GR32191, a highly potent and specific thromboxane A₂ receptor blocking drug on platelets and vascular and airways smooth muscle *in vitro*

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1 The thromboxane A₂ (TP)-receptor blocking activity and specificity of action of GR32191 ([1R-[1 α (Z),2 β ,3 β ,5 α]]-(+)-7-[5-([1,1'-biphenyl]-4-ylmethoxy)-3-hydroxy-2-(1-piperidinyl)cyclopentyl]-4-heptenoic acid has been evaluated in human platelets and various smooth muscle preparations, both vascular and non-vascular, from a range of species including man.

2 Utilising a platelet counting method to assess aggregation the drug was found to antagonise, in a surmountable manner, human platelet aggregation produced by the TP-receptor agonists, U-46619, EP171 and SQ26655, in whole blood and physiological buffer, with pA₂ values of approximately 8.3 and 8.7 in the two media respectively. In the presence of GR32191 the rate of aggregation induced by U-46619 was slowed.

3 The effect of GR32191 upon U-46619-induced platelet shape change and aggregation in platelet-rich plasma was evaluated utilising a turbidometric technique. Both shape change and aggregation were antagonised by GR32191. At relatively high concentrations of the drug a slowing of aggregation and shape change to U-46619 was seen and an unsurmountable antagonism became apparent.

4 The action of GR32191 upon human platelets was specific with platelet aggregation induced by adenosine 5'-diphosphate, platelet activating factor, vasopressin and adrenaline and the inhibitory effects of prostacyclin (PGI₂), prostaglandin D₂ (PGD₂) and N-ethylcarboxamide-adenosine (NECA) being unaffected by concentrations of the drug as high as 10 μ M. Furthermore, at concentrations of up to 100 μ M, the drug itself produced no shape change or aggregation, of human platelets.

5 GR32191 also specifically and potently antagonised in a competitive, surmountable manner the contractile actions of U-46619 upon human vascular smooth muscle and antagonised U-46619-induced contractions of vascular and airways smooth muscle preparations from rat, dog, guinea-pig and rabbit with varying potency. This is discussed in terms of possible heterogeneity of TP-receptors.

6 GR32191 therefore represents a highly potent and specific TP-receptor blocking drug. This profile of action, coupled to its long duration of effect in man described elsewhere, make it an ideal drug tool for elucidating the physiological and pathophysiological role of thromboxane A₂.

Introduction

With the discovery of thromboxane A₂ (TXA₂, Hamberg *et al.*, 1975), and identification of its potent platelet aggregatory and vasoconstrictor actions, attempts were soon in progress to synthesize structural analogues of these compounds as stable mimetics and antagonists. Compounds, such as

U-46619 (Bundy, 1975), were very quickly discovered which appeared to mimic the actions of TXA₂ and in some situations those of prostaglandin H₂ (PGH₂). The action of such compounds suggested the existence of a specific receptor through which TXA₂ exerts its biological effects (see MacIntyre, 1981) and this prostanoid receptor type has been termed a TP-receptor (Kennedy *et al.*, 1982).

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Many of the early attempts to synthesize TP-receptor blocking drugs yielded partial agonists or compounds which also possessed TXA₂ synthase inhibiting activity. The first compound which appeared to meet the criteria of a specific TP-receptor blocking drug was 13-azaprostanoic acid (13-APA) (Le Breton *et al.*, 1979) which specifically antagonised human platelet aggregation to arachidonic acid, PGH₂ and U-46619 but did not affect TXA₂ synthesis. However the compound was very weak and not suitable for *in vivo* studies. Nicolaou *et al.* (1979) originally observed a similar profile to that of 13-APA for pinane thromboxane A₂ (PTA₂). This compound, however, also possessed thromboxane synthase inhibitory activity and has subsequently been shown to exhibit weak partial TP-receptor agonist activity on human platelets and in addition to elevate cyclic AMP (Armstrong *et al.*, 1985).

