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Pharmacological profile of the novel P_{2T}-purinoceptor antagonist, FPL 67085 *in vitro* and in the anaesthetized rat *in vivo*

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1 The role of endogenous ADP in platelet aggregation *in vivo* remains unclear due to the lack of suitable P_{2T}-antagonist probes. This paper describes the potency, selectivity and specificity of the novel P_{2T}-purinoceptor antagonist, FPL 67085 (2-propylthio-D-β,γ-dichloromethylene ATP) both *in vitro* and in the anaesthetized rat *in vivo*.

2 FPL 67085 (3-30 nM) produced concentration-dependent rightward displacement of the concentration-effect (E/[A]) curve for ADP-induced aggregation of human washed platelets with no effect on ADP-independent aggregation at $\leqslant$ 10 μM.

3 Logistic fitting of ADP E/[A] data indicated that the antagonist effect of FPL 67085 did not consistently accord with simple competition: in some preparations depression of the asymptote was seen. Schild analysis of data combined from all preparations, regardless of the antagonist profile observed, gave an apparent pK_B of 8.9 (slope parameter 0.90).

4 The potency of FPL 67085 was unaffected by the P_1-purinoceptor antagonist, 8-sulphophenyltheophylline, was similar (IC_{50} 0.6-3.8 nM) in human and rat washed platelets or whole blood and, in rat blood, did not change following 2-30 min incubation at 37°C.

5 FPL 67085 was a weak ($pA_{50} \sim 4.2$) partial agonist in tissues containing P_{2X}- or P_{2Y}-purinoceptors, indicating some 30,000 fold selectivity for the P_{2T}-subtype.

6 In anaesthetized rats, intravenous infusion of FPL 67085 produced rapidly-reversible, dose-related inhibition of ADP-induced platelet aggregation measured *ex vivo* (ID_{50} 1.3 μg kg^{-1} min^{-1}) with no significant effect on haemodynamics or circulating cell counts.

7 Thus, FPL 67085 is a potent, specific and selective inhibitor of ADP-induced platelet aggregation both *in vitro* and *in vivo*. As such, it represents a novel pharmacological tool to define the role of endogenous ADP in thrombosis and the potential of P_{2T}-purinoceptor antagonists as a novel class of infusible anti-thrombotic agents for acute use in man.

Keywords: FPL 67085; P_{2T}-purinoceptor antagonist; ADP; platelet aggregation; human platelets; rat platelets; whole blood aggregation

Introduction

Platelet aggregation plays a pivotal role in normal haemostasis and in arterial thrombosis and is implicated in the pathogenesis of myocardial infarction, unstable angina and stroke and in the thrombotic complications of acute interventions such as thrombolysis and angioplasty (Fuster *et al.*, 1992). A role for adenosine diphosphate (ADP) in thrombosis and haemostasis was postulated over 20 years ago (Gaarder *et al.*, 1961). ADP-induced platelet aggregation can be demonstrated *in vitro* in platelet-rich plasma (PRP), suspensions of washed platelets and in whole blood (Born, 1962; Born & Cross, 1964; Cardinal & Flower, 1980) and *in vivo* upon intravenous (i.v.) administration of exogenous ADP to anaesthetized animals (Page *et al.*, 1982). However, the contribution of endogenous ADP to platelet aggregation *in vivo*, and hence to arterial thrombosis, has remained poorly defined. This has been due largely to the lack of potent and selective antagonists for P_{2T}-purinoceptors, the receptor which subserves the effect of ADP on platelets (Gordon, 1986). While adenosine triphosphate (ATP) is a competitive P_{2T}-purinoceptor antagonist (Macfarlane & Mills, 1975), it is, by definition, a non-selective P_2-purinoceptor ligand and is metabolically unstable. These properties limit the use of ATP in receptor classification studies *in vitro* and make it quite unsuitable as a probe for evaluating the contribution of endogenous ADP to platelet aggregation and thrombosis *in vivo*.

In a previous paper (Humphries *et al.*, 1994b), we described the pharmacology of the novel ATP analogue, FPL 66096, a potent (pK_B 8.7) P_{2T}-purinoceptor antagonist in preparations of human washed platelets with some 9000 fold selectivity for the P_{2T}-subtype compared to vascular P_{2X}- and P_{2Y}-purinoceptors *in vitro*. FPL 66096 is one of a series of novel compounds in which the anhydride link between the β and γ phosphates of ATP is replaced with a methylene link. This modification substantially reduces susceptibility of nucleotides to metabolism by ectonucleotidase enzymes (Welford *et al.*, 1986) and, therefore, makes possible investigation of the effect of selective P_{2T}-purinoceptor antagonism *in vivo* without the attendant complication of liberation of potential P_{2T}-purinoceptor agonists (the corresponding ADP-analogues). From the same series of compounds, FPL 67085 (2-propylthio-D-β,γ-dichloromethylene ATP) (Figure 1) was selected for evaluation *in vivo* and a preliminary report of our findings has been presented elsewhere (Humphries *et al.*, 1994a). In the present paper, we provide a more detailed account of the pharmacological profile of FPL 67085 both *in vitro* and *in vivo*.

Methods

Platelet aggregation in vitro

Blood sampling Blood was obtained from healthy male and female human volunteers by venepuncture or by cardiac puncture from male Sprague-Dawley rats (510-670 g, Charles

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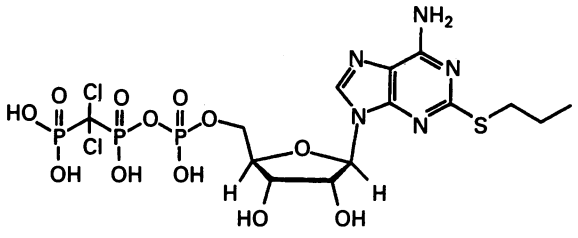

Figure 1 Chemical structure of FPL 67085.

