原　著

BN 52021 and related compounds : A new series of highly specific PAF-acether receptor antagonists isolated from *Ginkgo biloba L.*

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Key words : PAF acether, binding assay, platelet aggregation, PAF-acether antagonist, BN 52021

I. INTRODUCTION

Platelet activating factor (PAF, PAF-acether, AGEPC, APRL) is a naturally occurring etherphospholipid (1-O-alkyl-2 (R)-acetyl-glyceryl-3-phosphorylcholine) (**Fig. 1**) that is generated by specific activation of rabbit basophils[1], human and rabbit polymorphonuclears (PMNLs)[2~4], human macrophages[5] and eosinophils[6].

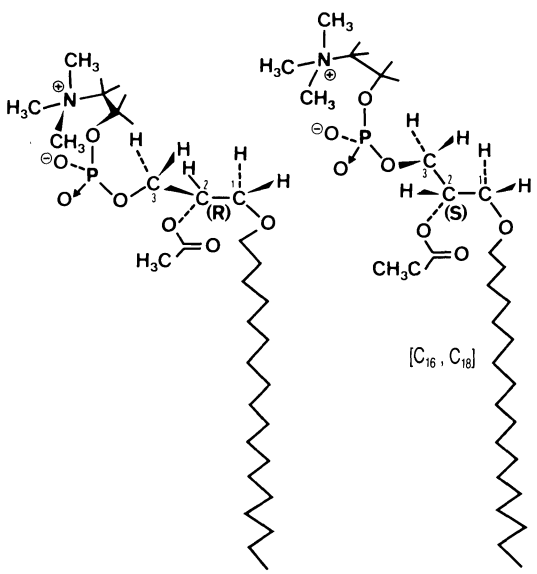

Fig. 1 PAF-acther (R and S)

Generation of PAF-acether during anaphylaxis and/or shock leads to chemotaxis, aggregation and degranulation of polymorphonuclear leukocytes[7]. On the other hand, PAF-acether induces bronchoconstriction and contraction of smooth muscle[8][9], being one of the most powerful platelet activators known inducing platelet shape change, aggregation and secretion (for review see ref.10). Furthermore, PAF-acether reduces coronary blood flow and contractile force of isolated guinea-pig heart[11], leading to cardiac anaphylaxis. In addition, it induces marked extravasation[12], hypotension[13] and shock[14].

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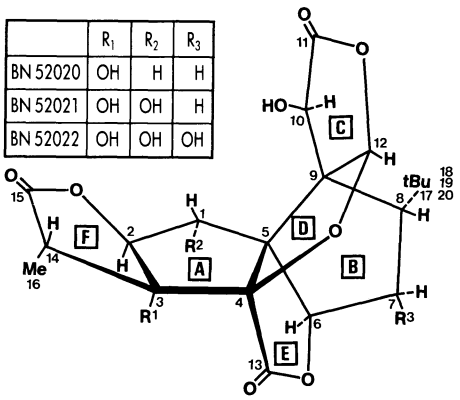

Fig. 2 PAF-acether antaganists isolated from *Ginkgo biloba L.*: Chemical structure

However, direct evidence for a role of PAF-acether in such patho-physiological processes was not available until now, in part due to the lack of specific antagonists.

These research tools are becoming available related either to PAF-acether framework[15)16)] or to lignans[17)18)]. In this article we describe the PAF-acether antagonistic properties of new highly specific inhibitors : BN 52021 and related compounds (Ginkgolides, see **Fig. 2**) isolated and chemically identified from *Ginkgo biloba L.*

These results have in fact just given a scientific basis to traditional Chinese medicine which describes *Ginkgo biloba L.* as "good for heart and lungs (chest dilatation)".

II. MATERIALS AND METHODS

Materials

PAF-acether (1-O-alkyl-2(R)-acetyl-glycero-3-phosphorylcholine) was purchased from Bachem (Bubendorf, Switzerland) and stored at −80°C in a 0.5% bovine serum albumin solution.

Ginkgo biloba extract (GBE 761) is a standardized extract prepared from dried and stabilized leaves of *Ginkgo biloba L.* (IPSEN Labs., France). The Ginkgo tree ("Icho" in Japanese) occupies a unique position in botany for several reasons[19)] : it is the sole representative of its family and is not linked to any other living plant : its origin extends as far back as the Paleozoic. The order Ginkgoales was once widely distributed throughout the world. In the last few million years all species excepting *Ginkgo biloba L.* have become extinct, the other species being found only as fossils in petrified woods ; moreover, *Ginkgo biloba L.* itself is believed to have remained unchanged for the last million years or so. The Ginkgo tree is thus called a "living fossil" or fossil tree[19)].

The extraction of the different antagonists (Ginkgolides) was performed according to the procedure previously described by Okabe et al[20)]. Further details concerning the isolation, purification and characterization (500 MHz NMR, X ray cristallography .....) of PAF-acether antagonists were published elsewhere[21)].

BN 52021 was identified as :

9H-1, 7a-(Epoxymethano)-1H, 6aH-cyclopenta[c]furo[2, 3-b]furo-[3′, 2′ : 3, 4] cyclopenta

[1, 2-d] furan-5, 9, 12-(4H)-trione, 3-tert-butylhexahydro-4, 4b-11-trihydroxy-8-methyl (Ginkgolide B, see **Fig. 2**).

