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PAF BINDING SITES

CHARACTERIZATION BY [^{3}H]52770 RP, A PYRROLO[1,2-*c*]THIAZOLE DERIVATIVE, IN RABBIT PLATELETS

CHRISTINE ROBAUT, GABRIELLE DURAND, CLAUDE JAMES,* DANIEL LAVE,* PIERRE SEDIVY, ANNE FLOCH, SERGE MONDOT, DANIEL PACOT,* ICILIO CAVERO and GÉRARD LE FUR†

Rhône-Poulenc Santé, Centre de Recherches de Vitry, Departments of Biology, and *Chemistry, 13 Quai Jules Guesde, 94403 Vitry-sur-Seine Cédex, France

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Abstract—52770 RP, the *N*-(3-chlorophenyl)-3-(3-pyridinyl)-1H,3H-pyrrolo[1,2-*c*]thiazole-7-carboxamide, displaces in a potent, specific and competitive manner [^{3}H]PAF from its binding sites on rabbit platelets. Since 52770 RP is not structurally related to PAF and has low liposolubility with respect to PAF, it was selected as a potential radioligand for PAF receptor sites. [^{3}H]52770 RP displayed high-affinity, specificity, as well as saturable and displaceable binding to a single class of recognition sites in intact platelets and crude platelet membranes. In these preparations, the values of binding parameters were, respectively, 8.5 and 7.6 nM for K_d, 0.2 pmol/5 × 10^7 platelets and 3.66 pmol/mg protein for B_{max} and 0.96 and 0.91 for nH. Inasmuch as the (+)-52770 RP was 300-fold more potent than the (−)-isomer at displacing [^{3}H]52770 RP in intact platelets, the studied binding site manifested stereospecific discrimination. A variety of pharmacological agents including pro- and anti-aggregant compounds did not exhibit affinity for [^{3}H]52770 RP binding sites. In contrast, PAF, some of its active analogues and several recognized PAF antagonists (BN 52021, brotizolam, L-652,731, triazolam), displaced the [^{3}H]52770 RP binding. Studies carried out using [^{3}H]PAF demonstrated that 52770 RP was approximately 4- and 200-fold more potent than L-652,731 and BN 52021 respectively, as a PAF-receptor antagonist. In washed rabbit platelets, the rank order of potency (K_i) for several analogues of 52770 RP, to displace [^{3}H]PAF from its binding sites, was highly correlated ($r = 0.96$) to their ability to antagonize [^{3}H]52770 RP binding. In functional studies, 52770 RP antagonized not only the PAF-induced aggregation in washed rabbit platelets but also the hypotension evoked by PAF in the anesthetized rat. In this respect, it was 26 and 2 times more potent than L-652,731, respectively. In conclusion, [^{3}H]52770 RP might represent a novel interesting tool for furthering our understanding of the role of PAF binding sites in pathophysiological processes.

The platelet activating factor (PAF-acether: in this paper referred to as PAF) is not only a potent pro-aggregant for platelets and leukocytes but can also produce hypotension, depression of myocardial contractility and bronchoconstriction. Furthermore, PAF is released into the blood during anaphylactic and endotoxin shocks, and is responsible for certain functional manifestations (e.g. hypotension, leucopenia, thrombopenia, hemoconcentration) associated with these pathological conditions [1].

Rabbit [2] and human [3] platelets have been shown to possess specific binding sites for [^{3}H]PAF. This binding was displaced in a competitive manner by BN 52021 [4], CV-3988 [5], L-652,731 [6] and 48740 RP [7]: these compounds also antagonize PAF-evoked aggregation in rabbit and human platelets. Furthermore, an excellent correlation appears to exist between the potencies of agonists and antagonists of PAF receptors, determined by either radioligand binding or platelet aggregation studies. Thus, the binding sites on platelets are likely to correspond to receptor sites involved in the mediation of cellular responses.

The aim of this study was to characterize [^{3}H]52770 RP, a pyrrolothiazole derivative, as a potent and specific ligand of PAF recognition sites on rabbit platelet preparations which possess a high number of binding sites for [^{3}H]PAF [8]. In fact, the new compound, as shown in this report, is 500 times more active than a previously described member of pyrrolo[1,2-c]thiazole derivatives, 48740 RP [7], in displacing [^{3}H]PAF from its binding sites. It should be noted that 52770 RP is an antagonist of PAF-induced hypotension or platelet aggregation (see Results section).

PAF, being a phospholipid, has a very high liposolubility which is often associated with extensive non-specific binding and difficulties of manipulation (high adherence to glassware). Thus, the availability of [^{3}H]52770 RP as a novel radioligand for PAF receptors with a non-phospholipid chemical structure may present advantages over [^{3}H]PAF.

A novel ligand for PAF recognition sites, [^{3}H]dihydrokadsurenone, was described [9] very recently when the present study was virtually com-

† Present address: Sanofi Recherche, 37 Avenue Pierre 1er de Serbie, 75008 Paris, France

pleted. In the Discussion section, we will comment on the findings reported therein.

MATERIALS AND METHODS

Washed rabbit platelets. Platelets were prepared according to the method described by Ardlie *et al.* [10]. Male New Zealand rabbits (hybrid Hy 2000, Achard de la Vente, 61350 Passais-la-Conception, France) weighing 2.5 kg were used. Blood was withdrawn from the marginal ear artery, collected in tubes containing an aqueous solution of citric acid (9 mM), sodium dihydrogen phosphate (1.75 mM) and dextrose (5.6 mM), then immediately centrifuged (120 *g*, 15° for 20 min) to prepare platelet-rich plasma (PRP) wherefrom a pellet was obtained by further centrifugation (1000 *g*, 15°, 15 min). The pellet was first washed with a Tyrode solution containing bovine serum albumin (BSA: 0.35% w/w) $MgCl_2$ (2 mM), EGTA (0.2 mM) and then with the same Tyrode solution minus EGTA. The platelets were finally suspended in the assay buffer solution of the following composition in mM: NaCl 140, KCl 2.7, NaH_2PO_4 0.4, $MgCl_2$ 2, $NaHCO_3$ 12, Tris–HCl 10, dextrose 6.2 and BSA 0.25% (w/w) (pH = 7.4). The final suspension was adjusted to contain 4×10^8 platelets/ml.