River) anaesthetized with urethane (2 ml of 40% solution) administered intraperitoneally (i.p.). For use in washed platelet experiments, blood was anticoagulated with 1/10 volume, 3.2% trisodium citrate. For aggregation measurements in whole blood, heparin (10 u ml^{-1} final concentration) was used as anticoagulant.

Preparation of washed platelets Suspensions of human or rat washed platelets were prepared from citrated blood by differential centrifugation as described in detail by Humphries *et al.* (1994b), with prostacyclin (PGI_2, 300 ng ml^{-1}) used to stabilize platelets during the washing procedure. The final platelet pellet was resuspended initially in 10 ml (human) or 2 ml (rat) calcium-free Tyrode solution (CFT, composition (mM): NaCl 137.0, $NaHCO_3$ 11.9, NaH_2PO_4 0.4, KCl 2.7, $MgCl_2$ 1.1, D-glucose 5.6) gassed with 95% O_2/5% CO_2 and maintained at 37°C. Further CFT was added, as required, to adjust the final platelet count to 2×10^5 μl^{-1}, prior to storage at 4°C with air excluded.

To allow subsequent recovery from PGI_2-inhibition of normal function, platelets were used in aggregation studies no sooner than 2 h after final re-suspension.

Rabbit ear artery

The use of the rabbit isolated ear artery preparation for assessment of compound activity at P_{2X}-purinoceptors has been described in detail by O'Connor *et al.* (1990). Briefly, endothelium-denuded arterial rings (5 mm), obtained from male New-Zealand White rabbits (Froxfield, 2.5–3.5 kg), were mounted horizontally, under isometric conditions, on fine tungsten wire hooks in 20 ml organ baths containing modified Krebs buffer of the following composition: (mM) NaCl 117.6, NaH_2PO_4 0.9, $NaHCO_3$ 25.0, $MgSO_4$ 1.2, KCl 5.4, $CaCl_2$ 2.5, D-glucose 11.1 and indomethacin (2.8 μM). Tissues, maintained at 37°C and gassed continually with 95% O_2/5% CO_2, were subjected to an initial force of 1.0 g and allowed to equilibrate for 1 h, during which time the resting force was adjusted to achieve a final value of 0.5–1.0 g.

Guinea-pig aorta

The use of this preparation for assessment of P_{2Y}-agonist and antagonist activity has been outlined previously (Dainty *et al.*, 1992; Humphries *et al.*, 1994b). Endothelium-intact rings (3 mm) of the thoracic aorta, obtained from male Dunkin-Hartley guinea-pigs (Charles-River, 300–350 g), were mounted horizontally, under isometric conditions, on fine tungsten wire hooks in 10 ml organ baths, containing modified Krebs solution maintained under the conditions described above. Tissues were subjected to an initial force of 2 g and allowed to equilibrate for 15 min. After this time, resting force was adjusted to 2 g at 0, 3, 6 and 9 min and the bathing solution exchanged at these times. The tissues were then allowed to equilibrate for a further 15 min prior to use. As with ear artery preparations, responses were recorded as changes in force using isometric transducers (Ormed) and displayed on Advance Bryan chart recorders.

Urethane-anaesthetized rats

Male Sprague-Dawley rats (Charles River, 470–550 g) were anaesthetized with urethane (3 ml of 20% solution i.p.). Intravenous (i.v.) anaesthetic supplements (40% solution) were administered as required *via* a caudal vein cannula. Body temperature was maintained at 37 ± 0.5°C with a heating lamp. Animals were allowed to breathe spontaneously and a tracheotomy was performed to ensure a patent airway. A cannula (size: 3 French Gauge (FG)), containing heparinized saline (25 u ml^{-1}) was introduced into the left femoral artery and connected to a transducer (Gould/Statham) to record pulsatile blood pressure (BP). Care was taken to avoid systemic administration of heparin from this cannula. Mean BP and heart rate (HR) were derived electronically from the pulsatile signal and all 3 variables were recorded on a calibrated pen recorder (Devices/Lectromed). Cannulae containing non-heparinized saline were introduced into the left common carotid artery (3FG) and left jugular vein (2FG) for withdrawal of arterial blood samples and i.v. administration of compounds. Upon completion of surgery, a 30 min stabilization period was allowed before starting the experimental protocol. Animals with a BP < 50 mmHg at the end of this period were not used.

Experimental protocols

Human washed platelets Aliquots (440 μl) of platelet suspension were added to siliconized aggregation cuvettes containing 10 μl $CaCl_2$ solution (final concentration 1 mM), maintained at 37°C and stirred at 900 r.p.m. in a Biodata PAP4 aggregometer. Human fibrinogen (10 μl) was then added to give a final concentration of 0.2 mg ml^{-1} of clottable protein. Recording of aggregation was then started and baseline light transmission monitored for 20 min before addition of a single submaximal concentration of ADP (30 or 100 μM) in a volume of 10 μl. FPL 67085 (0.1–100 nM) or vehicle (saline, 30 μl) was added 15 min before ADP. The effect of the non-selective P_1-purinoceptor antagonist, 8-sulphophenyltheophylline (8-SPT, 300 μM, 20 min incubation) on the anti-aggregatory potency of FPL 67085 was also investigated. Inhibitory concentration-effect (E/[A]) curves for FPL 67085 were constructed in a time-matched manner in the absence and presence of 8-SPT, with each point obtained in duplicate. Aggregation responses were recorded as the maximum rate of increase (slope) in light transmission through the platelet suspension, following addition of ADP, using the PAP4 slope reader.