BN 52020 was identified as :

9H-1, 7a-(Epoxymethano)-1H, 6aH-cyclopenta[c] furo [2, 3-b] furo-[3′, 2′: 3, 4] cyclopenta [1, 2-d]furan-5, 9, 12(4H)-trione, 3-tert-butylhexahydro-4, 7b-dihydroxy-8-methyl (Ginkgolide A, see **Fig. 2**).

BN 52022 was identified as :

9H-1, 7a-(Epoxymethano)-1H, 6aH-cyclopenta[c]furo[3′, 2′: 3, 4]cyclopenta[1, 2-d]furan-5, 9, 12(4H)-trione, 3-tert-butylhexahydro-2, 4, 7b, 11-tetrahydroxy-8-methyl (Ginkgolide C, see **Fig. 2**).

GBE 761, BN 52021 and congeners (IHB-IPSEN Institute for Therapeutic Research, France) were dissolved in dimethyl sulfoxide (DMSO).

Tritiated ligands for binding assays were purchased from Amersham or NEN. Thrombin, adenosine diphosphate (ADP), and collagen were obtained from Sigma (St Louis, USA) and A 23187 from Boehringer (FRG).

Kadsurenone was a gift of Dr Shen (MSD, Rahway, USA).

Methods

[^{3}H]PAF-Acether Assays

The inhibition of [^{3}H]PAF-acether binding to the PAF-acether receptor on isalated rabbit plasma membranes was performed according to Shen et al.[22] :

Preparation of washed rabbit platelets

Washed rabbit platelets were prepared as follows : platelets were freed from plasma soluble components in platelet-rich plasma (PRP) by two times Ficoll Paque (Pharmacia) separation and washed rabbit platelets were resuspended in basic Tyrode's solution with 0.10 mM (Sigma Chemical Co.) at pH 6.5 and a concentration of $2\text{-}3\times10^9$ platelets/m*l*.

Preparation of rabbit platelet plasma membranes

The platelets prepared above were washed and resuspended in a solution of 150 mM NaCl, 4 mM $MgCl_2$, 10 mM Tris pH 7.5 and 1 mM EDTA and lysed by repeated freezing with liquid nitrogen and thawing. The lysed membrane suspension was then layered on top of a discontinuous sucrose density gradient of 12% (w/v) and 27% (w/v) sucrose and centrifuged at 63,500 g for 5 hours. The membrane fractions collected from bands between 12 % and 27% were carefully collected and stored at −80°C.

PAF-receptor binding assays

100 μg of platelet plasma membranes was added to a final 1 m*l* of solution containing 1 pmole [^{3}H]PAF-acether and a known amount of BN 52021 and related compounds in a medium containing 150 mM NaCl, 10 mM Tris, 0.25% BSA (Solution A) pH 7.5 at 0°C. After incubation at 0°C for 1 hour the mixture was filtered through a Whatman GF/C glass fiber filter under vacuum. Each tube was washed rapidly with a total of 20 m*l* of ice-cold Solution A. The membranes prepared consistently showed *ca.* 35% specific PAF-acether receptor binding. The percent inhibition of PAF-acether receptor binding was expressed as :

$$\% \text{ Inhibition} = \frac{\text{Total binding} - \text{Total binding with antagonist}}{\text{Specific binding}} \times 100$$

Table 1 Receptors binding parameters

RECEPTOR 3H LIGAND (nM) BLANK (nM)	ANIMAL TISSUE	BUFFER
ALPHA-1 ADRENERGIC WB 4101 (0.1) phentolamine (1000)	rat cerebral cortex	tris-HCL (50 mM)
ALPHA-2 ADRENERGIC clonidine (0.6) phentolamine (1000)	rat cerebral cortex	tris-HCL (50 mM)
BETA-1 ADRENERGIC DHA (1.0) propanolol (1000)	rat cerebral cortex	tris-HCL (50 mM)
BETA-2 ADRENERGIC DHA (1.0) propanolol (1000)	rat cerebellum	tris-HCL (50 mM)
DOPAMINE-2 spiperone (0.5) +butaclamol (1000)	rat corpus striatum	tris-salt*
SEROTONIN-1 serotonin (0.6) serotonin (10000)	rat frontal cortex	tris-salt**
SEROTONIN-2 spiperone (0.25) serotonin (100000)	rat frontal cortex	tris-salt**
GABA muscimol (5.0) gaba (100000)	rat cerebellum	HEPES (50 mM)
OPIATE (μ) naloxone (0.5) levallorphan (1000)	guinea-pig forebrain	tris-HCL (50 mM)
OPIATE (δ) dadle (0.3) levallorphan (1000)	guinea-pig forebrain	tris-HCL (50 mM)
OPIATE (κ) EKC (0.16) levallorphan (1000)	guinea-pig forebrain	tris-HCL (50 mM)
HISTAMINE-1 pyralamine (2.0) triprolidine (2000)	guinea-pig cerebellum	NaK phosphate (50 mM)
HISTAMINE-2 tiotidine (2.0) cimetidine (10000)	guinea-pig cerebral cortex	NaK phosphate (50 mM)
BENZODIAZEPINE (CNS) flunitrazepam (1.0) clonazepam (1000)	rat cerebral cortex	NaK phosphate (50 mM)
BENZODIAZEPINE (non-neuronal) RO5-4864 (0.5) RO5-4864 (1000)	rat forebrain	tris-HCL (50 mM)
TRH methyl TRH (2.0) methyl TRH (1000)	rat forebrain	Na phosphate (20 mM)
MUSCARINIC ACH QNB (0.05) atropine (1000)	rat corpus striatum	NaK phosphate (50 mM)
IMIPRAMINE imipramine (1.2) desipramine (10000)	rat cerebral cortex	tris-salt***
DESIPRAMINE desipramine (1.5) desipramine (10000)	rat cerebral cortex	tris-salt***
DOPAMINE-1 flupenthixol (2.0) +butaclamol (1000)	rat corpus striatum	tris-salt*
ADENOSINE-1 CHA (1.5) 2-CA (10000)	rat forebrain	tris-HCL (50 mM)
CALCIUM ANTAGONIST nitrendipine (0.2) nifedipine (1000)	rat cerebral cortex	tris-HCL (50 mM)
*50 mM tris-HCL 120 mM Na Cl 5 mM KCl 2 mM CaCl 1 mM Mo Cl2 0.1% ASCORBIC ACID	**50 mM tris-HCL 4 mM CaCl2 0.01 mM PARAGYLINE - 0.1% ASCORBIC ACID	***50 mM tris-HCL 120 mM Na Cl 5 mM KCl