It was at about this time that we described the pharmacology of the prostanoid, AH19437 (Coleman *et al.*, 1981) (Figure 1). The compound, although being an advance over 13-APA in terms of potency, was still relatively weak with a pA₂ against U-46619-induced aggregation of human platelets and contraction of rat isolated aorta of approximately 6.0. It was, however, specific being without effect up to relatively high concentrations upon all other prostanoid and non-prostanoid receptor types studied. Subsequent chemical modification of AH19437 (Collington *et al.*, 1983) led to the discovery of AH23848, a TP-receptor blocking drug almost two orders of magnitude more potent than the parent drug (Brittain *et al.*, 1985). However, AH23848 also displayed some TP-receptor partial agonist activity and for this and other reasons its development was halted (Lumley, 1986).

GR32191 (Figure 1) is a close analogue of AH23848 in which the TP-receptor partial agonist activity is absent and the TP-receptor blocking potency is enhanced. We describe here in detail the *in vitro* pharmacology of GR32191 upon human platelets and vascular and non-vascular smooth muscle from a range of species.

Methods

Preparation of platelets

Human blood was collected from healthy male volunteers and anticoagulated with trisodium citrate as described elsewhere (Keery & Lumley, 1988). Platelet-rich plasma (PRP) was prepared by centrifugation (Mistral 3000, MSE) of citrated whole blood (1340 *g* for 3 min at 25°C) and kept at room temperature until used. Human resuspended platelets were prepared from PRP (Keery & Lumley, 1988).

Figure 1 The development of TP-receptor blocking drugs; the chemical structures of AH19437, AH23848 and GR32191.

Platelet aggregation: counting method

Platelet aggregation was quantified by monitoring the fall in single platelet count produced by aggregatory agents in aspirin-treated aliquots of either whole blood or platelets resuspended in modified Krebs solution, using an Ultra-Flo 100 whole blood platelet counter (Becton-Dickinson) (Lumley & Humphrey, 1981; Keery & Lumley, 1988). Duplicate platelet counts were recorded before and at intervals following addition of an aggregating agent until a nadir in the platelet count was achieved. The intervals employed, which were based upon detailed time-

course studies were, 1 and 3 min (adenosine 5'-diphosphate (ADP), platelet-activating factor (Paf), vasopressin and adrenaline), 5 min (U-46619 and SQ26655), and 10 min (EP171). Percentage platelet aggregation and agonist EC_{50} values were calculated as described previously (Keery & Lumley, 1988).

To quantify the potency of TP-receptor blocking drugs such as GR32191, a control concentration-effect curve was first constructed to the TP-receptor agonist, e.g. U-46619. Concentration-effect curves, randomised over the course of a series of experiments, were then repeated each in the presence of one of three concentrations of receptor blocker (10 min pre-incubation). Finally a second control curve was constructed to allow correction for any spontaneous change in platelet sensitivity. In specificity experiments, aggregation concentration-effect curves to the agonist under study were constructed in the absence (control) and presence of GR32191, usually $10\text{ }\mu\text{M}$ (10 min incubation), followed by construction of a second control curve. To assess the effect of GR32191 upon the antiaggregatory effects of prostacyclin (PGI_2), PGD_2 and N-ethylcarboxamide adenosine (NECA), ADP was utilised as the agonist. Aggregation concentration-effect curves were constructed to ADP in the absence and presence of up to two concentrations of the anti-aggregatory agent (pre-incubated with whole blood for 5 min) which were then repeated in the additional presence of GR32191 ($10\text{ }\mu\text{M}$, 10 min pre-incubation). Finally at the end of the experiment a second ADP control curve was constructed.

Data from these experiments were expressed in the form of concentration-ratios (CR) as described previously (Keery & Lumley, 1988).