In a separate series of experiments, baseline light transmission was monitored for a shorter period (10 min) prior to addition of ADP (1–1000 μM) in a volume of 10 μl. FPL 67085 (3–30 nM) or 30 μl vehicle was added 2 min before ADP. Although only a single concentration of agonist and antagonist was added to each cuvette, use of 8-aggregometer channels enabled construction of 4, time-matched E/[A] curves for ADP-induced aggregation, with duplicate measurements for each point. Thus, in each experiment, a complete analysis of antagonism was conducted. In these experiments, aggregation responses were recorded as the maximum increase (extent) in light transmission.

P_{2T}-specificity of the anti-aggregatory effect of FPL 67085 (0.1–10 μM, 15 min incubation) was tested against responses to the thromboxane A_2-mimetic, U46619 (0.1–10 μM), rendered ADP-independent by inclusion of the non-selective P_2-purinoceptor antagonist, suramin (100 μM). E/[A] curves for U46619 were constructed as described for ADP after a 20 min monitoring of baseline light transmission. Suramin was added 1 min prior to FPL 67085 in a volume of 10 μl.

Rat washed platelets The anti-aggregatory potency of FPL 67085 (0.01–10 nM, 15 min incubation) was assessed against aggregation produced by a standard submaximal concentration of ADP (3 μM) added to aliquots of platelet suspension in micro-cuvettes. Baseline light transmission was monitored for

20 min before addition of ADP. To accommodate the use of microcuvettes, addition volumes (μl) were adjusted proportionately from those described for human washed platelets as follows: platelet suspension, 172; $CaCl_2$, 4; fibrinogen, 4; FPL 67085, 10.

Whole blood aggregation The anti-aggregatory potency of FPL 67085 was assessed against aggregation produced by a standard submaximal concentration of ADP (1 or 3 μM) in 0.5 ml samples of heparinized human or rat blood, diluted 1:1 with saline and incubated at 37°C for 2 min in a Chrono-log aggregometer. FPL 67085 (human: 0.03–10; rat: 0.3–30 nM) or vehicle (saline, 30 μl) was then added, the cuvette transferred to the impedance measuring well and the sample stirred at 900 r.p.m. for a further 2 min before addition of ADP in a volume of 20 μl. Aggregation responses were obtained in duplicate and recorded as the maximum increase in impedance (ohms) within 6 min of addition of ADP.

In a separate series of experiments, FPL 67085 was incubated with rat blood for periods of 2, 5, 10 or 30 min before 1:1 dilution of the blood sample with saline and measurement of ADP (3 μM)-induced platelet aggregation. The concentration of FPL 67085 added to undiluted blood (10–60 nM) was double that initially shown to inhibit aggregation by approximately 80% when added directly to diluted blood.

Rabbit ear artery Tissues were initially contracted with KCl (80 mM) to establish viability, followed by addition of acetylcholine (1 μM). Lack of a relaxant response to acetylcholine confirmed denudation of the endothelium. After washing, tissues were allowed to equilibrate for 45 min prior to construction of a cumulative E/[A] curve for the standard P_{2X}-agonist, D-α,β-methylene ATP (α,β-meATP, 0.03–10 μM). The tissues were then washed and, after a further 70 min equilibration period, a cumulative E/[A] curve was obtained for FPL 67085 (1–1000 μM). In control experiments, this protocol allows construction of consecutive E/[A] curves for α,β-meATP with no evidence of desensitization. Following subsequent washing, a further E/[A] curve to FPL 67085 (10–1000 μM) was obtained in the presence of α,β-meATP (30 μM) added 15 min prior to the first concentration of FPL 67085. All E/[A] curves were constructed in the presence of 8-SPT (300 μM), added 45 min before the agonist, to exclude the possible contribution of P_1-mediated effects.

Guinea-pig aorta Tissues were contracted to a stable plateau with phenylephrine (Phe, 10 μM) and the standard relaxant P_{2Y}-agonist, 2-methylthio ATP (2-MeSATP, 10 μM) added to confirm the presence of functional endothelium. After washing, tissues were again contracted with Phe (10 μM) and, when the induced tone had stabilized (at least 15 min), cumulative E/[A] curves were constructed to 2-MeSATP (1–3000 nM). After washing, and a 35 min stabilization period, tissues were again pre-contracted with Phe and an E/[A] curve constructed for FPL 67085 (0.003–10 mM), with each concentration left in contact with the tissue for at least 3 min or until any relaxant response had stabilized. To determine whether any observed relaxations were P_{2Y}-mediated, tissues were then washed and allowed to equilibrate for 35 min before pre-contraction with Phe and construction of a second curve to FPL 67085 in the presence of 2-MeSATP (10 μM). This concentration of 2-MeSATP abolishes, or produces at least a 2 orders of magnitude rightward shift and significant depression of, the 2-MeSATP E/[A] curve in this preparation. All E/[A] curves were constructed in the presence of 8-SPT (300 μM), added 35 min prior to Phe, to exclude the possible contribution of P_1-mediated effects.