where specific binding was defined as the total amount of [^{3}H]PAF-acether bound minus the nonspecific binding which was measured as the total binding of [^{3}H]PAF-acether in the presence of 1000-fold excess of unlabeled PAF-acether (1 nmole per tube).

[^{3}H] Ligand Binding Assays

Male rats or guinea-pigs were sacrificed, and their brains were removed and placed on ice for dissection. The appropriate brain region (see **Table 1**) was homogenized in ice-cold buffer with a polytron PT-10 (setting 6, 10 sec) and centrifuged at 30000 g for 10 min at 4°C. The supernatant was discarded, and the pellets were rehomogenized and centrifuged a second time. The final pellets were resuspended in buffer and used immediately in the binding assay.

Aliquots of the washed membrane preparations were added to culture tubes containing the [^{3}H] ligand and test compound. Each test compound was assayed in duplicate at four concentrations (10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} M), the binding reaction was terminated by rapid filtration through Whatman GF/B filters under reduced pressure. The filters were washed three times with 5 m*l* aliquots of ice-cold buffer, and the bound radioactivity trapped on the filters was counted by liquid scintillation spectrometry. Further details on the specific assay conditions are shown in **Table 1**. For each receptor, the specific binding was defined

as the total [^{3}H] ligand bound minus that bound in the presence of a saturating concentration of unlabeled receptor ligand (blank).

K_i values (inhibition constants) were determined from the equation

$$K_i = \frac{IC_{50}}{1+L/K_d}$$

where L is the radioligand concentration, and K_d is the appropriate equilibrium dissociation constant. The IC_{50} was derived from the inhibition data by linear regression of

$$\log\left(\frac{B}{B_t}-B\right)$$

versus I, where I is the concentration of inhibitor in moles per liter, B_t is the total amount of [^{3}H] ligand bound, and B is the amount bound at a given concentration of inhibitor. The IC_{50} is the antilog of I when the expression log $(B/B_t-B)=0$. Compounds that showed less than 10% inhibition of specific binding at 10^{-5} M were considered inactive.

Interference with Arachidonic Acid Metabolism

Cyclooxygenase pathway

Bovine seminal vesicle microsomes were used as the source of prostaglandin synthetase: the microsomes were suspended in Tris-HCl buffer (pH=8.2, 0.1 M) to obtain a protein concentration of 14 mg/m*l*. The enzyme homogenate was then incubated at 37°C with [^{14}C] arachidonic acid (AA) (Amersham, 58 mCi/mmol), mixed with unlabeled AA (10^{-5} M) in presence of 5 mM adrenaline bitartrate and 10 mM reduced glutathione in a total volume of 0.5 m*l*. After 15 min incubation at 37°C with the test compound, the enzymatic reaction was stopped by addition of 250 μl HCl (1N) and the prostanoids were extracted by ethyl acetate and evaporated to dryness; the residue redissolved in 50 μl of chloroform/methanol (1 : 1) was separated by TLC on silica gel (Merck Kieselgel 60 F 250° with a solvent system consisting of ethyl acetate/acetate/acetic acid (90 : 10 : 1). TLC plates were subsequently scanned for radioactivity and zones corresponding to PGE_2 and $PGF_{2\alpha}$ were quantified with an automatic TLC-linear analyser LB 2482 Berthold.

Lipoxygenase pathways

Human blood from a healthy subject was centrifuged at 1000 g for 25 min. The PRP was discarded, and the PMNL fraction was obtained following 6% dextran-500 sedimentation, centrifugation over Ficoll Paque, and ammonium chloride (160 mM)+Tris (170 mM) treatment. Then, the cells were suspended in a standard phosphate-buffered medium (PBS) containing (mM): Na^+, 146; K^+, 4; Cl^-, 142; HPO_4^{2-}, 2.5; $H_2PO_4^-$, 0.5; Mg^{2+}, 1; glucose, 10; (pH~7.5; determined osmolarity~284 mOsM). $CaCl_2$ and $MgCl_2$ were added to the PMNL suspension to obtain 2 mM and 0.5 mM final concentrations, respectively, and the suspensions were pre-incubated for 5 min at 37°C in polypropylene tubes. The cells were then stimulated with the Ca^{2+}-ionophore A 23187 (1.5 μM) or/and exogenous AA (1 μM) and incubated for a further 5 min period. Incubation was stopped by addition of one volume of a mixture of methanol/acetonitrile (1 : 1) containing 200 ng of PGB_2 (internal standard). A stock solution of ionophore was prepared in ethanol which never reached 0.5% (final concentration) in the cell suspensions.

