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EVALUATION OF PAF ANTAGONISTS USING HUMAN NEUTROPHILS IN A MICROTITER PLATE ASSAY*

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Abstract—This paper describes a testing model for the detection and evaluation of PAF antagonists, based on the inhibition of PAF-elicited elastase release by human neutrophils. Incubations are performed in microtiter plates in the presence of a specific fluorogenic elastase substrate allowing direct measurement of the exocytosis response by means of a 96-well fluorescence reader. Determinations of the IC_{50} values for five established PAF antagonists, Ro 19-3704, BN 52021, CV-3988, 48740 RP and kadsurenone, showed that the new model is comparable in sensitivity and discriminative capacity to other *in vitro* assays. From the effect of antagonists on the PAF concentration–response curve pA2 values could be calculated and information on the type of antagonism obtained. BN 52021 was found to behave as a competitive antagonist while Ro 19-3704 showed a more complex type of inhibition.

As a one-plate system, the test is simple to handle and highly reproducible, and appears therefore particularly useful for large drug screening programs.

Over the last few years, several publications have indicated that the alkyl phospholipid, platelet-activating factor (PAF), is a major mediator of inflammation and anaphylaxis with important pathogenetic effects in respiratory and cardiovascular disorders [1–3]. PAF is produced by several types of stimulated cells including neutrophils, mononuclear phagocytes, basophils, platelets and endothelial cells [4]. It causes aggregation and exocytosis in platelets and neutrophils [4], induces smooth muscle contraction [5], enhances vascular permeability [6, 7] and leads to bronchoconstriction, hypotension and renal failure [1–4, 8].

The identification of the structure of PAF by Benveniste *et al.* [9] and Demopoulos *et al.* [10] has stimulated the search for PAF antagonists and the evaluation of their biological and pharmacological properties. We have studied the effects of PAF on exocytosis and the respiratory burst in human neutrophils [11], and have recently explored the possibility to inhibit these responses with PAF antagonists. Using different types of molecules, the PAF analogues CV-3988 [12] and Ro 19-3704 [13], the synthetic inhibitor 48740 RP, and the natural compounds BN 52021 [14] and kadsurenone [15], we found that enzyme release by neutrophils is particularly sensitive to inhibition. We have therefore investigated the possibility of adopting this biological response for the assessment of PAF antagonists, and present here a simple testing model developed for this purpose.

MATERIALS AND METHODS

Materials. *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP), 1-O-hexadecyl-2-O-acetyl-*sn*-glycero-3-phosphocholine (PAF) and 1-O-hexadecyl-*sn*-glycero-3-phosphocholine (lyso-PAF) were purchased from Bachem AG, Bubendorf, Switzerland. Cytochalasin B was from Serva GmbH & Co, Heidelberg, F.R.G., and lactate dehydrogenase (LDH) from Boehringer, Mannheim, F.R.G. Leukotriene B_4 (LTB_4) was a gift of Dr J. Rokach, Merck Frosst Canada Inc., Pointe Claire-Dorval, Que, Canada. Human C5a was a gift of Dr C. A. Dahinden, Department of Clinical Immunology, University of Bern, Bern, Switzerland. The elastase substrate *N*-succinyl-L-alanyl-L-alanyl-L-prolyl-L-valine-7-amido-4-methylcoumarin was kindly provided by Dr T. G. Payne, Preclinical Research, Sandoz Ltd., Basel, Switzerland. The PAF antagonists were obtained from the following sources: BN 52021 from Dr P. Braquet, Institut Henri Beaufour, Le Plessis Robinson, France; Ro 19-3704 from Dr H. R. Baumgartner, F. Hoffmann-La Roche & Co Ltd., Basel, Switzerland; 48740 RP from Dr P. Sédivy, Rhône-Poulenc, Vitry, France; Kadsurenone from Dr T. Y. Shen, Merck Sharp & Dohme, Rahway, NJ, USA. CV-3988, a product of Takeda Chemical Industries Ltd., Osaka, Japan, was synthetized by Dr M. Lee, Sandoz Inc., East Hanover, NJ; Compound FEI 206-346 (1-[4-(nonyloxy)phenyl]-4-(1-piperidinyl)-1-butanone hydrogenoxalate) was synthetized by Dr A. Leutwiler, Wander Research Institute, a Sandoz Research Unit, Bern, Switzerland.