Urethane-anaesthetized rats Each animal received 3 × 20 min infusions of either FPL 67085 (0.08, 0.8 and 8 μg kg^{-1} min^{-1} i.v. free acid, mol wt. 648) or vehicle (saline 0.03 ml min^{-1} i.v.). Blood samples were taken immediately prior to the first infusion, at the end of each infusion and 20 min after cessation of the final infusion, for measurement of platelet aggregation *ex vivo*. At each sampling point, 1 ml arterial blood was withdrawn into a 1 ml syringe containing 0.1 ml heparinized saline (100 u ml^{-1}). Mean BP and HR values were noted before each sample was taken. Additional samples were taken at the end of the control and final infusion periods for measurement of platelet, red blood cell (RBC) and white blood cell (WBC) counts (Technicon H1).

Immediately after sampling, ADP-induced platelet aggregation was measured in duplicate in 0.5 ml blood samples diluted 1:1 with saline and incubated at 37°C for 4 min. For the final minute of this period, cuvettes were transferred to the impedance measuring well of a Chrono-log aggregometer and the sample stirred at 900 r.p.m. ADP (3 μM) was then added in a volume of 20 μl and aggregation recorded as the maximum increase in impedance (ohms) within 6 min of addition of ADP.

Effects on ADP-induced platelet aggregation were calculated as % inhibition of the pre-infusion response. The dose of FPL 67085 producing 50% inhibition (ID_{50}) was derived by graphical interpolation.

Analysis of data

Curve fitting With the exception of data obtained for U46619 in human washed platelets, E/[A] curve data were fitted to a logistic function (i) of the form:

$$E = \frac{\alpha[A]^m}{[A_{50}]^m + [A]^m} \qquad \text{(i)}$$

in which α, $[A_{50}]$ and m are, respectively, the asymptote, location and slope parameters. $[A_{50}]$ values were assumed to be log-normally distributed and they were estimated as such and quoted as $p[A_{50}]$ ($-\log[A_{50}]$) values.

Analysis of antagonism E/[A] curve data obtained for ADP-induced platelet aggregation in the absence and presence of FPL 67085 were fitted to equation (i). Parallelism was tested by one-way analysis of variance prior to fitting of E/[A] data describing the 4 curves obtained in individual experiments to the following form of the Schild equation (Black *et al.*, 1985):

$$\log_{10}[A_{50}] = \log_{10}[A_{50}^c] + \log_{10}(1 + [B]^n/K_B) \qquad \text{(ii)}$$

where $[A_{50}^c]$ is a control $[A_{50}]$, [B] is the concentration of FPL 67085, K_B is its equilibrium dissociation constant and n is the Schild plot slope. Other parameters are as defined for equation (i). Thus, an estimate of n and K_B was obtained from each experiment.

E/[A] parameters for U46619-induced platelet aggregation were calculated from manual plots of the E/[A] data and the effect of FPL 67085 on these parameters was tested either by one-way ANOVA (α) or the non-parametric Kruskal-Wallis test ($p[A_{50}]$).

Anti-aggregatory potency Differences in pIC_{50} values obtained for FPL 67085 under different conditions (human:rat; whole blood:washed platelets; +8-SPT:-8-SPT; 2:5:10:30 min incubation) were tested by one-way ANOVA.

Urethane-anaesthetized rats Between group comparisons of control values for aggregation, BP, HR, platelet, RBC and WBC counts were made with Student's unpaired *t* test. The significance of treatment-related changes from pre-infusion values was determined by repeated measures ANOVA followed by Dunnett's multiple-comparison test where appropriate.

Data are presented as mean ± s.e. of results obtained from individual human or rat blood donors, individual tissues or from individual anaesthetized rats. All fitting procedures used either BMDP Statistical Software installed on a VAX mainframe computer, KaleidaGraph software on a Macintosh II cx computer or Fig. P software on a Compaq PC. Other statistical analyses used InStat or t-EASE software. $P < 0.05$ was taken as indicative of a statistically significant difference in all cases.

Drugs and solutions

Drugs were obtained from the following sources: U46619 (9,11-dideoxy-9α, 11α-methanoepoxy-$PGF_{2\alpha}$), indomethacin, acetylcholine bromide, phenylephrine hydrochloride, PGI_2, human fibrinogen, and the disodium salts of ADP, β,γ-meATP and α,β-meATP, Sigma Chemical Co., Poole, Dorset; 8-SPT and 2-MeSATP, Research Biochemicals Inc., St. Albans, Herts; urethane, Aldrich Chemical Co., Gillingham, Dorset; heparin sodium, Evans medical, Horsham, W. Sussex; suramin was a gift from Bayer plc, UK.

The tetrasodium salt of FPL 67085 was synthesized by N.D. Kindon, Department of Medicinal Chemistry, Fisons plc, R & D Laboratories, Loughborough, Leics., extending previously described methodology for the preparation of 2-alkylthio, β,γ-dihalomethylene analogues of ATP (Yoshikawa *et al.*, 1967; Blackburn *et al.*, 1984).

Indomethacin was dissolved in 10% w/v Na_2CO_3 at 10 mg ml^{-1} with subsequent dilutions in Krebs buffer. A working dilution of PGI_2 (0.1 mg ml^{-1}) was made in saline from a stock solution of 1 mg ml^{-1} in ethanol, no more than 1 min before use. 8-SPT was dissolved at the working concentration (5.6 mg ml^{-1}) in 6% glucose. All other drugs were dissolved in distilled water or saline.