The possible effects of BN 52020, BN 52021 and BN 52022 were determined: drugs were added to the medium 10 min before addition of A 23187. AA metabolites were analyzed

Table 2 Gradient used for RP-HPLC of AA metabolites

Time (min)	Solvent A	Solvent B	Solvent C
Initial	100	0	0
5	70	30	0
15	40	60	0
25	0	100	0
35	0	100	0
37	0	0	100
55	0	0	100

Solvent A: methanol-acetonitrile-water, 25/25/50, v/v/v, containing 0.02% H_3PO_4 and 0.0025% dimethylsulfoxide; adjusted to pH 3.1 (apparent pH) with NH_4OH.
Solvent B: methanol-acetonitrile-water, 30/60/10, v/v/v, containing 0.02% H_3PO_4
Solvent C: methanol-acetonitrile-water, 30/50/20, v/v/v, containing 0.02% H_3PO_4, adjusted to pH 5.5 (apparent pH) with NH_4OH.

using a modification of a method related separately[23]. Briefly, denatured PMNL suspensions were centrifuged at 3000 g for 15 min to remove the precipitated material. Supernatants (2 m*l*) were acidified to pH 3.0 with H_3PO_4 and 1 m*l* portions were then injected on a HIBAR RP 18 cartridge (125×8 mm, 5 μm particles, Merck) previously equilibrated with solvent A (see below).

The various lipoxygenase (LO) products were eluted at a flow rate of 1 m*l*/min using the three solvent mixtures (A, B, C) shown in **Table 2**. All solvents were of HPLC grade. Elution of the various compounds was monitored using fixed wavelength ultraviolet photometers at 229 and 280 nm (Waters Scientific Model 441 with extended wavelength module). The pumps (Waters, model 6000 A) were controlled by a microprocessor (Waters, model 720).

Platelet Aggregation

In vitro experiments

Rabbit platelet aggregation was measured (PRP or washed platelets) turbidometrically using a 2 channels aggregometer (Coultronics chronolog) at 37°C with stirring at 1100 rpm. All platelet samples were prewarmed for five minutes exactly at 37°C and inhibitors or solvent (DMSO) were added two minutes before aggregation. Platelet aggregation was initiated by adding different doses of pro-aggregating substances (ADP, collagen, thrombin, A 23187 and PAF-acether).

For each aggregation obtained in the presence of inhibitor, the maximum amplitude was measured and the inhibition percentage was calculated by comparison with the maximum amplitude obtained with the control aggregations made in the presence of solvents; in each series of experiments a concentration-inhibition percentage curve was drawn. Using this curve, the inhibitor concentration inducing 50% inhibition was determined (IC_{50}). The means (± S.D.) of the inhibition percentages for each concentration of inhibitor and the means of the IC_{50} were calculated on at least three different lots of PRP.

Ex vivo experiments

Ex vivo aggregometry refers to experiments in which aggregometry is performed in vitro

on PRP samples obtained from rabbits dosed with drugs. In this study, inhibition of PAF-acether-induced platelet aggregation was investigated in conscious male New Zealand white rabbits, just before the administration of drugs (oral route) and at different times later (1, 2, 6 hours); blood samples were obtained from the auricular artery.

In Vivo Thromboformation Induced by Electrical Stimulation of the Rat Carotid Artery

Female CD Sprague-Dawley rats (180～210 g) were anaesthetised with urethane (5 m*l*/kg i. p. of a 25% solution in 0.9% saline). The left carotid artery was exposed for a length of approximately 2 cm and placed over shielded stainless steel electrodes 0.5 cm apart; a thermistor for recording arterial surface temperature was placed around the artery 1 cm distal to the electrodes; the thermistor was connected to a MX 2 recorder.

A current of 5 mA was passed through the arterial electrodes for 2 min using a Neurolog NL 800 constant current generator. The time from commencing electrical stimulation to a rapid and marked fall in surface temperature of artery was taken as the time for thrombus formation. If appropriate, the recording could be continued for up to 45 min after electrical stimulation. Animals received BN 52021 (20 mg/kg) or vehicle orally at a dose volume of 10 m*l*/kg, 50 min before anaesthesia.

PAF-Acether- or Formyl Methionine Leucine Phenylalamine (FMLP)-Induced Aggregation of Isolated Human PMNLs

After isolation of PMNLs (see above section), PMNL aggregation was monitored with a 2 channels aggregometer (Coultronic chronolog) at 37°C. Percent aggregation was calculated as above except that Hank's bovine serum albumin solution was used as the reference. PMNL aggregation was performed either with PAF-acether ($10^{-10}\rightarrow10^{-5}$ M) or FMLP ($10^{-10}\rightarrow10^{-5}$ M).

III. RESULTS

1. Inhibition of PAF-Acether-Receptors Binding by BN 52021 and Related Compounds

Fig. 3 presents the percent inhibition of [^{3}H]PAF-acether binding to the PAF-acether binding site on isolated rabbit plasma membranes in the presence of BN 52021 and related antagonists. BN 52021 was the most effective compound in displacing [^{3}H] PAF-acether with an IC_{50} of 2.5×10^{-7} M as compared with 9.4×10^{-7} M and 1.7×10^{-5} M for BN 52020 and BN 52022, respectively. In the same membrane preparation, under the same experimental conditions, the K_d of [^{3}H]PAF-acether bindng to its receptor site was $1.36\pm0.05\times10^{-5}$ M. By assuming competitive inhibition, the K_i value of BN 52021 was determined as 10^{-8} M accodring to the equation $K_i=IC_{50}/(1+L/K_d)$ where L is the PAF-acether concentration (1 nM).