Human neutrophils were prepared from donor blood stored overnight at 4° (Swiss Red Cross Laboratory, Bern, Switzerland). Single buffy coats diluted 4-fold with PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.9 mM $CaCl_2$, 0.49 mM $MgCl_2$) containing heparin (13 U/ml) were centrifuged through Ficoll-Hypaque gradients [16] and the neutrophils were isolated from the cell pellets according to Weening *et al.* [17]. The preparations

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contained 93.4 ± 2.0% neutrophils and 2.4 ± 1.1% eosinophils (N = 20).

Exocytosis assay

The elastase assay method described by Sklar *et al.* [18] was modified for use in 96-well microtiter plates. Fifty microlitres of neutrophil suspension (2×10^7 cells/ml), 50 μl of the elastase substrate *N*-succinyl-L-alanyl-L-alanyl-L-prolyl-L-valine-7-amido-4-methylcoumarin (0.125 μmol) and 50 μl of either a solution of a test compound in medium or medium alone (control) were dispensed into the wells of flat-bottom microtiter plates. After 10 min at room temperature 50 μl of cytochalasin B (20 μg/ml) were added and exocytosis was elicited 10 min later with 50 μl of stimulus. Stimulus addition to all 96 wells required about 1 min. Fluorescence reading was started 2 min after addition of stimulus to the first well and was repeated at 5 or 10 min intervals. The plates were kept at room temperature.

The incubation medium consisted of PBS containing 2.5 mg/ml bovine serum albumin (PBS/BSA). The following stock solutions were used: cytochalasin B 5 mg/ml, fMLP 10 mM, BN 52021 10 mM, kadsurenone 10 mM in DMSO; 48740 RP (hydrochloride) 10 mM in distilled water; PAF 10 mM and CV-3988 10 mM in PBS/BSA. Ro 19-3704 was dissolved in ethanol at 10 mM and immediately diluted to 1 mM with Ca^{2+}, Mg^{2+}-free PBS containing 40 mg/ml BSA. All further dilutions were made with PBS/BSA. The concentration of DMSO in the incubation mixture did not exceed 0.2%.

The 96-well microtiter plates were prepared according to the following scheme: Row 1 always contained unstimulated cells (blank) and row 2 stimulated cells but no test compound. The test compounds were added to rows 3 through 12 at the desired concentrations. Routinely, 4 antagonists at 5 concentrations were examined in quadruplicate on one plate. Obviously, other combinations with respect to number of compounds and concentrations are possible. In some experiments we used 2 PAF concentrations (rows A to D and E to H, respectively) and only 2 antagonists per plate.

When multiple concentrations of PAF were used (concentration–response experiments), the reaction was started by addition of the neutrophils. Fifty microlitres of elastase substrate (0.125 μmol) and 100 μl of PAF (0.03–10 μM final concentration) were dispensed into the wells of the microtiter plate. Neutrophils (1.5×10^7 cells/ml) were incubated separately with or without test compound for 10 min. Cytochalasin B (0.5 vol, 15 μg/ml) was then added, and after 10 min the reaction was started by dispensing 100 μl aliquots of these suspensions into the wells. In control experiments, identical results were obtained with this modified sequence of additions and the standard procedure described above.

Fluorescence measurements. Microtiter plates were read with a Titertek Fluoroskan (Eflab, Helsinki, Finland) equipped with an excitation interference filter of 355 nm (HBW 50 nm, ±10 nm) and interfaced with an Apple IIe computer. The computer was programmed to average the quadruplicate readings, subtract the blank, determine the net mean fluorescence increments between readings and calculate relative activities (percent of the activity obtained in the absence of test compounds). Initial experiments to assess the time course of elastase release were performed in a Perkin-Elmer LS-5 fluorimeter (Perkin-Elmer GmbH, Ueberlingen, F.R.G.) equipped with a magnetic stirring device and a thermostatted cuvette holder. The cuvette contained 2.5 ml of the reaction mixture, and the conditions were as described for the exocytosis assay (excitation: 370 nm, emission: 460 nm).