Platelet aggregation: optical aggregometer

The effect of GR32191 upon platelet aggregation and shape change induced by both U-46619 and ADP in human PRP was assessed in a six-channel Born-type aggregometer (Glaxo Bioengineering Department). Platelet aggregation was measured as an increase in light transmission and minimum and maximum light transmission set on a chart recorder (Ormed, MX6) using PRP and platelet-poor plasma, respectively. Platelet shape change was recorded as a decrease in light transmission, full scale deflection being calibrated using a sample of PRP which had undergone maximum shape change, but not aggregation, to U-46619 (usually 100 nM). Aliquots (0.3 ml) of aspirin-treated (2 mM) PRP in glass tubes (1.5 ml ; Payton Scientific Inc) were allowed to equilibrate at 37°C for 10 min without stirring. After this time stirring was initiated at 900 r.p.m. by means of a metal flea (Payton Scientific Inc) for 1–2 min, at which time the aggregating agent was added. For shape change

measurements, EGTA (4 mM) was added 1 min before the addition of the aggregating agonist to prevent aggregation occurring. The effect of GR32191 upon U-46619- and ADP-induced aggregation and shape change was assessed as described above for the platelet counting technique. Each aggregation and shape change response was allowed to proceed to its maximum and, the change in light transmission measured in arbitrary units at this point, expressed as a percentage of the largest change achieved in the first control curve. Results were again expressed as CR values (see above) using the mean EC_{50} value for the duplicate control concentration-effect curves.

Vascular and airways smooth muscle

Human pulmonary arteries were obtained from macroscopically normal lung segments removed by surgical resection approximately 24 h earlier from patients with lung carcinoma. Thoracic aortae were obtained from male Wistar rats ($200\text{--}300\text{ g}$), male Dunkin Hartley guinea-pigs ($300\text{--}500\text{ g}$) and male New Zealand white rabbits ($1.5\text{--}2.5\text{ kg}$). Dog saphenous veins were obtained from barbitone anaesthetized beagle dogs ($7\text{--}10\text{ kg}$, see Coleman, 1987). A perspex rod of suitable diameter was inserted into the vessels which, after clearing away non-vascular tissue, were cut spirally (Coleman, 1987). Hearts were removed from beagle dogs killed by an overdose of sodium pentobarbitone. The proximal 20 mm portion of the left anterior descending coronary artery was removed, cleared of cardiac muscle and connective tissue and divided into 3 mm rings. Guinea-pig and rat lung strips were dissected as described for guinea-pig (Coleman, 1987). Four vascular or lung strips from each animal were suspended by cotton threads and rings between wire hooks for isometric recording of tension changes, in 10 ml organ baths containing a modified Krebs solution (Keery & Lumley, 1988) at $37 \pm 0.5^\circ\text{C}$ and bubbled with a $95\%\text{ O}_2$ plus $5\%\text{ CO}_2$ mixture. The Krebs solution contained atropine ($0.8\text{ }\mu\text{M}$), phenoxybenzamine ($0.7\text{ }\mu\text{M}$) and indomethacin ($2.8\text{ }\mu\text{M}$) unless otherwise stated. Vascular preparations were subjected to initial resting tensions of $0.3\text{--}0.5\text{ g}$ and lung strips to a tension of 0.2 g .

An equilibration period of 20–30 min was followed by a particular priming sequence, involving one or two sequential exposures to KCl (30 mM) and then U-46619 (100 nM), which for each preparation enabled two reproducible U-46619 concentration-effect curves to be obtained subsequently. The bathing solution was repeatedly changed between each exposure to re-establish initial resting tension and then a cumulative control concentration-effect curve to U-46619 constructed on each preparation. In some cases (guinea-pig and rat lung strip) repro-

ducible responses to U-46619 could only be achieved by repeating up to three concentration-effect curves to the agonist, the final curve being used as the 'control'. Following washout of U-46619, three preparations were each exposed for one hour to a different concentration of GR32191 (0.03, 0.3 or 3.0 μM) and the fourth to its vehicle and a final U-46619 concentration-effect curve constructed. On each preparation the increase in developed tension (g) was expressed as a percentage of the maximum obtained in the 'control' U-46619 concentration-effect curve. Concentration-ratio values were obtained as previously described and corrected for spontaneous changes in sensitivity to the agonist by dividing the CR value from the test preparations by the CR value obtained from the vehicle-treated preparation (see Humphrey *et al.*, 1988). If this latter value did not lie between 0.5 and 2.0 the data for the whole experiment were discarded.