In the other receptor binding assays (see **Table 1.** for details) these substances were totally inactive in the range of 10^{-8} to 10^{-5} M.

2. Effect of BN 52021 and Related Compounds on Arachidonic Acid Metabolism

Whatever concentrations were ($10^{-7}\rightarrow10^{-3}$ M), BN 52021 and related compounds did not inhibit cyclooxygenase activity of the bovine seminal vesicle preparation. In human PMNLs, only a slight inhibition was observed for the highest dose used (10^{-4} M : 5-HETE = −22%,

$LTB_4 = -38\%$, 20-OH $LTB_4 = -27\%$, see **Fig. 4** for details).

3. Inhibition of Rabbit Platelet Aggregation (PRP) by GBE 761

PAF-acether induced rabbit platelet aggregation dose-dependently (data not shown). At a concentration of 2.5×10^{-7} M, PAF-acether induced a maximal aggregation which was inhibited dose-dependently by GBE 761. At 1.0 or 2.5 mg/m*l*, the inhibition was total. The IC_{50} was 3.7 μg/m*l*. The dose effect curves measuring the platelet aggregation as a function of increasing doses of PAF-acether were not parallel to each other, demonstrating the existence of several antagonists (data not shown).

This inhibition was only observed with PAF-acether, GBE 761 being totally inefficient with respect to the other pro-aggregating agents such as ADP (2.5 μM), thrombin (1 U/m*l*), collagen (0.2 mg/m*l*) and A 23187 (10 μM).

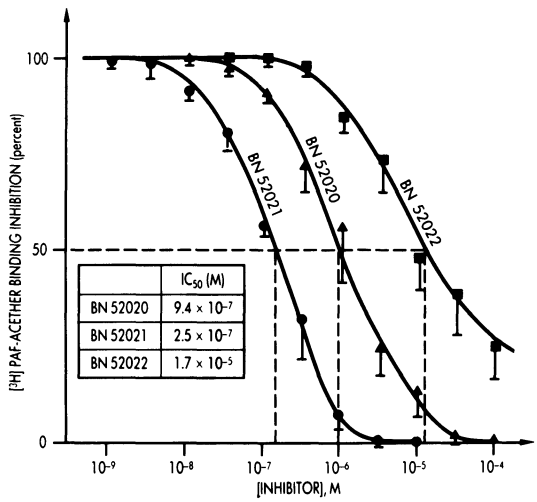

Fig. 3 Inhibition of the binding of [^{3}H]PAF-acether to its receptor by BN 52020, BN 52021 and BN 52022 (mean ± SD, n=6 for each point)

4. Inhibition of In Vitro Rabbit Platelet Aggregation (PRP) by BN 52021 and Related Compounds-Comparison with GBE 761 and Kadsurenone

The specificity with respect to PAF-acether was also found with the pure antagonists isolated from whole Ginkgo extract. BN 52021 was found to be the most efficient inhibitor of aggregation with an $IC_{50} = 1.88 \times 10^{-7}$ M for 2.5 nM PAF-acether (**Fig. 5**). In the same experiment, BN 52020 was approximately as efficient as Kadsurenone ($IC_{50} = 8.32 \times 10^{-7}$ M for BN 52020 as against 9.94×10^{-7} for Kadsurenone). As shown in **Table 3**, BN 52021 was found to be more efficient no matter what concentration of PAF-acether was used. No difference in the degree of inhibition was recorded with varying time of preincubation between 1 and 20 min (data not shown).

In a series of experiments performed with washed rabbit platelets, it was of interest to observe that the inhibitory

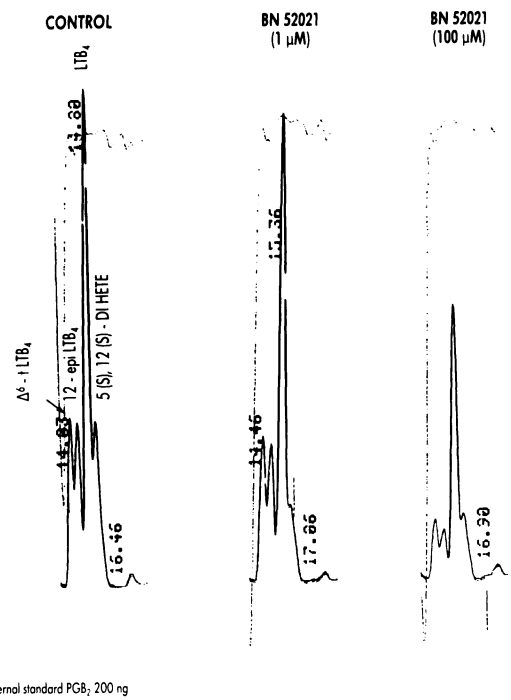

Fig. 4 Effect of BN 52021 on the production of LTB_4 and its isomers by A 23187-stimulated human leukocytes (HPLC profile)

Table 3 IC_{50} of PAF-acether antagonists isolated from *Ginko biloba L.* against PAF-acether-induced rabbit platelet aggregation (PRP); comparison with Kadsurenone.