Cytotoxicity test. Possible cytotoxic effects of the test compounds were evaluated by determining LDH release during the period of elastase measurement. Neutrophils were stimulated with PAF in the presence of PAF antagonists, elastase substrate and cytochalasin B, and the elastase activity released was measured for up to 30 min as described above. The 96-well microtiter plates were then centrifuged and 50 μl of each supernate were transferred to a fresh plate for assay of LDH activity according to Bergmeyer and Bernt [19]. To 50 μl of supernate were added 50 μl of a freshly prepared reagent mixture consisting of 1 part of 1.2 M Tris/HCl, pH 8.5 containing 7.5 mg/ml BSA and 0.15% Triton X-100, 1 part of 0.3 M sodium lactate and 1 part of a solution containing 9 mM NAD, 4.8 mM INT (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride) and 1.92 mM PMS (phenazine methosulfate). Absorbance at 492 nm was measured immediately after the last addition and at 2–5 min intervals thereon using an EIA Reader Model 2550 (Bio Rad Laboratories AG, Glattbrugg, Switzerland). The absorbance change per min was related to mU on the basis of standard amounts of commercial LDH. The assay was linear with time (5–20 min) and enzyme concentration (1–20 mU LDH/assay). Control experiments showed that the LDH determination in neutrophil lysates was not influenced by 0.5 mM elastase substrate, 1 μM PAF or 100 μM of any of the PAF antagonists used.

RESULTS

PAF-induced exocytosis

Exocytosis following stimulation of cytochalasin B-treated human neutrophils was quantified by determining the elastase released into the extracellular medium. Initial experiments (Fig. 1) showed that the release response to 1.0 μm PAF, at room temperature (20–22°), was complete in about 1 min (56 ± 2.6 sec, N = 3). At that time, the rate of substrate hydrolysis (or fluorescence increase) became linear indicating that elastase release had ceased. The rate of fluorescence increase was dependent on the PAF concentration used to stimulate the cells (0.1 to 1.0 μM), and was linear for up to 40 min (Fig. 2). Stimulation of the cells with other agonists, i.e. fMLP (Fig. 2), C5a or LTB_4 yielded similar results. The effect of fMLP was comparable to that described by Sklar *et al.* [18]. Using a different formylmethionyl peptide, these authors found that the exocytosis response of cytochalasin B-treated human neutrophils was complete within 30 sec at 37°.

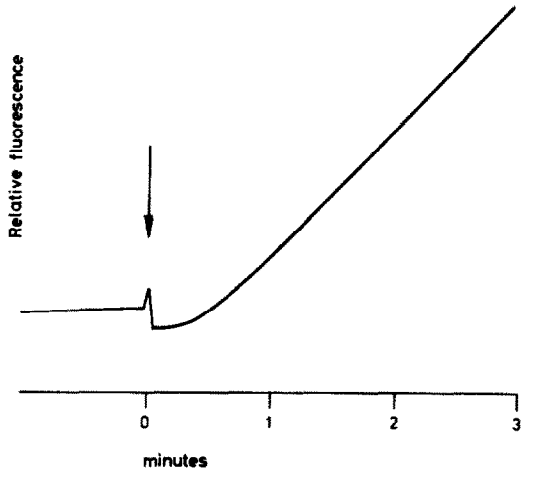

Fig. 1. Time course of elastase release. Elastase activity was measured continuously in a Perkin-Elmer LS-5 fluorimeter in a cuvette equipped with a magnetic stirring device. 10^7 neutrophils were incubated for 5 min at room temperature in 2 ml of PBS/BSA containing 1.25 μmol elastase substrate and 10 μg cytochalasin B. The release reaction was then induced with 0.5 ml PAF to a final concentration of 1.0 μM.