Crude platelet membrane preparation. The platelet pellet, described in the above section was suspended in ice-cold lysing buffer solution (5 mM EDTA, 5 mM Tris–HCl) and homogenized (Ultra-turrax model TP 18/2, Janke and Kunkel KG, Stauffen, Switzerland). The tubes of lysates were immersed in liquid nitrogen for 5 min and then left at room temperature for 20 min, and the resulting suspension was centrifuged (27,000 *g*, for 10 min at 4°). The recovered pellet was washed twice and then suspended in the assay buffer described in the above section to yield a final concentration of 1.5 to 3 mg membrane protein/ml. The protein concentration was measured, using BSA as standard, according to Lowry *et al.* [11].

[^{3}H]52770 *RP binding assay.* This assay was performed by using a filtration technique. Incubations were carried out in a 0.5ml final volume which contained washed rabbit platelets (10^8 platelets/ml) or membrane fractions (30 μg of membrane protein unless otherwise indicated) and [^{3}H]52770 RP (0.5–30 nM for saturation studies or 4 nM in competition assays) dissolved in the assay buffer. All incubations lasted 30 min (a time period sufficient to reach equilibrium) and were terminated by addition of a 2 ml ice-cold assay buffer followed by rapid vacuum filtration over Whatman GF/C glass-fiber filters in order to separate bound from free ligand. Each tube was quickly washed 3 times with 2 ml of ice-cold assay buffer. The filters were then dried and placed into vials containing 4.5 ml of the scintillation fluid (Ready Solv MP, Beckman). Radioactivity was measured using a scintillation counter (LKB, model Rack Beta 1218) at an efficiency of 50%. Non-specific binding was assessed in parallel experiments and defined as that portion of total binding not displaceable by 10 μM of 52629 RP, a compound which has a 3-methoxyphenyl, instead of the 3-chlorophenyl moiety present in the 52770 RP. The K_i values for 52629 RP against [^{3}H]PAF and [^{3}H]52770 RP binding on washed rabbit platelets were 13.6 ± 1.7 nM (N = 5) and 14.2 ± 2.4 (N = 5), respectively.

[^{3}H]*PAF binding assay.* These assays were routinely carried out by incubating a sample of 5×10^7 platelets in 0.5 ml total volume of the assay buffer described in the previous section, to which 0.5 nM [^{3}H]PAF alone or together with 1 μM 52629 RP was added. After the equilibrium had been reached at 20°, the reaction was stopped by rapid filtration over Whatman GF/C filters previously presoaked in the assay buffer. The incubation tube and the filter were then washed 3 times with 3 ml of ice-cold assay buffer. The filters were dried, placed in mini-scintillation vials containing 4.5 ml of Ready Solv MP and transferred to a liquid scintillation counter.

Determination of the inhibiting potencies of a variety of pharmacological agents and PAF agonists and antagonists. The specificity of [^{3}H]52770 RP binding was studied by determining whether a range of concentrations (generally 1 nM–10 μM) of various pharmacological agents could displace [^{3}H]52770 RP (4 nM) binding in rabbit platelets. The following compounds were used: adenosine diphosphoric acid (ADP, 1 μM), arachidonic acid, aspirin, atropine, 1-benzylimidazole, chloroquine, clonidine, collagen (1 mg/ml), dazoxiben, diazepam, dopamine, fibrinogen (3 mg/ml), imipramine (1 μM), ketoprofen, morphine, nordihydroguaiaretic acid, noradrenaline, phentolamine, PK 11195 (52028 RP), prazosin, propranolol, 5-hydroxytryptamine, sulfinpyrazone, U-46,619 and yohimbine.

The potency of 36 derivatives of 52770 RP, BN 52021, brotizolam, L-652,731 and triazolam were studied as displacers of [^{3}H]52770 RP and [^{3}H]PAF. These experiments were carried out by using a range of concentrations (10 pM–0.1 mM) which were incubated at equilibrium with either 0.5 nM of [^{3}H]PAF or 4 nM [^{3}H]52770 RP in a suspension containing 5×10^7 platelets. Similar studies were performed with a few PAF derivatives such as enantio-PAF, lyso-PAF, 2-*O*-methyl lyso-PAF and 2-*O*-benzyl lyso-PAF.

Determination of the antagonistic properties of 52770 RP and L-652,731 against PAF-evoked hypotension in anesthetized rats and PAF-induced aggregation in washed rabbit platelets. Male Sprague–Dawley rats (Charles River Laboratory, Saint Aubin-lés-Elbeuf, France) weighing 200–240 g were anesthetized with sodium pentobarbital (60 mg/kg i.p.). The carotid artery was cannulated for the measurement of blood pressure with a transducer (Statham P34 Id) connected to an appropriate amplifier. The pressure signals were recorded on a polygraph (Graphtec UR 3101). Intravenous infusions were given into cannulated femoral veins. PAF (0.5 μg/kg/min i.v.) was infused for 20 min. Ten min after initiating this administration, a concomitant 5 min i.v. infusion of either solvent (0.25 ml/kg/min), 52770 RP (0.2 or 0.4 mg/kg/min) or L-652,731 (0.4 or 0.8 mg/kg/min) was undertaken. The solvent was saline alone for 52770 RP and 1.25% dimethylformamide (v/v) in saline for L-652,731.

Washed rabbit platelets were prepared as

described above. The assay buffer in which platelets were suspended was of the following composition (mM): NaCl 140, KCl 2.7, $CaCl_2$ 1.0, $MgCl_2$ 2.2, NaH_2PO_4 0.5, $NaHCO_3$ 12, Tris–HCl 10, dextrose 6.2, BSA 0.25% and apyrase 0.2 mg/ml. Platelet aggregation was carried out in an Icare aggregometer (Marseille, France) using a previously described method [12]. Concentration–response curves were constructed for PAF (0.4–76.0 nM) in the absence and presence of several concentrations (17 nM–12 μM) of 52770 RP, L-652,731 and triazolam.