	PAF-ACETHER (nM)		
	2.5	5.0	10.0
BN 52020	$8.32\ 10^{-7}$	$1.32\ 10^{-6}$	$9.34\ 10^{-6}$
BN 52021	$1.88\ 10^{-7}$	$3.20\ 10^{-7}$	$5.75\ 10^{-7}$
BN 52022	$1.53\ 10^{-6}$	$2.54\ 10^{-5}$	$3.21\ 10^{-4}$
KADSURENONE	$9.94\ 10^{-7}$	$1.40\ 10^{-6}$	$1.81\ 10^{-5}$

activity of the antagonist persisted after further washing (data not shown).

Analysis of [^{3}H]PAF-acether binding to washed platelets was performed in the presence of 5 μM BN 52021. The antagonist added to platelets together with [^{3}H]PAF-acether prevented in part the total binding when compared to the control test performed in the presence of the solvent. Addition of an excess of cold PAF-acether (10 nM) in place of BN 52021, exhibited exactly the samc effect (**Fig. 6**). Calculations from these data showed that BN 52021 totally suppressed the spe-

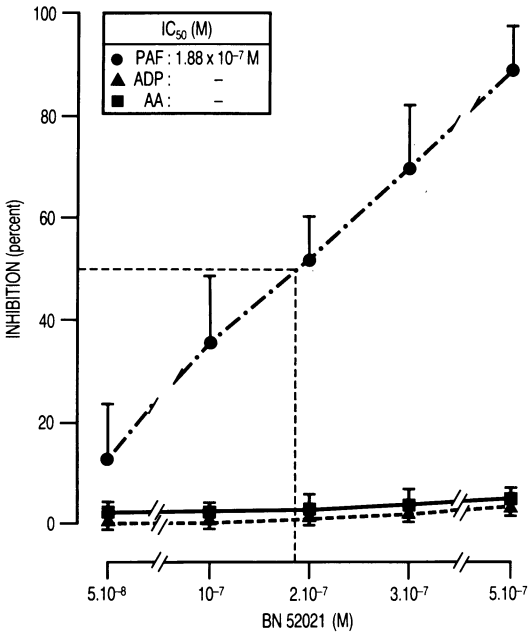

Fig. 5 Effects of BN 52021 on rabbit platelet aggregation (PRP) triggered by ADP, AA or PAF-acether. Each point represents the mean (± 1 SD) of aggregation inhibition percentages of rabbit platelets after 2 min incubation with BN 52021. ADP, AA and PAF-acether concentrations were chosen so as to trigger sub-maximal aggregations in presence of solvent.

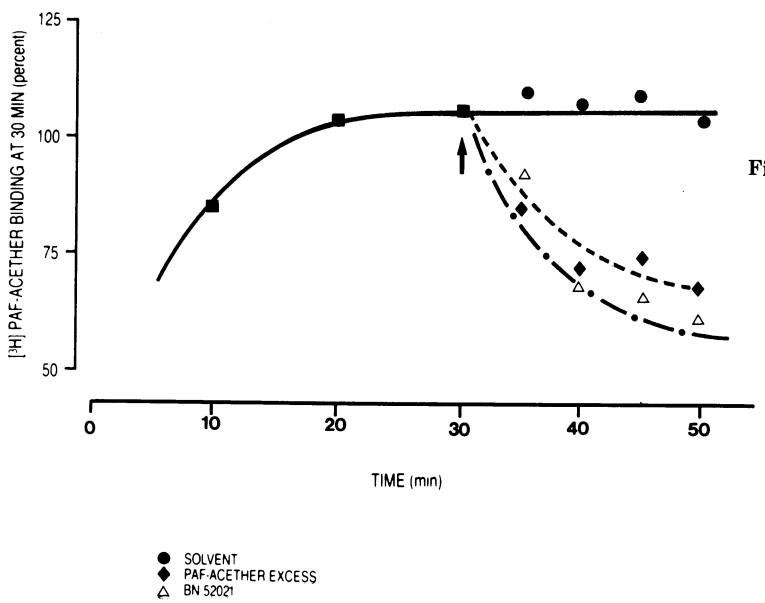

Fig. 6 Effect of BN 52021 on the binding kinetic of [^{3}H] PAF-acether to intact washed rabbit platelets. The full lines represent the kinetics of the total binding observed in the presence of 10 nM cold PAF-acether (◆), 5 μM BN 52021 (△) or its solvent (●). Each point is the mean of duplicate determinations in one experiment representative of three.

Table 4 Inhibition of ex vivo rabbit platelet aggregation (PRP) by BN 52021. The drug was administered in gum (15 mg/kg orally). n=6.

ANTAGONIST mg/kg, PO	PAF-acether (nM)	% OF MAXIMAL AGGREGATION TIME (h)			
		control	1	2	6
-	1.25	45.5 ± 8.9	29.9 ± 4.9	33.7 ± 4.1	30.9 ± 6.4
	2.50	62.4 ± 4.9	52.6 ± 6.7	56.9 ± 6.3	53.3 ± 8.2
BN 52021 (15)	1.25	46.1 ± 5.6	2.6 ± 1.6[b]	2.8 ± 1.8[b]	10.1 ± 4.4[a]
	2.50	62.3 ± 6.3	14.6 ± 4.5[b]	14.7 ± 6.1[b]	29.5 ± 8.2[a]
BN 52041[c] (15)	1.25	48.2 ± 5.8	0[b]	0.8 ± 0.6[b]	12.8 ± 4.9[a]
	2.50	61.4 ± 5.8	3.6 ± 1.9[b]	11.4 ± 4.0[b]	31.1 ± 7.7[a]

Mean ± SD; a: $p<0.01$; b: $p<0.001$; c: BN 52041 is a mixture of BN 52021 and BN 52020 (40:60)

Table 5 Effects of oral administration of BN 52021 and GBE 761 on in vivo thromboformation induced by electrical stimulation of the rat carotid artery. The drugs were administered in a suspension in gum. Mean±SD, n=6.