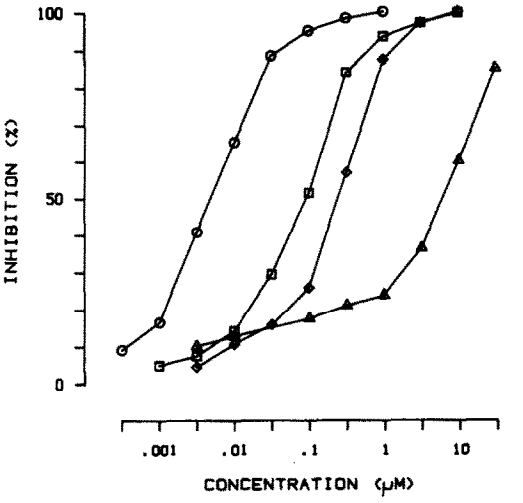

Fig. 3. Effect of PAF antagonists on PAF-induced elastase release. The antagonists were: Ro 19-3704 (○), BN 52021 (□), CV-3988 (◇) and 48740 RP (△). PAF was used at 0.1 μM.

Evaluation of PAF antagonists

Five compounds that have been shown to have antagonistic activity in various biological systems were tested for their ability to inhibit PAF-induced elastase release from cytochalasin B-treated neutrophils. Figure 3 shows the results of a typical experiment. Parallel sigmoidal curves relating percent inhibition to antagonist concentration were obtained with two structural analogues of PAF, Ro 19-3704 and CV-3988, the natural compound BN 52021 and the synthetic compound 48740 RP. Lyso-PAF, the biologically inactive PAF metabolite, lacked an appreciable effect (less than 20% inhibition at 10 μM). The results of a series of similar experiments are summarized in Table 1 which shows the mean antagonist concentration causing 50% inhibition of response (IC_{50}) elicited by 0.1 and 1.0 μM PAF. The natural compound kadsurenone was included in addition to the antagonists presented in Fig. 3. The relatively high standard deviations reflect the different responsiveness of the individual neutrophil preparations both to PAF and the antagonists. A similar degree of variation was observed in a study on the inhibition of human platelet aggregation [20]. The IC_{50} values depended on the PAF concentration used to elicit release. At 0.1 μM PAF, Ro 19-3704 appeared to be the most potent antagonist, while at

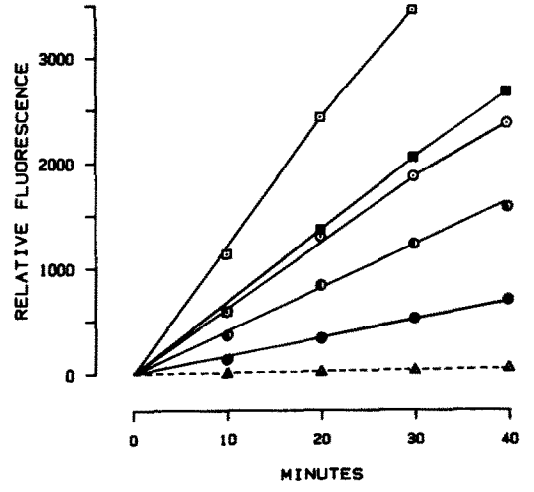

Fig. 2. Elastase release assay in 96-well plates; dependence on stimulus concentration. 10^6 neutrophils were preincubated for 15 min at room temperature in 0.2 ml PBS/BSA containing 0.125 μmol elastase substrate and 1 μg cytochalasin B. 50 μl of PAF or fMLP were then added to start the release reaction. Increments in fluorescence at 10 min intervals are shown. Final micromolar concentrations were 0.1 (●), (0.3 (◐), 1.0 (○) for PAF; and 0.01 (■), 0.03 (□) for fMLP. Unstimulated control (△).

Table 1. Inhibition of PAF-induced elastase release by PAF antagonists

Antagonist	IC_{50} (nM) PAF 0.1 μM (N)	PAF 1.0 μM (N)
Ro 19-3704	9 ± 6 (12)	174 ± 27 (6)
BN 52021	125 ± 60 (7)	228 ± 94 (6)
CV-3988	270 ± 147 (5)	4910 ± 614 (4)
Kadsurenone	438 ± 233 (5)	2520 ± 1200 (3)
48740 RP	6400 ± 1400 (6)	>10,000 (6)

The IC_{50} values were obtained from experiments as shown in Fig. 3. PAF was used at 0.1 and 1.0 μM. Values represent means ± SD with the number of experiments using different neutrophil preparations in brackets.