Results

The effect of FPL 67085 on ADP-induced aggregation in human and rat washed platelets and whole blood in vitro

FPL 67085 produced concentration-dependent inhibition of ADP-induced aggregation of human and rat platelets *in vitro*. The anti-aggregatory potency of FPL 67085 (IC_{50} 0.6–3.8 nM) was similar for human and rat platelets, whether tested in suspensions of washed platelets or in whole blood and, in human washed platelets, was not affected by 8-SPT (300 μM) (Table 1).

The degree of inhibition of ADP (3 μM)-induced platelet aggregation produced by FPL 67085 (10–60 nM) did not change significantly ($P > 0.05$) when the antagonist incubation time in undiluted rat blood was increased from 2 to 30 min: 85±4 and 87±4% (mean±s.e., $n=4$), respectively.

Characterization of the anti-aggregatory effect of FPL 67085

ADP produced concentration-dependent aggregation of human washed platelets with a $p[A_{50}]$ value of 5.1±0.1 (s.e., $n=10$) and this E/[A] relationship was displaced to the right in a concentration-dependent manner by FPL 67085 (3–30 nM). In Figure 2a, curves obtained by unconstrained fitting of E/[A] data to the logistic function (i) are shown superimposed on mean (±s.e.) datum points obtained for ADP in the absence and presence of FPL 67085, from 10 replicate experiments. Statistical analysis of the E/[A] parameters (Table 2) obtained from unconstrained logistic fitting indicates that the rightward displacements produced by FPL 67085 did not differ significantly from parallelism as assessed by comparison of values of m. However, in some experiments, FPL 67085 caused an apparently concentration-dependent reduction in the maximal response, α. By the criterion of curve parallelism, therefore, the effect of FPL 67085 did not consistently accord with simple competitive antagonism. However, analysis of the rightward displacements of the E/[A] curves (using equation (ii)) indicated that the Schild plot slope criterion was met

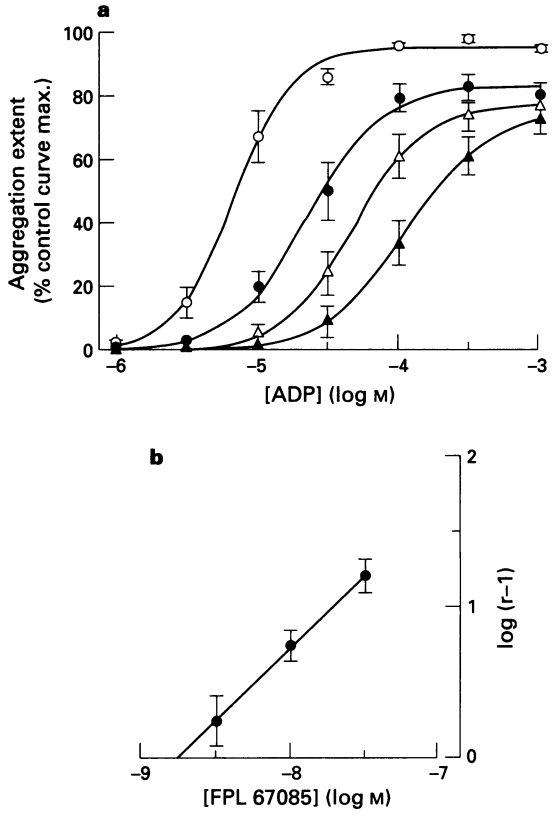

Figure 2 Analysis of antagonism by FPL 67085 of ADP-induced aggregation of human washed platelets. (a) Computer-generated lines obtained using the logistic function (i) (see text) are shown superimposed on mean datum points for E/[A] curves obtained for ADP following 2 min incubation with either FPL 67085 (● 3, △ 10 or ▲ 30 nM) or vehicle (○). Results are presented as mean responses (±s.e., $n=10$), expressed as a % of the maximum response to ADP in the control curve. (b) Corresponding Schild plot of the effect of FPL 67085 on E/[A] curves for ADP. Points show mean concentration-ratio (r) data plotted as (r-1) values (±s.e.) for 10 replicate experiments.

Table 1 The effect of FPL 67085 on ADP-induced platelet aggregation in suspensions of human and rat washed platelets and in whole blood *in vitro*

	Washed platelets		*Whole blood*
Species	*−8-SPT*	*+8-SPT*	*−8-SPT*
Human	9.05±0.12	8.60±0.09	8.89±0.22
Rat	9.21±0.11	not tested	8.42±0.24

Results are presented as mean±s.e. pIC_{50} values ($n=4-24$) for inhibition of platelet aggregation induced by a sub-maximal concentration of ADP.

Table 2 E/[A] curve parameters for ADP-induced aggregation of human washed platelets in the absence and presence of FPL 67085

[FPL 67085] (nM)	*Parameter* $p[A_{50}]$	α(%)	m
0	5.16±0.08	97±1	2.73±0.44
3	4.67±0.08**	85±4*	2.75±0.39
10	4.33±0.09**	79±5**	2.93±0.93
30	3.93±0.1**	77±5**	1.93±0.18

Values are means (±s.e.) of parameter estimates made by fitting the logistic function (i) (see text) to individual E/[A] data-sets obtained from 10 replicate experiments. *$P<0.05$ and **$P<0.01$ compared to parameter values from control E/[A] curve: ANOVA (m) followed by Bonferroni's multiple comparison test ($p[A_{50}]$); Kruskal-Wallis non-parametric ANOVA followed by Dunn's multiple comparisons test (α).