The specificity of action of GR32191 (3–100 μM , one hour contact) was tested against 5-hydroxytryptamine (5-HT), KCl or histamine. Phenoxybenzamine was omitted from the Krebs solution bathing tissues exposed to 5-HT. Pairs of preparations from the same animal were studied for each agonist. A priming sequence of two KCl (30 mM) responses, only, was adopted on all preparations which was followed by construction of two agonist concentration-effect curves, the first in the absence, the second in the presence of GR32191 or its vehicle. The CR value obtained from the GR32191-treated preparation was corrected as described above using the CR from the vehicle-treated preparation.

Effects at other prostanoid and non-prostanoid receptors

In addition to testing its effects upon IP- and DP-receptors on human platelets, GR32191 (10 μM) was also tested for agonist and antagonist effects upon EP₁-, EP₂- and EP₃-receptors (guinea-pig fundus, cat trachea, guinea-pig vas deferens, prostaglandin E₂ (PGE₂) standard agonist) and FP-receptors (dog and cat iris, PGF_{2 α} standard agonist) using methods previously described (Coleman *et al.*, 1987; Coleman, 1987). The compound (10 μM) was also tested (preparation; standard agonist; methodology reference) for agonist and antagonist effects upon α_1 - (rabbit aorta; noradrenaline; Furchgott & Bhadrakom, 1953), β_1 - (rat spontaneously beating right atrium and electrically stimulated left atrium; isoprenaline; Blinks, 1965; 1967) and β_2 -adrenoceptors (electrically stimulated guinea-pig trachea; isoprenaline; Coleman & Nials, 1986), upon 5-HT₁- and 5-HT₂-receptors (dog saphenous vein and rabbit aorta respectively; 5-HT; Humphrey *et al.*, 1988), D₂-dopamine receptors (rabbit ear artery; quinpi-

role; Steinsland & Hieble, 1978) and M₁- (rat cortex; pirenzepine; Hammer *et al.*, 1986) and M₂-muscarinic receptors (rat salivary gland and left atrium; N-methylscopolamine; Hammer *et al.*, 1986).

Expression of results

Data were expressed as arithmetic or geometric mean values with 95% confidence intervals. Individual data were compared by means of paired or unpaired Student's *t* test. Data from interaction studies between TP-receptor agonists and antagonists were analysed by the method of Arunlakshana & Schild (1959).

Drugs used

GR32191 (hydrochloride salt, Glaxo Group Research Ltd) was prepared as a 1 mM stock solution by dissolving in distilled water. Tri-sodium citrate (129 mM; BDH), (–)-adrenaline (10 mM; Sigma), 5-hydroxytryptamine creatinine sulphate (10 mM; Sigma), potassium chloride (4 M; BDH) were all dissolved in distilled water at the concentration shown in parentheses; subsequent dilutions were made in 150 mM NaCl solution (saline). In the case of adrenaline, ascorbic acid was included in the stock solution and dilutions at a final concentration of 1 mM. Atropine sulphate (4 mM; Sigma), phenoxybenzamine hydrochloride (1 mM, Smith Kline and French), histamine acid phosphate (1 mM; BDH), vasopressin (1 mM; Sigma), EP171 ([1R-[1 α ,2 β (Z),3 α (1E,3R*),4 α]]-7-[4-[(4-fluorophenoxy)-3-hydroxy-1-butenyl]-7-oxabicyclo [2.2.1] hept-2-yl]-5-heptenoic acid, 0.1 mM; gift), EGTA (ethyleneglycol-bis-(β -aminoethyl ether) N,N,N',N'-tetracetic acid, 400 mM; Sigma) were dissolved in saline; dilutions were also prepared in saline. The EGTA solution also contained 20 mM HEPES (N-2-hydroxyethyl piperazine-N'-2-ethanesulphonic acid; Sigma) and the pH of the solution adjusted to 7.4 with 5 N NaOH. Prostaglandin E₂ (10 mM; Ono, SQ26655 ([1S-(1 α ,2 α (Z),3 β (1E,3R*),4 α]]-7-[3-(3-hydroxy-1-octenyl)-7-oxabicyclo [2.2.1]hept-2-yl]-5-heptenoic acid, 10 mM; gift), BM 13.505 (4-[2-[(phenylsulphonyl)amino]ethyl]phenoxyacetic acid, 10 mM; Glaxo Group Research Ltd) and BM 13.177 (4-[2-[(4-chlorophenyl)sulphonyl]amino]ethyl]benzeneacetic acid, 10 mM; Glaxo Group Research Ltd) and indomethacin (10 mM; Sigma) were dissolved in 10% w/v sodium bicarbonate solution and diluted with saline to give a final bicarbonate concentration of 1% w/v. Dilutions were prepared in saline. Prostaglandin F_{2 α} (dinoprost trimethamine, Upjohn) was supplied as a 5 mg ml⁻¹ solution and dilutions were again prepared in saline. AH19437 and 13-azaprostanoic acid (10 mM, Glaxo