1.0 μM PAF it was practically equiactive with BN 52021. Figure 4 shows the dependence of elastase release on the concentration of PAF. The response increased with the amount of agonist added, and did not tend to level off even at the very high concentration of 10 μM PAF. Similar responses to PAF were reported by Shen *et al.* [15] who assayed for beta-glucuronidase release. The effects of two antagonists, BN 52021 and Ro 19-3704, are also shown. A concentration-dependent, parallel shift of the curves to the right was observed with BN 52021 (Fig. 4A), indicating that the inhibition was surmountable with increasing agonist concentration. A pA2 of 7.39 ± 0.09 and a slope of -0.99 ± 0.11 for the regression line of the Schild plot was obtained in four experiments performed with different neutrophil preparations (values determined at the 50 and 70% level of the uninhibited response to 1 μM PAF). BN 52021 thus appears to qualify as a competitive antagonist in agreement with the report by Nunez *et al.* [20] who used a platelet aggregation assay. The results obtained with Ro 19-3704 are shown in Fig. 4B. Ro 19-3704 was found to be a potent antagonist at low PAF concentrations. Inhibition, however, was readily overcome by higher PAF concentrations resulting in agonist concentration–response curves that were steeper than the control, suggesting a more complex type of antagonism.

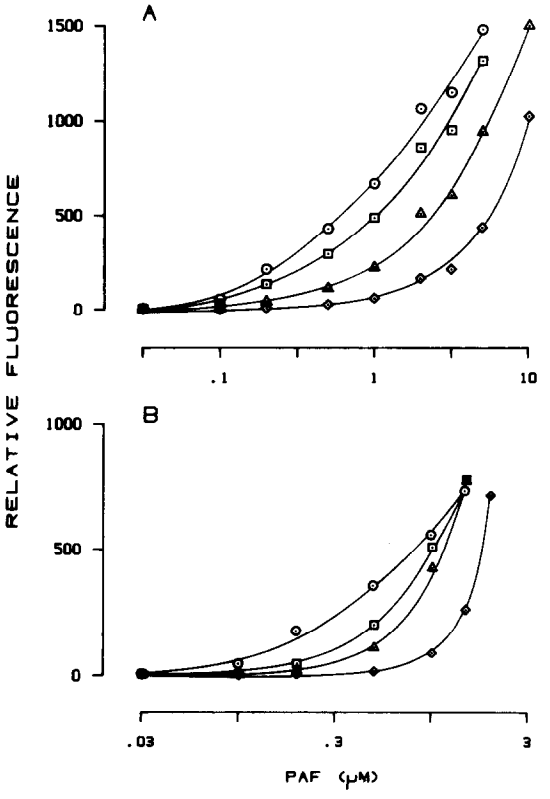

Fig. 4. Elastase release induced by increasing concentrations of PAF in the presence and absence of BN 52021 (A) or Ro 19-3704 (B). Final micromolar concentrations were 0.03 (□), 0.1 (△) and 0.3 (◇) for BN 52021 and 0.01 (□), 0.03 (△) and 0.1 (◇) for Ro 19-3704; (○) release in the absence of antagonist.

In a number of experiments the antagonists were tested in the absence of stimulus. No elastase release was observed in the concentration range used (see Fig. 3) indicating that none of the compounds exhibited agonistic activity. The action of PAF antagonists was selective for PAF-induced release. No inhibition was observed when exocytosis was elicited with fMLP, C5a or LTB_4. This is shown in Fig. 5 for Ro 19-3704. Corresponding results were obtained with BN 52021, CV-3988 and 48740 RP. Similarly, kadsurenone showed no effect on LTB_4-induced release. However, a 30–50% inhibition of the response to fMLP was observed at the highest concentration tested (10 μM). The finding that elastase release induced by stimuli other than PAF was not affected indicates that the PAF antagonists do not interfere with the process of exocytosis and do not inhibit elastase. Common drugs with antiinflammatory, membrane-stabilizing, local anesthetic and calcium antagonistic activities (i.e. indomethacin, phenylbutazone, chlorpromazine, tetracain, verapamil and cyproheptadine) were also tested as possible inhibitors of PAF-induced elastase release, but no effects were obtained at concentrations up to 10 μM.