(0.90±0.06). An 'apparent pK_B' estimate (8.9±0.1, mean±s.e.) was, therefore, made from analysis of data from 10 replicate experiments. A Schild plot derived from this analysis is presented for display purposes in Figure 2b and E/[A] curves from 2 experiments representing the extremes of the effect of FPL 67085 contributing to the analysis are shown in Figure 3.

Specificity and selectivity in vitro

In human washed platelets, FPL 67085 (0.1–10 μM) had no significant effect on the position or asymptote of the E/[A] curve obtained for U46619 (0.1–10 μM) in the presence of suramin (100 μM) (Figure 4). In the standard P_{2X}-containing preparation of the rabbit isolated ear artery, FPL 67085 was an agonist with a mean p[A_{50}] of 4.2±0.2 (s.e., $n=5$). E/[A] curves did not achieve the α,β-meATP maximum (α=59±12%, mean±s.e., $n=5$) and were abolished in the presence of α,β-meATP (30 μM). In the standard P_{2Y}-containing preparation of the guinea-pig aorta, FPL 67085 was also a weak agonist (p[A_{50}]=4.2±0.1, mean±s.e., $n=4$) and E/[A] curves did not reach the same maximum as that produced by 2-MeSATP (α=73±2%, mean±s.e., $n=4$). Relaxant responses to FPL 67085 were only partially inhibited following occupation-desensitization with a high concentration of 2-MeSATP (10 μM).

The effects of i.v. infusion of FPL 67085 in urethane-anaesthetized rats

Platelet aggregation ex vivo Control aggregation responses to ADP (3 μM) in blood from saline- or FPL 67085-infused animals were not significantly different: 21.8±1.7 and 24.3±1.8 ohms, respectively (mean±s.e., $n=4$–6). The effect of i.v. infusion of FPL 67085 on aggregation measured *ex vivo* is presented graphically in Figure 5. FPL 67085 (0.08–8 μg kg^{-1} min^{-1}, i.v.) produced dose-related inhibition of ADP-induced platelet aggregation with a geometric mean ID_{50} of 1.3 μg kg^{-1} min^{-1} (range 0.7–3.6, $n=4$). Twenty minutes after cessation of infusion of the highest dose of FPL 67085, ADP-induced aggregation had returned fully to pre-infusion levels. Aggregation responses in the control, saline-infused ($n=6$), group did not change significantly throughout the experimental protocol ($P>0.05$).

Haemodynamics and cell counts BP, HR, platelet, RBC and WBC count measurements are summarized in Table 3. There was no significant difference ($P>0.05$) in baseline values between saline- and FPL 67085-infused animals. A total of 60 min infusion of FPL 67085 (20 min at each of 0.08, 0.8 and 8 μg kg^{-1} min^{-1}, i.v.) or saline (0.03 ml min^{-1}, i.v.) produced no significant change in BP, HR, platelet, RBC or WBC counts compared to corresponding pre-infusion control values ($P>0.05$).

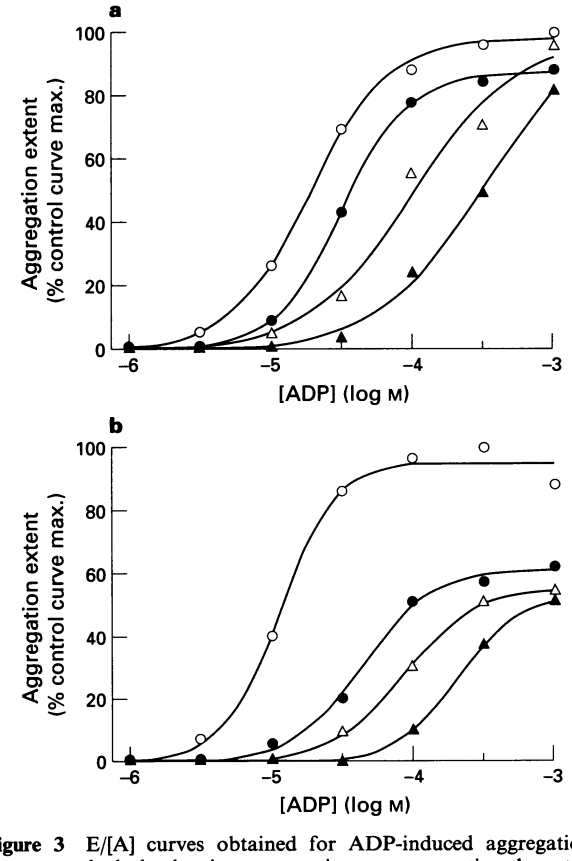

Figure 3 E/[A] curves obtained for ADP-induced aggregation of human washed platelets in two experiments representing the extremes of the effect of FPL 67085 on the ADP-asymptote: (a) no depression; (b) depression. Responses to ADP were obtained following 2 min incubation with either FPL 67085 (● 3, △ 10 or ▲ 30 nM) or vehicle (○) and are expressed as a % of the maximum response to ADP in the control curve.

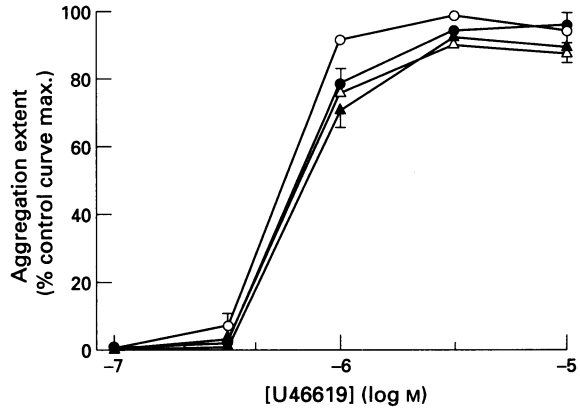

Figure 4 E/[A] curves obtained for U46619-induced aggregation of human washed platelets in the presence of suramin (100 μM). Results are presented as mean responses (±s.e., $n=4$), expressed as % of the maximum response to U46619 in the control curve. Responses were obtained following 15 min incubation with either FPL 67085 (● 0.1, △ 1 or ▲ 10 μM) or vehicle (○).