Analysis of data. Results are reported as means ± SEM unless otherwise indicated. Several experimental data were plotted according to Scatchard and fitted with a non-weighed, iterative, least-squares non-linear regression program [13] run on an IBM PC. IC_{50} values were derived from equilibrium binding studies by using original data plotted according to log $(B_o–B)/B$ against log concentration of competitor, where B_o represents the concentration of ligand specifically bound in the absence of the competitor and B is the concentration of ligand specifically bound in the presence of antagonists. The results of Fig. 6 were fitted with a least-square linear regression.

For the PAF-induced aggregation studies, the pA_2 values, which measure the potency of the antagonists, were calculated according to Arunlakshana and Schild [14].

Drugs. Drugs used were: ADP (Diagnostica Stago, Asnières, France), arachidonic acid from porcine liver (Sigma Chemical Co., St Louis, MO), acetylsalicylic acid (RP Santé, Vitry-sur-Seine, France), atropine (Prolabo, Paris, France), 1-benzylimidazole (Aldrich, Strasbourg, France), BN 52021 (IHB, Le Plessis, France), brotizolam (Boehringer Ingelheim, Ingelheim am Rhein, F.R.G.), BW 755C HCl (RP Santé), chloroquine sulfate (RP Santé), clonidine HCl (RP Santé), type I Collagen from bovine Achilles tendon (Sigma), dazoxiben HCl (UK 37,248-01, Pfizer, Sandwich, U.K.), diazepam (Hoffman-La Roche Ltd., Basel, Switzerland), dopamine HCl (RP Santé), fibrinogen (fraction I rabbit plasma) (Sigma), imipramine HCl (Ciba-Geigy Ltd., Basel, Switzerland), ketoprofen (RP Santé), L-652,731 (RP Santé), morphine HCl (Ets. Darasse, Paris, France), noradrenaline bitartrate (Sigma), nordihydroguaiaretic acid (RP Santé) PAF (1-*O*-octadecyl-2-*O*-acetyl-*sn*-glycero-3-phosphorylcholine) (Bachem, Torrance, CA), [^{3}H]PAF : 80 Ci/mmol (Amersham, Bucks, U.K.), enantio-PAF (3-*O*-octadecyl-2-*O*-acetyl-sn-glycero-1-phosphorylcholine) (Bachem), lyso-PAF (1-*O*-octadecyl-sn-glycero-3-phosphorylcholine) (Bachem), phentolamine methanesulfonate (Ciba-Geigy), 52028 RP (RP Santé), prazosin HCl (Pfizer), propranolol HCl (ICI, Paris, France), 5-hydroxytryptamine créatinine sulfate (RP Santé), sulfinpyrazone (Ciba-Geigy), triazolam (Upjohn, Kalamazoo, MI), U-46,619 (Upjohn), yohimbine (Ets Darasse, Paris). All the concentrations mentioned in the text refer to the bases of the compounds.

Chemical preparation of [^{3}H]52770 *RP.* The *N*-(2-^{3}H-3-chlorophenyl)-3-(3-pyridinyl)-1H,3H-pyrrolo[1,2-*c*]thiazole-7-carboxamide, [^{3}H]52770 RP, was prepared by catalytic tritium exchange of iodine in position 2 of the chlorophenyl ring. The obtained compound was purified by chromatography on partisil 20-ODS-3 by using a methanol/water mixture (65/35). The purified product had a specific activity of 28 Ci/mmole (1.036 Tbq/mole) and a radiochemical purity of 99%. The integrity of the ligand during the experimental procedure was assessed by a high-pressure liquid chromatography for which the solvent system was 50% acetonitrile, 50% water at a flow of 1.5 ml/min on a ZORBAX ODS Column (7 μM). Under these experimental conditions, the retention time of 52770 RP was 11.8 min.

Chemical separation of (+)- and (−)-52770 RP. The enantiomers of 52770 RP were prepared by formation of diastereoisomeric salts from racemic 3-(3-pyridinyl)-1H,3H-pyrrolo[1,2-c]-thiazole-7-carboxylic acid and either *d*-α-methylbenzylamine or *l*-α-methylbenzylamine. The resolved salts were then converted to free enantiomeric acids, which, after transformation to acid chlorides, were condensed with 3-chloroaniline to give the (+)-52770 RP ($[\alpha]_D^{20} = +97° \pm 1.6°$ in 1.1% w/v solution in dimethylformamide; m.p. = 164°) and the (−)-52770 RP ($[\alpha]_D^{20} = -97° \pm 1.5°$ in 1% w/v solution in dimethylformamide; mp. = 164°).

RESULTS

Binding of [^{3}H]52770 *RP to washed rabbit platelets*

Total, specific and non-specific bindings of [^{3}H]52770 RP (4 nM) were found to increase linearly over platelet concentrations ranging from 7×10^7 to 4×10^8/ml. Specific binding of [^{3}H]52770 RP (4 nM) represented 86 ± 3% (N = 7) of the total binding to intact platelets (10^8/ml) and was less than 2% of the total radioactivity added to the incubating medium. After a 30 min equilibration period, 98% of the recovered radioactivity was determined by HPLC analysis to be unchanged [^{3}H]52770 RP.

Kinetic characteristics of [^{3}H]52770 *RP binding.* The association of [^{3}H]52770 RP to rabbit platelets was rapid inasmuch as equilibrium was reached within the initial 15 min of the incubation time at 20° (Fig. 2).

The specifically bound [^{3}H]52770 RP was totally displaced by 10 μM of 52629 RP (a potent PAF antagonist of the same chemical family as the ligand) in less than 30 sec, indicating that the binding of [^{3}H]52770 RP was reversible. However, the extreme rapidity of this phenomenon did not allow the determination of a sufficient number of experimental data

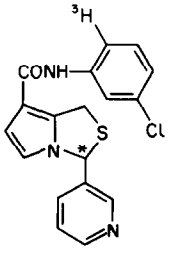


Fig. 1. Chemical structure of [^{3}H]52770 RP. The asterisk indicates the position of the asymetric carbon.