Reproducibility

In this study, all determinations were done in quadruplicate. The reproducibility of parallel determinations was established in a set of five randomly selected experiments. The relative differences of the single values from the mean of the four readings were calculated, and an overall variation between 1.5 and 7.8% was found. This analysis suggested that reliable assays could be done in duplicate. The possibility to evaluate PAF antagonists on this basis

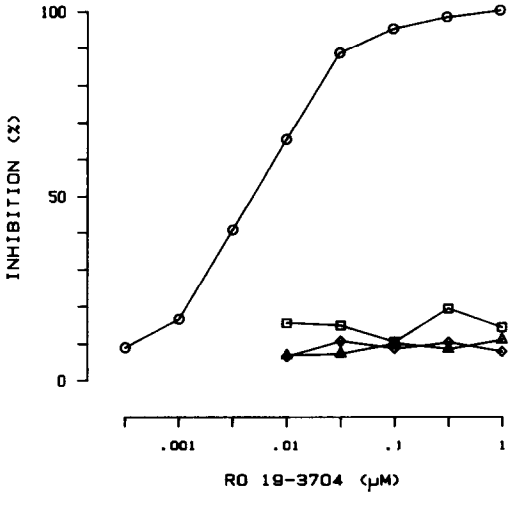

Fig. 5. Lack of effect of PAF antagonists on elastase release induced by fMLP, C5a and LTB_4. The effect is shown for Ro 19-3704. In the absence of test compound the elastase activity released by 0.1 μM PAF (○), 0.01 μM fMLP (□), 0.1 μM LTB_4 (△) and 0.001 μM C5a (◇) amounted to 1445, 1223, 1967 and 3073 fluorescence units/30 min, respectively.

was tested by comparing the results obtained with four PAF antagonists in parallel sets of duplicates. Figure 6 shows the results obtained with BN 52021. Both inhibition curves determined in duplicate are almost superimposable and in good agreement with quadruplicate determinations from three separate experiments performed for the calculation of the IC_{50} values (Table 1). Similar degrees of reproducibility were obtained with Ro 19-3704, CV-3988 and kadsurenone upon stimulation of the cells with either 0.1 or 1.0 μM PAF.

Cytotoxicity assessment

LDH release was measured in the cell-free supernatants following elastase determination (see Materials and Methods). Table 2 shows the results obtained in a representative experiment. None of the PAF antagonists exhibited cytotoxicity as indicated by the low levels of LDH which were close to those of the drug-free controls. The results obtained with two compounds with known cytolytic activity, chlorpromazine and FEI 206-346, are also shown.

Table 2. Assessment of cytotoxicity

Test compound	Conc. (μM)	LDH release (% of cellular content) PAF 0.1 μM	PAF 1.0 μM
None		4.9	5.3
Ro 19-3704	10	4.8	5.6
	100	6.2	6.9
BN 52021	10	5.4	5.8
	100	4.6	5.1
CV-3988	10	4.9	6.7
	100	5.7	7.1
Kadsurenone	10	4.8	5.3
	100	4.9	5.5
48740 RP	10	5.0	5.3
	100	5.6	5.8
Chlorpromazine	10	5.8	6.0
	100	23.8	28.4
FEI 206-346	10	5.9	6.8
	30	15.3	20.0
	100	69.4	77.5

Representative experiment out of at least three for each compound. 100% LDH was released by addition of 0.05% Triton-X-100.