GROUP	ORAL TREATMENT	DOSE (mg/kg)	GROUP MEAN TIME TO THROMBUS FORMATION (minutes)	% INCREASE IN TIME TO THROMBUS FORMATION COMPARED WITH CONTROLS
1	VEHICLE (1% TRAGACANTH)	—	8.95 ±0.80	—
2	BN 52021	20	12.16* ±1.27	35.9
3	GBE 761	200	10.15 ±1.14	13.4

cific PAF-acether binding to platelets.

Whatever PAF-acether concentration was used, a good correlation was observed between the ratio:

$$\frac{IC_{50}\ (\text{antagonist})}{IC_{50}\ (\text{GBE 761})}$$

and the relative concentration of each of the antagonists in the whole extract. Increasing concentration of PAF-acether (10^{-7} M → 3×10^{-6} M) clearly shifted to the right the PAF-acether aggregation curves (**Fig. 7**).

5. Inhibition of Ex Vivo Rabbit Platelet Aggregation (PRP) by BN 52021

This experiment was perfomed only with the most efficient inhibitor (BN 52021) and BN 52041, a mixure of 52021 and 52020 (40 : 60).

As shown in **Table 4**, the antagonistic effect vs PAF-acether was found in platelets from pre-treated animals, BN 52021 or BN 52041 having been administered orally (15 mg/kg) at various times (h) before the blood sampling. At t=1 or t=2, a significant inhibition ($p<0.001$) of platelet aggregation was recorded. At t=6, this inhibition was still detectable ($p<0.01$). Here again, the antagonists were only effective against PAF-acether-induced aggregation and not against that caused by ADP, collagen, thrombin or A 23187.

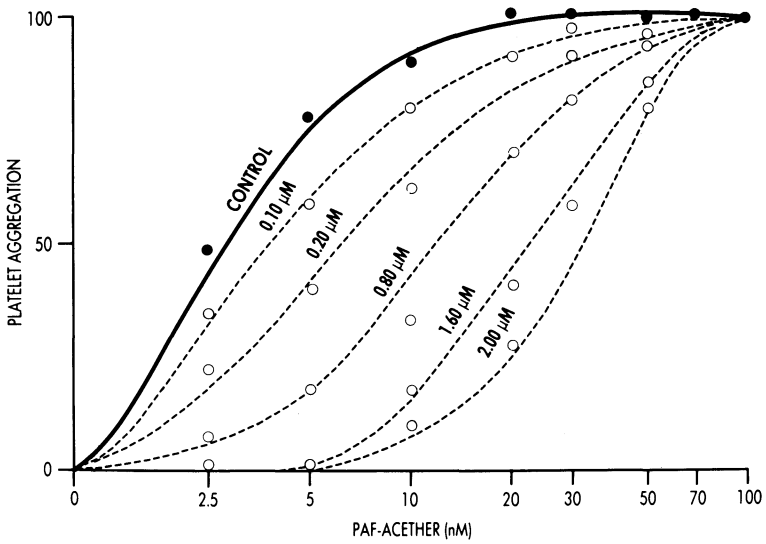

Fig. 7 Shift of PAF-acether concentration-effect curve in presence of BN 52021. Dose-response curve in the absence of BN 52021 (control) and in its presence at various concentrations. Each point is the mean of 6 experiments.

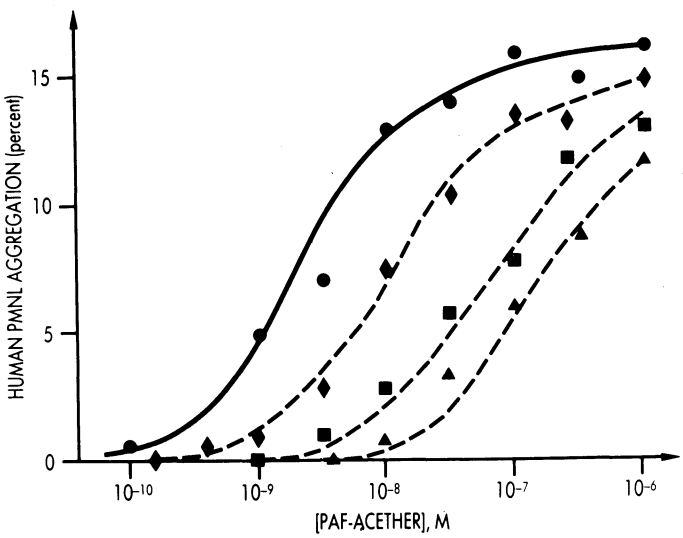

Fig. 8 Effect of BN 52021 on PAF-acether-induced aggregation of human PMNLs. In this assay, 5 nM of PAF-acether was required to produce 50% inhibition of human PMNI aggregation. Control (●), BN 52021 5×10^{-7} M (◆), BN 52021 5×10^{-6} M(■), BN 52021 10^{-6} M(▲).