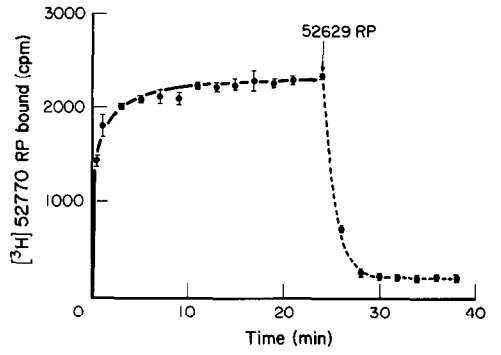


Fig. 2. Time-course of [^{3}H]52770 RP binding to intact platelets. Platelets (5×10^7) were incubated at 20° for various periods of time with 4 nM [^{3}H]52770 RP. An arrow indicates the time at which 52629 RP (10 μM) was added to initiate the dissociation process. Each point is the mean ± SE calculated from 3 experimental determinations.

points to perform a kinetic analysis for the calculation of the dissociation rate constant.

Equilibrium binding studies. Studies of saturation binding of [^{3}H]52770 RP (0.5–30 nM) to washed rabbit platelets revealed that the non-specific binding, as assessed by using 10 μM of 52629 RP, increased linearly with concentrations of [^{3}H]52770 RP. In contrast, the specific [^{3}H]52770 RP binding exhibited full saturability (Fig. 3). In a separate series of experiments performed in triplicate on different preparations of platelets (N = 5), the apparent equilibrium dissociation constant (K_d) and the maximum binding capacity of [^{3}H]52770 RP were 8.5 ± 2.9 nM and 0.201 ± 0.050 pmol/5 × 10^7 platelets, respectively. This yielded approximately 2420 binding sites/platelet. The Scatchard analysis of data (Fig. 3 inset)

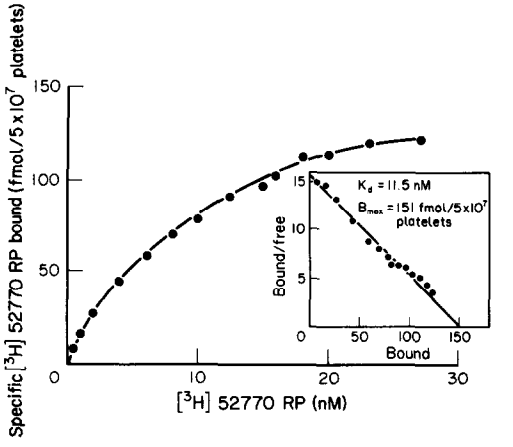


Fig. 3. Saturability of the [^{3}H]52770 RP binding to washed rabbit platelets. Platelets (5×10^7) were incubated in the presence of increasing concentrations of [^{3}H]52770 RP (0.5–30 nM) for 30 min at 20°. This experiment was repeated 5 times and the reported curve was drawn from results obtained with a single representative determination. Non-specific binding was measured in the presence of 52629 RP (10 μM). Inset: Scatchard analysis of the specific binding of [^{3}H]52770 RP calculated from saturation isotherms. Data are from a representative experiment.

revealed a linear plot consistent with one binding site (Hill coefficient: nH = 0.96 ± 0.14). Since this analysis may lead to wrong conclusions when a narrow range of ligand concentrations is used [15], the results represented in Fig. 3 were fitted with a non-linear iterative, least-squares procedure (Marquard algorithm) which yielded the following binding parameters: K_d = 11.5 nM; B_{max} = 0.151 pmol/5 × 10^7 platelets and nH = 0.92. Thus, in accord with the Clark equation [16], the equilibrium binding experiments confirmed the previous conclusion that [^{3}H]52770 RP interacted with a single population of binding sites.

Displacement of [^{3}H]*PAF and* [^{3}H]52770 *RP binding by PAF, 52770 RP enantiomers and* L-652,731. PAF displaced the bound [^{3}H]52770 RP from intact rabbit platelets. The concentration required to inhibit the specific binding by 50% (IC_{50}) was 4.10 ± 1.05 nM (K_i = 2.7 ± 0.3 nM) (N = 4) (Fig. 4. and Table 1). It should be noted that a small part (13 ± 2%, N = 4) of [^{3}H]52770 RP specific binding was not displaced even by 10 μM PAF. Thus, this non-displaceable portion of binding can lead to a small overestimation (314 sites/platelet) of the B_{max} value. For this reason, the use of 1 μM PAF as displacer should be preferred to that of unlabelled 52770 RP or one of its chemical derivatives in order to measure more appropriately non-displaceable binding (blank value). Lyso- and enantio-PAF (100 μM), two close derivatives of PAF devoid of significant biological activity via PAF receptors, inhibited the specific [^{3}H]52770 RP binding by only 21 ± 4% and 15 ± 3% (N = 3), respectively. Similarly, the 2-*O*-methyl lyso-PAF and 2-*O*-benzyl lyso-PAF (Table 1) were poor displacers of either [^{3}H]PAF and [^{3}H]52770 RP. To assess whether [^{3}H]52770 RP binding exhibited stereospecificity in rabbit platelets, unlabelled racemic 52770 RP and its (+)- and (−)-enantiomers were compared and found to produce concentration-dependent inhibitions of the specific binding of [^{3}H]52770 RP with IC_{50} values of 14.9 ± 2.3 nM, 10 ± 2.8 nM and 2000 ± 456 nM (N = 3), respectively (Fig. 4). Their respective apparent inhibition constants (as determined by the formula of Cheng and Prussof) were 11.2, 7.5 and 1500 nM. Thus, the (+)-enantiomer is 200–350 times more potent than the (−)-enantiomer in displacing the [^{3}H]52770 RP specifically bound to the rabbit platelets. Furthermore, the K_i value of unlabelled 52770 RP was not significantly different from the K_d value obtained in saturation studies.