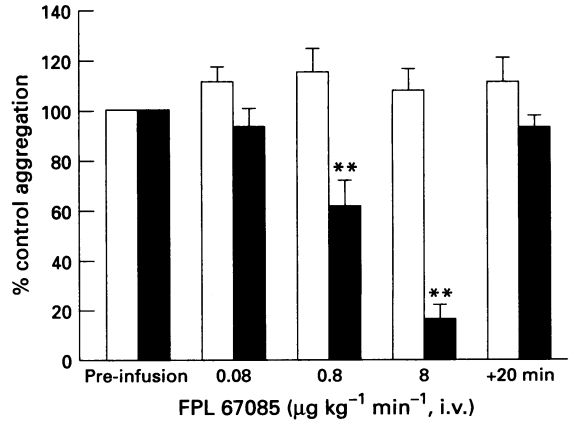

Figure 5 The effect of intravenous infusion of either FPL 67085 (0.08–8 μg kg^{-1} min^{-1}, solid columns, $n=4$) or vehicle (saline, 0.03 ml min^{-1}; open columns, $n=6$) on ADP (3 μM)-induced platelet aggregation (mean±s.e.) measured *ex vivo* in whole blood from urethane-anaesthetized rats. Blood samples were taken immediately prior to the first infusion, at the end of each infusion and 20 min after cessation of the final infusion. **$P<0.01$ compared to pre-infusion control (Dunnett's multiple-comparison test).

Table 3 The effect of intravenous infusion of FPL 67085 on blood pressure, heart rate and circulating cell counts in urethane-anaesthetized rats

	Saline		*FPL 67085*	
Variable	*Control*	*+60 min*	*Control*	*+60 min*
BP	73±4	62±5	84±10	76±5
HR	331±17	317±19	345±17	325±9
PLT	475±42	382±69	413±19	451±60
WBC	15.7±1.2	15.6±1.4	16.8±1.8	15.3±0.8
RBC	8.0±0.5	7.4±0.3	7.2±0.3	6.7±0.1

Abbreviations and units: BP: blood pressure (mmHg); HR: heart rate (beats min^{-1}); PLT: platelet count ($\times 10^3 \mu l^{-1}$); WBC: white blood cell count ($\times 10^3 \mu l^{-1}$); RBC: red blood cell count ($\times 10^6 \mu l^{-1}$). Values are means (±s.e.) before and after 3×20 min infusions of either FPL 67085 (0.08, 0.8 and 8 $\mu g kg^{-1} min^{-1}$, i.v. $n=4$) or vehicle (saline, 0.03 $ml min^{-1}$, $n=6$).

Discussion

In this study, we have demonstrated that FPL 67085 is a potent inhibitor of ADP-induced aggregation of human and rat platelets both in suspensions of washed platelets and in whole blood *in vitro*. Furthermore, we have shown that i.v. infusion of FPL 67085 results in potent and rapidly reversible inhibition of platelet aggregation *in vivo* in the anaesthetized rat.

We have shown previously that 2-propylthio-substitution on a β,γ-dihalomethylene analogue of ATP yields a compound (FPL 66096) with high affinity for the human platelet P_{2T}-purinoceptor *in vitro* (Humphries *et al.*, 1994b). In the present study, using FPL 67085, we have extended this observation to washed platelet preparations from a second species and also demonstrated that high anti-aggregatory potency is retained in whole blood from both human subjects and rats. In some experiments, antagonism of the ADP-induced aggregation of human washed platelets by FPL 67085 was non-surmountable and, under these circumstances, a pK_B should not be quoted. However, since the displacements of ADP E/[A] curves accorded with the Schild slope criterion, it may be reasonable to conclude that, whatever the curve depression was due to, it did not contribute to the rightward displacements of the E/[A] curves. Making this assumption, we estimated an 'apparent pK_B' of 8.9. It is not clear why, in some experiments in this study, the profile of FPL 67085 was fully consistent with simple competition while, in others, clear depression of the ADP asymptote was observed. Investigation of this variable behaviour of FPL 67085 is in progress. Whatever the explanation, this profile of antagonism adds further to the complexity of effects of 2-alkylthio-substituted analogues of ATP on ADP-induced platelet aggregation: FPL 66096 (2-propylthio-D-β,γ-difluoromethylene ATP) acts as an apparently competitive P_{2T}-purinoceptor antagonist (pK_B 8.7) in human washed platelets; 2-MeSATP, 2-ethylthio ATP and 2-methylthio-β,γ-methylene ATP are non-surmountable, low efficacy inhibitors of ADP-induced platelet aggregation in human PRP (Cusack & Hourani, 1982; Hourani *et al.*, 1986; Humphries *et al.*, 1994b). While differences in ionic environment and protein concentration may, in part, explain different results obtained in preparations of washed platelets and PRP, the reason for the somewhat different profiles of FPL 67085 and FPL 66096, tested under the same conditions, remains unclear. However, what is clear is the important affinity-conferring role played by the 2-propylthio substituent, a structural feature of both compounds, when compared to the other 2-alkylthio substituents mentioned above.