Group Ltd), EP092 ([1 α ,2 β (Z),3 α ,4 α]-(+)-7-[3-[1-[[[phenylamino]thioxomethyl]hydrazono]ethyl]bicyclo[2.2.1]hept-2-yl]-5-heptenoic acid, 1 mM; gift), SQ29548 ([1R-[1 α ,2 β (Z),3 β ,4 α]]-7-[3-[[[phenylamino] carbonyl] hydrazino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid, 1 mM; gift) and SQ28668 ([1 α ,2 α (Z)3 α (1E,3S*4R*)4 α]-7-[3-(3-hydroxy-4-phenyl-1-pentenyl)-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid, 1 mM; gift) were dissolved in absolute ethanol and saline added to give a final concentration of ethanol of 6% v/v; dilutions were prepared in saline. Details of the preparation of U46619, AH23848, aspirin, NECA (5'-N-ethylcarboxamide adenosine), PGI₂, PGD₂, ADP (adenosine-5'-diphosphate) and Paf DL- α -phosphatidylcholine, β -acetyl- γ -O-hexadecyl are given elsewhere (Keery & Lumley, 1988). All other reagents used were Analar grade. In the text, concentrations of drugs used in whole blood experiments are expressed as the concentrations in whole blood with no correction for haematocrit. All drugs were kept on ice during an experiment.

Results

Activity of GR32191 on human platelets in whole blood and resuspended in physiological buffer

In human whole blood, U-46619 (0.03–1.0 μ M) induced aggregation of platelets, as measured by the disappearance of single platelets, with a mean (95% confidence interval, n = number of experiments) EC₅₀ value of 0.21 (0.11–0.42) μ M (n = 6). GR32191 10–100 nM produced a progressive rightward displacement of the U-46619 concentration-effect curve for platelet aggregation. Concentration-ratios for U-46619 of 3.2 (1.9–5.5), 9.4 (4.0–22.2) and 34.7 (16.8–71.5) were obtained at GR32191 concentrations of 10, 30 and 100 nM, respectively. No depression of the maximum of the U-46619 concentration-effect curve was observed at the highest concentration of GR32191 used in these experiments, although a lengthening of the time to achieve peak aggregation was seen (Figure 2). Analysis of the data yielded a mean pA₂ value of 8.2 (7.9–8.5) (n = 6) and a slope of the regression of 1.29 (1.13–1.45) which was significantly greater than unity. To determine whether equilibrium had been achieved with GR32191 (30 nM) under these experimental conditions a comparison was made of 10 and 30 min incubations of the drug upon U-46619-induced platelet aggregation. No significant difference was observed with the two incubation times (mean (n = 5) with U-46619 CR values of 15.2 and 14.0, respectively).

GR32191 (10–100 nM) also antagonised platelet aggregation in whole blood induced by two other potent TP-receptor agonists, namely SQ26655 and

Figure 2 The effect of GR32191 (30 nM) upon the rate of U-46619-induced platelet aggregation in human whole blood. Curves are the mean from 4 experiments with vertical lines indicating s.e.mean. Control U-46619 (1 μ M) response (○); response to U-46619 (30 μ M) in the presence of GR32191 (30 nM) (▲); response to U-46619 (1 μ M) in the presence of GR32191 vehicle (●).