A striking parallelism appeared to exist between the [^{3}H]52770 RP and [^{3}H]PAF bindings (Fig. 4) since the K_i against the latter ligand for (±), (+) and (−)-52770 RP were 7, 5 and 3360 nM, respectively. Furthermore, L-652,731 [6], in concentrations ranging from 0.1 nM to 0.1 mM, inhibited the binding of [^{3}H]52770 RP with a K_i of 36 ± 6 nM (N = 3), a value which is of similar magnitude as that (24 ± 3.2 nM, N = 3) obtained against [^{3}H]PAF binding.

The specificity of [^{3}H]52770 RP was also assessed. The ligand bound to washed rabbit platelets was not significantly displaced by a 10 μM concentration of a variety of pharmacological agents such as noradrenaline, clonidine, prazosin, propranolol, yohimbine, phentolamine, atropine, morphine, 5-

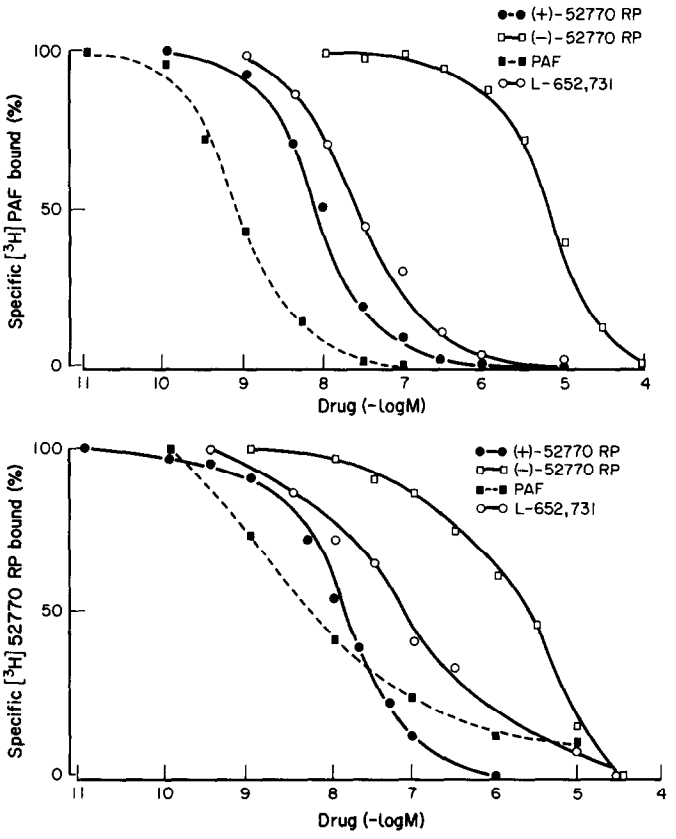


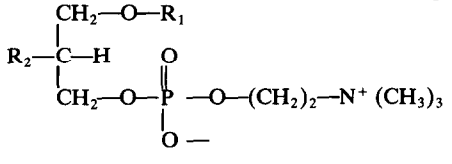
Fig. 4. Displacement of specific [^{3}H]PAF (0.5 nM) (upper panel) and [^{3}H]52770 RP (4 nM) binding (lower panel) by PAF, (+)-52770 RP, (−)-52770 RP and L-652,731 in washed rabbit platelets incubated at 20°. Each point is the average of results from at least 3 independent experimental determinations performed in triplicate.

Table 1. Comparison of potency (expressed as K_i) of some PAF-acether analogues to displace [^{3}H]PAF and [^{3}H]52770 RP specific binding in washed rabbit platelets

Compound		R_1	R_2	[^{3}H]PAF K_i (nM)	[^{3}H]52770 RP K_i (nM)
52770 RP				11.2	7
PAF	sn	$C_{18}H_{37}$	O—CO—CH_3	0.89	2.7
Lyso-PAF	sn	$C_{18}H_{37}$	H	> 10 000	> 10 000
Enantio-PAF	sn	$C_{16}H_{33}$	O—CO—CH_3	> 10 000	> 10 000
2-*O*-Methyl Lyso PAF	rac	$C_{18}H_{37}$	O—CH_3	235	765
2-*O*-Benzyl Lyso-PAF	rac	$C_{18}H_{37}$	O—CH_2—C_6H_5	10 720	35 370

The inhibitory effects of 52770 RP, unlabelled PAF and various analogues against specific [^{3}H]PAF and [^{3}H]52770 RP binding was determined in washed rabbit platelets. Inhibition constants were obtained using the Cheng–Prussof formula. The reported results are means of 3–5 independent experiments performed in triplicate. The K_i values were calculated by using original data plotted according to log $(B_o - B)/B$ vs log concentration of the competitor.

The abbreviations sn and rac mean stereospecific numerotation and racemic compound, respectively.



hydroxytryptamine, diazepam, 52028 RP, dopamine, chloroquine, aspirin, ketoprofen, nordihydroguaiaretic acid, 1-benzylimidazole, sulfinpyrazone, U-46,619, dazoxiben, arachidonic acid, ADP (1 μM), imipramine (1 μM), collagen (1 mg/ml) and fibrinogen (3 mg/ml).

Determination of the nature of the antagonism of [^{3}H]52770 *RP against PAF.* Specific binding of [^{3}H]52770 RP (0.5–30 nM) to rabbit platelets was studied in the absence and presence of three increasing concentrations of PAF (1, 10 and 100 nM). Scatchard analysis of the binding results indicated that PAF increased the K_d value of [^{3}H]52770 RP binding, whilst having no effect on the maximum number of binding sites. The Lineweaver–Burk plot analysis (Fig. 5) demonstrated that PAF inhibition was of a competitive nature. The K_m values (determined from the least-square linear regression analysis) for [^{3}H]52770 RP in the absence or presence of 1, 10 or 100 nM PAF were 8.3, 10.7, 18.8 and 32.2, respectively. These values did not significantly differ from the K_ds obtained by Scatchard analysis.

