the idea that the compound is acting by interference with the neutrophil receptor (Dewald & Baggiolini 1987; Marquis et al 1989). In the competition experiments with the sulphoquinovosyl diacylglycerol and the hetrapazine BN 50730, a plateau at approximately 55% of the total binding was, furthermore, obtained, in contrast with PAF itself and the PAF analogue Ro 19-3704, which at higher concentrations also replaced part of the binding that was defined as non-specific (data not shown). This indicates that the sulphoquinovosyl diacylglycerol and BN 50730 act more specifically at the PAF receptor. Finally, the relatively low IC50 value of the sulphoquinovosyl diacylglycerol also implicates a receptor-mediated effect.

In conclusion, we have by fractionation guided by PAF-induced exocytosis isolated 1,2-di-*O*-palmitoyl-3-*O*-(6-sulpho-α-D-quinovopyranosyl)-glycerol from an extract of the fern *Polypodium decumanum* (calaguala) with clinical use in psoriasis. We suggest that this compound acts by PAF-receptor antagonism in intact human neutrophils and that the action could be relevant to the reported medical use of the fern.

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