6. Effect of BN 52021 on Thrombus Formation Induced by Electrical Stimulation of the Rat Carotid Artery

This experiment was performed only with the most efficient inhibitor, BN 52021 and GBE 761. The results are summarized in **Table 5**. As compared with control a statistically significant increase in time to thrombus formation was obtained with BN 52021 at 20 mg/kg,

administered orally, 30 min before the challenge.

7. Inhibition of Aggregation of Human PMNLs by BN 52021

Human PMNLs aggregated dose-dependently with PAF-acether or FMLP between 10^{-10} and 10^{-5} M. In the presence of increasing concentrations of BN 52021 there is a shift in the aggregation curves (**Fig. 8**), evidencing a dose-dependent inhibition of PAF-acether induced PMNL aggregations. Conversely, BN 52021 ($10^{-7} \rightarrow 10^{-4}$ M) did not inhibit aggregation induced by FMLP.

IV. DISCUSSION

Several authors have suspected that PAF-acether is a powerful mediator of anaphylaxis and shock (see for instance ref. 14-15). The recent development of specific inhibitors allowed us to investigate here such a hypothesis. In this paper, we describe a new series of compounds with specific inhibitory properties which are chemically defined molecules extracted from the leaves of *Ginkgo biloba L.*, identified as BN 52020, BN 52021 and BN 52022. These compounds are usually named Ginkgolides A, B and C, respectively. The first isolation of these terpenoids was performed from Ginkgo by Furukawa in 1932[24]. However it was only at the end of the 1960's that the Japanese team of Nakanishi identified their chemical structures[25~27]. Ginkgolides are unique cage molecules which are C_{20} compounds, incorporating a tert-Bu group and six 5-membered rings including a spiro [4. 4] nonane system, a tetrahydrofurane cycle and three lactonic groups. Few works had been devoted to these compounds since the pioneering works of Nakanishi et al.[25~27]. The studies we performed using 500 MHz NMR[28] and X ray crystallography[29] analysis allowed us to confirm the structures proposed 15 years previously and deduced mainly by chemical methods.

In order to characterize the anti-PAF-acether properties of BN 52021 and related compounds, we performed several investigations on PAF-acether/membrane interactions using rabbit platelets and human leukocytes. Firstly, we demonstrated that these compounds strongly interact with PAF-acether receptor since they inhibit the binding of [^{3}H] PAF-acether. BN 52021 (Ginkgolide B) was the most efficient with an IC_{50} close to 10^{-7} M. Conversely, BN 52022 (Ginkgolide C), chemically characterized by the presence of a hydroxyl group in the vicinity of the tert-Bu, was considerably less active, suggesting that the presence of a hydrophilic group close to the lipophilic moiety counteracts the antagonistic property. A simlar observation was recorded in the Kadsurenone series in which the introduction of a hydrophilic group in the vicinity of the allyl moiety also reduced the antagonistic power (T. Y. Shen, pers. com.). Our results on the inhibition of rabbit platelet aggregation further confirmed the binding data : once again BN 52021 was the most efficient compound with an IC_{50} close to 10^{-7} M for the various doses of PAF-acether used, whereas BN 52022 was the least efficient. Furthermore, this effect was specific since no inhibition was observed with the other pro-aggregating agents (ADP, collagen, thrombin, A 23187). The specificity of the action of BN 52021 was particularly evident when it was observed that this antagonist displaced the [^{3}H] PAF-acether from its receptor like non-labeled PAF-acether. The potent binding capacity of BN 52021 to PAF-acether receptor was also demonstrated by the fact that when platelets are treated by the antagonist and then washed, the activity remained practically unchanged. The anti-PAF-acether properties of BN 52021 and related compounds was also

found ex vivo in rabbits preventively treated by oral route (15 mg/kg) : 6 hours after administration of the drugs, the efficiency of PAF-acether in aggregating platelets was significantly lower than that of the control group. These data show that the products are rapidly absorbed and with a long lasting effect.

The efficiency of BN 52021 in increasing the time to thrombus formation suggests that PAF-acether may be involved in electrically-induced thrombogenesis in rat although the platelets of this species do not contain high affinity receptors for PAF-acether. Our results corroborate the data obtained by Bourgain et al.[30], demonstrating that BN 52021 was able to inhibit electrically-induced thrombus formation in the guinea-pig mesenteric artery.

The antagonist effect is not mediated by interaction with the arachidonic acid metabolism since none of the products inhibited either cyclooxygenase or lipoxygenase activity.

BN 52021 is thus a new specific anti-PAF-acether compound, which can be used as a tool for a better understanding of the role of PAF-acether in various biological systems. One of the first applications will be to study the participation of this mediator in the platelet activation process, both in vitro and in vivo. It is noteworthy that recently inhibitory effects of CV 3988 were reported in two experimental models in the rat i. e. one kidney, one clip hypertension after unclipping[31] and endotoxin shock[32]. For the latter model, Kadsurenone also proved to be active[33]. Using BN 52021, inhibitory effects were also observed against (i) bronchoconstriction induced either by PAF-acether or by the specific antigen in sensitized guinea pigs[34][35], (ii) IgG-induced hypotension in rats[36] (iii) *Salmonella enteritidis*-induced endotoxemia[37] and (iv) heart allograft rejection in rats[38].

In conclusion, BN 52021 and other PAF-acether antagonists constitute promising pharmacological tools for exploring PAF-acether involvement in different models of anaphylaxis, shock or thrombosis.

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