Determination of the inhibiting potencies of several compounds against [^{3}H]52770 *RP and* [^{3}H]*PAF binding in washed rabbit platelets.* The existence of a possible correlation between the recognition site for [^{3}H]52770 RP and the PAF receptor site was demonstrated by studying the effects of 36 derivatives of 52770 RP (unpublished results), BN 52021 [4], brotizolam [17], L-652,731 [6] and triazolam [8]. BN 52021 and brotizolam antagonized in a competitive manner the binding of [^{3}H]PAF with K_i values of 3353 ± 97 and 22.3 ± 2.3 nM (N = 3), respectively. The negative logarithms of K_is against [^{3}H]PAF were highly correlated ($r = 0.96$, N = 41, $P < 0.001$) to the corresponding K_is against [^{3}H]52770 RP (Fig. 6). On the basis of their K_i values, 52770 RP is approximately 4 and 300 times more potent than L-652,731 and triazolam, respectively, as an antagonist of [^{3}H]PAF and [^{3}H]52770 RP.

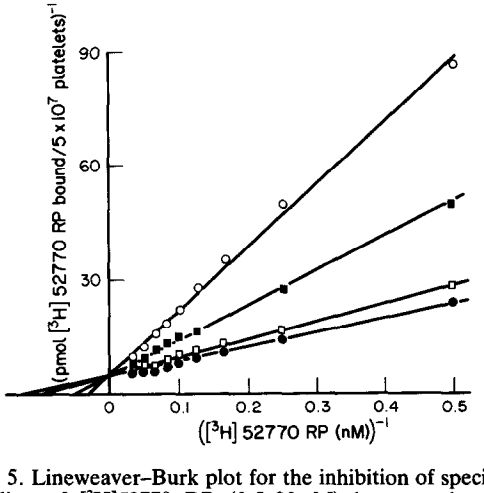


Fig. 5. Lineweaver–Burk plot for the inhibition of specific binding of [^{3}H]52770 RP (0.5–30 nM) by several concentrations of PAF (□, 1 nM; ■, 10 nM; ○, 100 nM; ●, control) in intact rabbit platelets. Non-specific binding was determined using 10 μM of 52629 RP. The fitting of the results with a least-squares linear regression analysis allowed the calculation of the following $1/K_d$ values: 0.093 (□), 0.053 (■), 0.031 (○) and 0.12 (●).

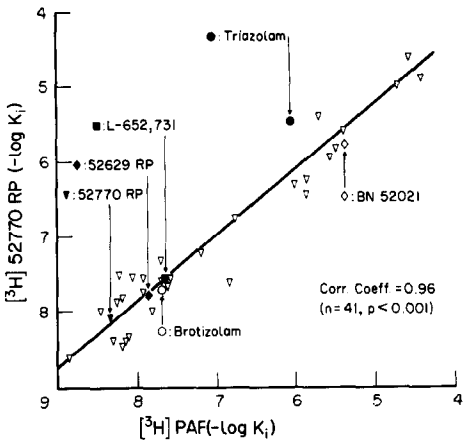


Fig. 6. Correlation between the antagonistic potencies of 36 structural analogues of 52770 RP (▽), L-652,731 (■), triazolam (●), BN 52021 (◇) and brotizolam (○), to displace the binding of 0.5 nM [^{3}H]PAF and to inhibit specific binding of [^{3}H]52770 RP in rabbit platelets. Drugs were tested in duplicate at 6–9 concentrations. The K_i values were calculated from the Cheng and Prusoff equation whereas IC_{50} values were determined from an indirect Hill plot. The reported points were fitted with a least-squares linear regression ($r = 0.96$, N = 41, $P < 0.001$).

Binding of [^{3}H]52770 *RP to rabbit platelet plasma membranes*

To assess whether part of the measured binding of [^{3}H]52770 RP to intact platelets was due to an intracellular uptake process, [^{3}H]52770 RP binding was determined on rabbit platelet plasma membranes. The non-specific binding was defined as the amount of binding that was not inhibited by 10 μM of 52629 RP.

Kinetic characteristics of [^{3}H]52770 *RP binding.* There was a good linearity between the extent of specific binding and the platelet plasma membrane protein content over a wide concentration range (0–200 μg/ml) of membrane proteins (data not shown). The association of [^{3}H]52770 RP to rabbit platelet plasma membranes at + 4° was relatively rapid, reaching equilibrium levels within 20–30 min and remaining unchanged for the subsequent 60 min. This indicates that neither a loss of receptors nor a degradation of ligand occurred within the incubation time. Transformation of these data according to the method of Frost and Pearson [19] provided an estimated association velocity constant (K_{+1}) of 0.0107 nM^{-1} min^{-1} for [^{3}H]52770 RP. This binding was completely reversible by the addition of 10 μM 52629 RP, a close derivative of 52770 RP. The rate constant for the dissociation (K_{-1}) was 0.067 min^{-1}. Thus, the kinetically determined dissociation constant ($K_d = K_{-1}/K_{+1}$) at the equilibrium is 6.26 nM.

Equilibrium binding studies. At 20°, the specific binding of 1 nM [^{3}H]52770 RP to rabbit platelet membranes reached equilibrium in less than 10 min. In steady-state conditions, [^{3}H]52770 RP at 20° bound to platelet membranes in a saturable manner (Fig. 7). Scatchard analysis of the saturation isotherms (mean of 5 independent determinations) provided a K_d value of 7.61 ± 0.79 nM and a B_{max} value

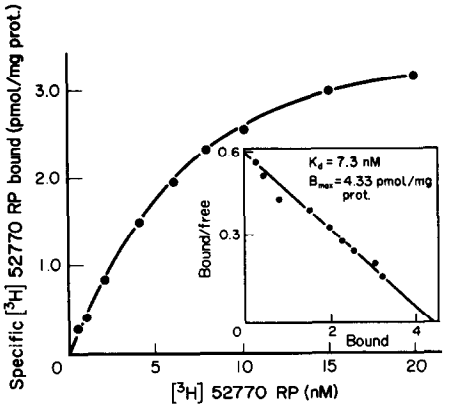


Fig. 7. Saturability of [^{3}H]52770 RP binding in rabbit platelet membranes. Membranes (50 μg/ml) were incubated in the presence of increasing concentrations of [^{3}H]52770 RP (0.5–20 nM) for 20 min at 20°. Experiments were repeated 7 times and the represented results are from one single determination. Non-specific binding (in the presence of 10 μM 52629 RP) was always less than 3% of total binding. Inset: Scatchard analysis of the specific binding of [^{3}H]52770 RP performed from saturation isotherms. The reported affinity constant (K_d) and the maximum number of binding sites (B_{max}) were calculated from this representative experiment.