EP171, with a similar potency to that seen against U-46619 (Table 1). No depression of the maximum response to EP171 was observed in these experiments but some slight depression, at the highest concentration of GR32191 used, was observed with SQ26655. In addition, at this concentration of GR32191 a slowing of the aggregatory response to SQ26655 was also observed. However, no such effect was observed when EP171 was used as the agonist.

The estimation of receptor blocking potency is complicated in whole blood by the presence of plasma proteins. The interaction of GR32191 with U-46619, SQ 26655 and EP171 was therefore investigated in a preparation of human platelets resuspended in modified Krebs solution. In this medium there was an enhancement in the potency of U-46619 for inducing platelet aggregation but, interestingly, not in that of the other two agonists, SQ26655 or EP171 (Table 1). As in whole blood, GR32191 (3–30 nM) produced a progressive rightward displacement of the U-46619 aggregation concentration-effect curve (Figure 3a). Mean U-46619 CRs of 3.1 (2.2–4.3), 13.7 (4.5–41.7) and 42.7 (15.5–117.2) were obtained with 3, 10 and 30 nM GR32191, respectively. These experiments yielded an apparent mean pA₂ value of 8.79 with a slope of the Schild regression of 1.31 (Table 1). When compared with the value obtained in whole blood this shows an approximate 2–3 fold increase in potency of GR32191 in this medium, indicating that the drug is susceptible to binding by plasma proteins. A similar enhancement of potency of GR32191 was obtained in resuspended platelets using SQ26655 and EP171 as the agonist; these results are summarised in Table 1.

Table 1 The potency of GR32191 upon human platelet aggregation induced by various TP- and non-TP-receptor agonists

Medium	Agonist	Agonist EC_{50} (nM)	GR32191		(n)
			pA_2	Slope	
Whole blood ^a	U-46619	230 (110–470)	8.23 (7.90–8.56)	1.27 (1.09–1.44)	(6)
	SQ26655	32 (24–43)	8.32 (8.15–8.50)	1.61 (1.28–1.93)	(9)
	EP171	1.9 (1.5–2.3)	8.30 (8.26–8.35)	1.13 (1.06–1.20)	(4)
	ADP	900 (690–1170)	< 5.0 ^b	—	(9)
	PAF	57 (45–71)	< 5.0 ^b	—	(4)
	Adrenaline	1460 (950–2250)	< 5.0 ^b	—	(8)
	Vasopressin	117 (80–169)	< 5.0 ^b	—	(4)
Resuspended platelets ^a	U-46619	57 (41–80)	8.79 (8.63–8.94)	1.31 (1.0–1.58)	(4)
	SQ26655	27 (2.5–281)	8.64 (8.06–9.22)	1.55 (1.22–1.88)	(4)
	EP171	1.8 (1.6–2.0)	8.77 (8.69–8.86)	1.25 (1.14–1.35)	(4)
	ADP	1115 (870–1430)	< 5.0 ^b	—	(4)

Values are the mean (95% confidence interval) from *n* experiments.

^a Platelet counting method.

^b CR value of < 2.0 obtained at 10 μ M GR32191.

In both whole blood and in resuspended platelets GR32191 (10 μ M) was without effect upon ADP-induced aggregation (Table 1). In addition in whole blood at this concentration the compound was also without effect upon platelet aggregation induced by adrenaline (0.1–3.0 μ M), Paf (10–300 nM) or vasopressin (0.01–1.0 μ M) (Table 1). The antiaggregatory effects of PGI₂, PGD₂ and NECA assessed against

Figure 3 The effect of GR32191 upon (a) U-46619-induced aggregation of human resuspended platelets and (b) contraction of human isolated pulmonary artery strips. Curves are the mean from 4 and 10 experiments in (a) and (b) respectively; vertical lines show s.e.mean. In (a) U-46619 control concentration-effect curves were performed at the beginning (○) and end (●) of the experiments and compared with those performed in the presence of 3 (▲), 10 (■) and 30 (▼) nM GR32191, respectively. In (b) in each experiment a control U-46619 concentration-effect curve was initially constructed on all four strips of pulmonary artery and the pooled data shown (○), *n* = 40; three of the four strips were then exposed to GR32191 30 (▲), 300 (■) and 3000 (▼) nM respectively, for one hour before repeating the U-46619 curves. The fourth preparation received GR32191 vehicle only (●).