DISCUSSION

We have described a testing system for the detection and evaluation of PAF antagonists, which is based on the inhibition of elastase release by human neutrophils stimulated with PAF. The test has important advantages over existing methodology. The use of a fluorimetric assay for elastase permits the measurement of exocytosis in the presence of the stimulated cells at the pH of the incubation medium. Photometry using chromogenic substrates, e.g. *N*-tert-butoxycarbonyl-L-alanine-*p*-nitrophenyl ester, was found to be much less sensitive and more prone to disturbance by the cells. Alternative markers of release such as beta-glucuronidase [15] and other acid glycosidases require the transfer of cell-free supernates for assay on a second plate. High capacity is an outstanding feature of the model. Four antagonists can be evaluated in quadruplicate at five concentrations on one 96-well plate. Higher numbers may be tested under less stringent conditions by performing the assays in duplicate. A further advantage is versatility. Several controls can be included in the same test set up. A test compound may lower the elastase reading other than through PAF antagonism: It may inhibit the process of granule fusion or other steps of the release mechanism, may cause cell damage with consequent liberation of elastase inhibitors [18], or may inhibit elastase directly. Alternatively, a test compound could have agonistic activity. All these possibilities can be checked on the assay plate itself by either omitting PAF or using a different stimulus, e.g. fMLP. Although the primary goal of the study was to set up a simple procedure to screen for PAF antagonists, our method may also be used to obtain information on the nature of the antagonism, as demonstrated in the experiments performed with BN 52021, an apparently competitive antagonist, and Ro 19-3704 which showed a more complex type of inhibition. A more extensive characterization, however, would require additional experiments including receptor binding studies.

In the past, evaluation of PAF antagonists *in vitro* has been performed mainly using rabbit or human platelets which aggregate and secrete in response to PAF [7, 12, 13, 15, 20–22]. Rabbit platelets have been the favored model because of their high sensitivity to PAF [23]. The first report in which human neutrophils were used was a study on the properties of kadsurenone [15]. Since the release of granule enzymes like elastase is easy to quantify and large numbers of samples can be tested in parallel, the neutrophils appear to be a convenient cellular model for large screening programs.

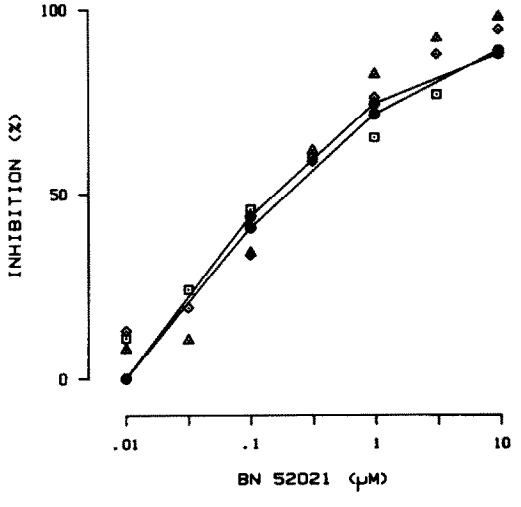

Fig. 6. Reproducibility of PAF antagonist evaluation; results with BN 52021. Inhibition curves from two parallel determinations in duplicate using the same neutrophil preparation (●) are shown together with quadruplicate determinations performed on different days with three different neutrophil preparations (△, □, ◇). PAF concentration was 0.1 μM.

Table 3. Inhibition of platelet aggregation and neutrophil exocytosis by PAF antagonists

Test system*	PAF	IC_{50} (μM) CV-3988	Kadsurenone	BN 52021	References
Rabbit platelets†	0.1 nM	0.2			22
	0.2–0.5 nM	0.1	0.6		7, 21
Human platelets†	7.5 nM	1.0	0.8	2.2	20
Human platelets‡	2.0 μM	27.6	19.6	3.3	20
Human neutrophils	0.1 μM		2.3		15
	1.0 μM		14.5		15
	0.1 μM	0.27	0.44	0.13	§
	1.0 μM	4.91	2.52	0.23	§

* Platelets, inhibition of aggregation; neutrophils, inhibition of azurophil granule enzyme release.
† Washed patelets.
‡ Platelet-rich plasma.
§ This study.

The inhibitory potencies reported for three PAF antagonists by different laboratories are compared with our own data in Table 3. The values obtained in our testing system are similar to those reported for the inhibition of platelet aggregation. The potency ranking appears to depend on the concentration of PAF used, as already pointed out in the Results (Table 1). Overall, however, CV-3988 and kadsurenone have similar antagonistic activity, while BN 52021 is more active at high PAF concentration. The IC_{50} values reported by Shen *et al.* [15] for kadsurenone as inhibitor of PAF-induced exocytosis of human neutrophils are higher than those obtained in this study. This could be due mainly to the fact that our experiments were performed at room temperature rather than at 37°.

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