of 3.66 ± 0.62 pmol/mg protein (Fig. 7 inset). Since there were 17.9×10^8 platelets/mg of protein, this B_{max} corresponds to 0.098 pmol/5×10^7 platelets, which is 2-fold smaller than the B_{max} from intact platelets. The calculated Hill coefficient was 0.91 ± 0.08, suggesting that in crude platelet membranes, as already shown in intact platelets, [^{3}H]52770 RP binds to a single population of sites at least for the concentrations used (0.5–20 nM).

Displacement of [^{3}H]52770 *RP binding with PAF,* 52770 *RP enantiomers and L-*652,731. As shown in Fig. 8, unlabelled racemic 52770 RP competitively inhibited the specific binding of [^{3}H]52770 RP to rabbit platelet membranes with a K_i value of 8.5 ± 1.7 nM (N = 4). This agrees with the K_d obtained from the saturation experiments (7.61 ± 0.79 nM) (N = 5). (+)-52770 RP was found to be one of the most potent displacers of [^{3}H]52770 RP binding studied, being 150 to 200-fold more potent than the (−)-enantiomer. Their respective K_i values were 4.1 ± 0.8 (N = 5) and 589 ± 125 nM (N = 5). L-652,731, a PAF antagonist, displaced the binding of [^{3}H]52770 RP with a K_i of 42.5 ± 8 nM (N = 3) in platelet membranes. This value was close to that determined in whole platelets. In contrast, the displacing potency of PAF was approximately 4-fold smaller in rabbit platelet membrane preparation (K_i = 10.4 ± 2.7 nM, N = 3) than in intact platelets (K_i = 2.07 ± 0.89 nM, N = 3).

Functional studies on the PAF receptor blocking properties of 52770 *RP and* L-652,731

Effects of 52770 *RP and* L-652,731 *on PAF-induced hypotension in pentobarbital anesthetized rats.* In this preparation a 20 min i.v. infusion of PAF (0.5 μg/kg/min) produced a sustained fall in mean carotid artery blood pressure. A concomitant infusion of 52770 RP and L-652,731 started 10 min after the PAF administration antagonized in a dose-related manner the PAF-evoked hypotension, 52770 RP being approximately twice more potent than L-652,731 (Fig. 9).

Effects of 52770 *RP, L-*652,731 *and triazolam on PAF-induced aggregation in washed rabbit platelets.* Addition of PAF to a platelet (4×10^8/ml) suspension produced concentration–aggregation response curves. 52770 RP, L-652,731 and triazolam, added to the platelet preparation 10 min before the above aggregation with PAF, displaced the control curves rightwards without affecting the slopes and maxima. The calculated pA_2 values using the Schild plots were found to be 7.71 ± 0.05 (N = 5) for 52770 RP, 6.29 ± 0.07 (N = 5) for L-652,731 and 5.76 ± 0.08 (N = 5) for triazolam. Furthermore, the slopes of these plots were closed to unity (0.88 ± 0.05, 0.87 ± 0.03 and 1.01 ± 0.08 respectively), indicating that the studied antagonisms were probably of competitive nature. Thus, on the basis

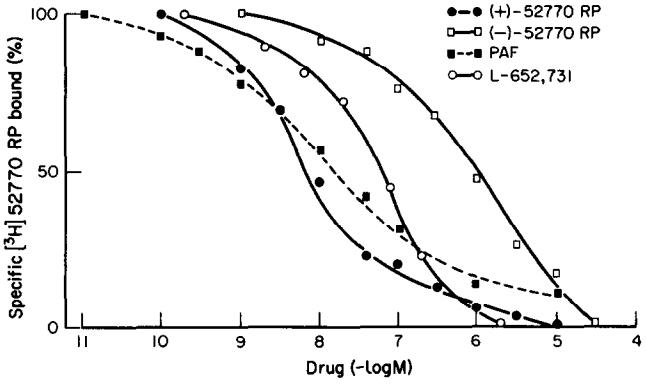


Fig. 8. Displacement of specific [^{3}H]52770 RP (4 nM) binding by PAF, (+)- and (−)-52770 R and L-652,731 in rabbit platelet membranes at 20°. Each point is the average of results from at least three independent experiments performed in triplicate.