In common with other 2-alkylthio-substituted analogues of ATP, including FPL 66096, the anti-aggregatory effect of FPL 67085 was specific to P_{2T}-purinoceptors. Firstly, the possibility of direct or indirect agonist activity at platelet P_1-purinoceptors (A_{2a}) was excluded by use of the non-selective P_1-purinoceptor antagonist, 8-SPT. At a concentration previously shown (Humphries *et al.*, 1994b) to attenuate markedly the inhibition of aggregation produced by the P_1-purinoceptor agonist, 5'-N-ethylcarboxamidoadenosine in this system, 8-SPT had no effect on the anti-aggregatory potency of FPL 67085. Secondly, at a concentration 8,000 fold in excess of its apparent K_B, FPL 67085 had no effect on aggregation produced by the thromboxane A_2-mimetic, U46619, in the presence of suramin, a response shown previously to be ADP-independent (Humphries *et al.*, 1994b).

FPL 67085 was highly selective for the P_{2T}-purinoceptor subtype, showing no agonist or antagonist activity at P_{2X} or P_{2Y}-purinoceptors at a concentration 300 fold higher than its apparent K_B against ADP-induced aggregation of human washed platelets. Concentration-related contractions of the rabbit isolated ear artery produced by higher concentrations of FPL 67085 were prevented in the presence of a high concentration (30 μM) of α,β-meATP, implicating a P_{2X} mechanism. In preparations of the guinea-pig isolated aorta, pretreatment with 2-MeSATP did not inhibit relaxant responses to high concentrations of FPL 67085 to an extent consistent with the relaxations being wholly mediated by a P_{2Y} mechanism. It was, therefore, concluded that, in the guinea-pig aorta, relaxations produced by high concentrations of FPL 67085 involve both P_{2Y}- and an as yet undefined component, as observed previously with FPL 66096 (Humphries *et al.*, 1994b). The high affinity of FPL 67085 for the P_{2T}-purinoceptor (apparent pK_B 8.9) compared with its potency in P_{2X} and P_{2Y}-containing tissues ($p[A_{50}] \sim 4.2$), indicates that it has an approximately 4.5 orders of magnitude selectivity for the P_{2T}-purinoceptor.

In addition to experiments in human washed platelets, which would have detected activity at prostaglandin (TP, IP) or P_1 (A_{2a})-purinoceptors, the high degree of specificity of FPL 67085 has been confirmed in a wide range of functional and binding assays containing nucleotide- (P_{2U}), adenosine- (A_1, A_{2b}), catecholamine- (α_1, α_2, β_2, D_2), 5-hydroxytryptamine- (5-HT_2), angiotensin- (AT_1) and prostaglandin- (EP_2) receptors (unpublished observations).

The high potency, selectivity, and specificity of FPL 67085 for P_{2T}-purinoceptors *in vitro* made it a suitable candidate for progression to studies *in vivo*, and these features were subsequently confirmed during i.v. infusion of FPL 67085 in urethane-anaesthetized rats. In these experiments, FPL 67085 produced dose-dependent inhibition of ADP-induced platelet aggregation measured in whole blood *ex vivo*. Aggregation was inhibited by 50% at a dose of 1.3 $\mu g kg^{-1} min^{-1}$, i.v. while no effects on BP, HR or circulating cell counts were seen at $\leqslant 8 \mu g kg^{-1} min^{-1}$. The anti-aggregatory effect of infusion of FPL 67085 (8 $\mu g kg^{-1} min^{-1}$, i.v.) was fully reversed within 20 min of cessation of infusion, indicating extremely rapid metabolism or clearance in this anaesthetized rat preparation. However, when measured in rat blood *in vitro*, the effect of a concentration of FPL 67085, chosen to produce approximately 80% inhibition of ADP-induced platelet aggregation, did not change significantly when the incubation time was increased from 2 to 30 min. The stability of FPL 67085 in blood, as indicated by measurement of inhibition of platelet aggregation, contrasts with the relatively rapid breakdown of ATP in this tissue ($t_{\frac{1}{2}}$ 10 min) (Trams *et al.*, 1980). This indicates that, in rat blood over a period of 30 min, FPL 67085 is resistant to breakdown by ectonucleotidases which are present on the surface of blood cells (Pearson, 1985), confirming the reduced

susceptibility to this route of metabolism conferred by a β,γ-methylene link in the tri-phosphate chain (Welford *et al.*, 1986). Thus, the rapid offset kinetics of FPL 67085 observed *in vivo* cannot be explained by metabolic inactivation by blood components but rather must reflect an alternative metabolic process or a clearance mechanism *in vivo*.

Using FPL 67085, we have had the first opportunity to evaluate the effects of a highly potent, specific and selective P_{2T} purinoceptor antagonist *in vivo*. The pharmacodynamic and functional pharmacokinetic profile of FPL 67085 indicate that it may have clinical potential as a potent, infusible anti-platelet agent for acute use. In the present study, we have shown that i.v. infusion of this P_{2T}-purinoceptor antagonist inhibits ADP-induced platelet aggregation measured *ex vivo*. The potential of FPL 67085 and other P_{2T}-purinoceptor antagonists as novel anti-thrombotic agents will depend critically on the contribution of endogenous ADP to thrombosis *in vivo*. This question is now being addressed in animal models of thrombosis, using FPL 67085 as a probe that may also ultimately represent a suitable candidate for progression to the clinic.

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