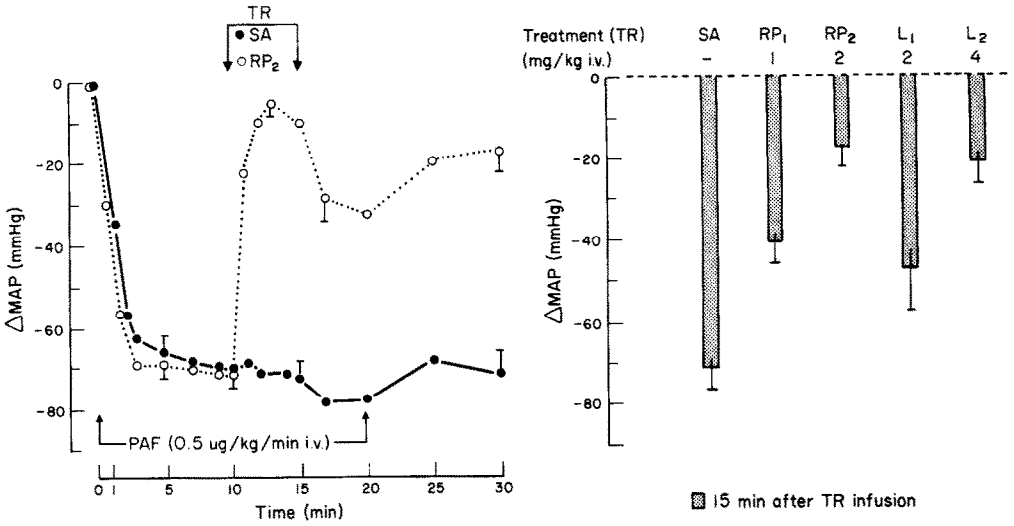


Fig. 9. Antagonism by 52770 RP and L-652,731 of PAF-evoked decrease in mean carotid artery blood pressure (ΔMAP) in pentobarbital anesthetized rats (N = 5–7/group). *Left panel*: time-course of the hypotensive effect of PAF and its antagonism by 52770 RP (2 mg/kg i.v. given over 5 min). Right panel: PAF hypotensive effects measured 15 min after the administration of saline (SA), 52770 RP (RP_1: 1 mg/kg; RP_2: 2 mg/kg) or L-652,731 (L_1: 2 mg/kg; L_2: 4 mg/kg). 52770 RP and L-652,731 inhibited significantly the effects of PAF.

of the pA_2 values, 52770 RP is approximately 26 and 89 times more potent than the L-652,731 and triazolam, respectively.

DISCUSSION

The present study shows that on intact rabbit platelets 52770 RP, a pyrrolo[1,2-*c*]thiazole derivative chemically related to 48740 RP [7], can displace [^{3}H]PAF (0.5 nM) from its high-affinity binding sites with a very low K_i (7 nM). This finding allows us to conclude that 52770 RP can be considered as one of the most potent synthetic antagonists of [^{3}H]PAF binding. In fact, the K_i for 52770 RP is, respectively, 3, 120, 240, 320, 400 and 500 times smaller than that of the PAF antagonists L-652,731, triazolam, BN 52021, brotizolam, 48740 RP [7] or SRI 63-072 [20]. Interestingly, our results on L-652,731 (K_i = 24 nM) confirm those already published (K_i = 9.8 nM) on the ability of this compound to displace [^{3}H]PAF binding [6]. Furthermore, whereas triazolam has been reported to antagonize the PAF-evoked human platelet aggregation [18], to our knowledge, its inhibition of [^{3}H]PAF binding is described herein for the first time. Finally, functional studies demonstrated that 52770 RP is 26 and 89 times more potent than L-652,731 and triazolam respectively, in antagonizing PAF-evoked aggregation in washed rabbit platelets. Its inhibitory activity against PAF-induced hypotension was twice as great as that of L-652,731. All these findings encouraged us to prepare [^{3}H]52770 RP as a potential, potent radioligand of [^{3}H]PAF binding sites. [^{3}H]52770 RP binding was characterized by high-affinity, specificity, saturability and reversibility in both intact rabbit platelets and crude platelet membrane preparations. Furthermore, this ligand was found to bind to a single class of recognition sites.

The similarity of the apparent dissociation constants (K_d), determined kinetically on platelet membrane preparations (6.2 nM) and from equilibrium binding experiments in either intact platelets (8.5 nM) or platelet membranes (7.6 nM) indicates that the affinity of [^{3}H]52770 RP is not modified by a platelet lysis procedure. Thus, it is reasonable to infer that the binding of this novel ligand occurs at membrane sites and is not dependent on mechanisms associated with intracellular structures. This conclusion is further strengthened by the failure of chloroquine, an inhibitor of lysosomal uptake [21], to affect the [^{3}H]52770 RP binding.

The maximum number of PAF-receptor sites determined by using [^{3}H]PAF in parallel matched studies carried out in our laboratory (K_d = 1.21 ± 0.44 nM; B_{max} = 0.194 ± 0.014 pmol/5 × 10^7 platelets corresponding to 2336 ± 163 sites/platelet, N = 10) was virtually identical to that of [^{3}H]52770 RP recognition sites (2420 ± 269/platelet). These findings in addition to the ability of reciprocal competitive displacement by PAF and 52770 RP in rabbit platelet preparations suggest that both agents interact with the same membrane site. This conclusion is further supported by compounds possessing PAF-receptor agonistic (Table 1) [22] or antagonistic (Fig. 6) properties exhibiting a rank order of potency to displace [^{3}H]PAF from its binding sites, which was not significantly different from that to antagonize [^{3}H]52770 RP binding. Enantio- and lyso-PAF which, in contrast to PAF, had poor platelet aggregating activity [22], failed to display affinity for the recognition sites of the used ligands.

The (+)-52770 RP was 700 and 300-fold more potent than (−)-52770 RP in displacing [^{3}H]PAF and [^{3}H]52770 RP binding, respectively. Thus, the studied binding site is characterized by a distinct stereospecific discrimination. Furthermore, there

was an excellent correlation between the K_i values of 36 closely related analogues of 52770 RP determined by using either [^{3}H]PAF or [^{3}H]52770 RP on washed rabbit platelets.

A variety of pharmacological agents including aggregating agents such as ADP, collagen, fibrinogen, noradrenaline and 5-hydroxytryptamine did not compete with [^{3}H]52770 RP, implying that the binding site for this ligand on rabbit platelets is not shared by compounds devoid of affinity for PAF-receptor.

In conclusion, our results are consistent with the proposal that the pharmacological requirements of the [^{3}H]52770 RP recognition sites are of the same nature as those of [^{3}H]PAF-receptor sites.

The recently described radioligand, [^{3}H]dihydrokadsurenone, was suggested to have an affinity for only a part of the PAF binding site [9]. Thus, it is possible that [^{3}H]52770 RP has an affinity for a site, within the PAF receptor, different from that of the latter PAF antagonist. This suggestion is supported by our unpublished results that the binding of [^{3}H]52770 RP was not modified, in contrast to [^{3}H]dihydrokadsurenone binding, by changes in the ionic composition (substitution of 150 mM NaCl with 10 mM $MgCl_2$) of the assay buffer [9].

Thus, [^{3}H]52770 RP, being a receptor antagonist chemically unrelated to the natural phospholipid PAF, might represent a new, useful and interesting radioligand to further our knowledge on the role of PAF binding sites in certain pathophysiological processes.

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