Table 2 The antiaggregatory effects of prostacyclin (PGI₂), prostaglandin D₂ (PGD₂) and 5'-N-ethylcarboxamide adenosine (NECA) assessed against ADP-induced platelet aggregation in the absence and presence of GR32191 (10 μ M)

Anti-aggregatory agent (μ M)	ADP concentration-ratio	
	absence of GR32191	presence of GR32191
PGI ₂ (0.003)	14	9.4
	(4.4-45)	(3.4-26)
(0.01)	118	147
	(52-267)	(50-436)
PGD ₂ (0.1)	5.6	4.6
	(2.6-12)	(2.8-7.4)
(0.3)	71	64
	(12-401)	(8.9-467)
NECA (1.0)	14	14
	(6.9-28.4)	(7.1-27.4)

Values shown are geometric mean values (95% confidence intervals) of 4-6 determinations

ADP-induced platelet aggregation were also unaffected by GR32191 (10 μ M, Table 2). Furthermore, GR32191 up to concentrations of 10 μ M was without effect upon human single platelet count in either whole blood or resuspended platelets.

Activity in platelet-rich plasma

The profile of action of GR32191 was also assessed in human platelet-rich plasma (PRP) using a turbidometric technique. With this technique U-46619 induced platelet aggregation with a mean EC₅₀ value of 0.5 (0.4-0.6) μ M (n = 7). GR32191 (3-100 nM) antagonised U-46619-induced platelet aggregation. At the lower concentrations a parallel rightward displacement of the U-46619 concentration-effect curve was observed. For example at a concentration of 10 nM a U-46619 CR value of 5.7 (3.0-10.9) (n = 6) was obtained with no depression of the maximum response being observed. However, at a concentration of 100 nM a marked depression in the U-46619 maximum was seen which was also associated with a slowing of the aggregatory response.

U-46619-induced platelet shape change was monitored separately. The agonist potently induced a shape change response (mean EC₅₀ value of 0.04 (0.01-0.09) μ M (n = 4)) which was antagonised by GR32191. The profile of this antagonism appeared to be more like that seen using the platelet counting method, with GR32191 (3-100 nM) producing parallel rightward displacements of the agonist concentration-effect curve with no depression of the maximum effect. Interestingly, quantitative analysis

of one such experiment yielded a pA₂ value of 8.78 and a slope of the Schild regression of 0.89. However, at higher concentrations of GR32191 (300 nM), a depression of the maximum of the shape change concentration-effect curve and slowing of the response was observed. In contrast to its effects upon U-46619, GR32191 (10 μ M, n = 2) was without effect upon either aggregation or shape change induced by ADP in human PRP. In addition GR32191 itself, up to concentrations of 100 μ M produced no shape change response or direct aggregatory effects in human PRP.

Vascular smooth muscle

U-46619 was a potent contractile agonist on all preparations of vascular smooth muscle studied (Table 3). GR32191 antagonised these U-46619-induced contractions in preparations from a variety of species including man (Table 3). The antagonism observed appeared to be of a competitive surmountable nature, except on the rabbit aorta where a slope of the Schild regression significantly less than unity was obtained. U-46619 concentration-effect curves upon human pulmonary artery strips in the absence and presence of GR32191 are shown in Figure 3b. The experiments, in which U-46619 mean (n = 10) CRs of 5.6 (3.4-9.3), 52 (27-98) and 521 (306-886) were obtained with 30, 300 and 3000 nM GR32191 respectively, yielded a mean pA₂ value of 8.18. GR32191 was of comparable potency to this upon vascular smooth muscle from rat, guinea-pig and dog, but appeared to be weaker upon rabbit aorta (Table 3).

The profile of action of GR32191 was examined further against a range of TP-receptor agonists upon the rat isolated aorta (Table 4). All of the agonists tested, namely SQ26655, EP171, PGD₂, PGE₂, PGF_{2 α} and PGI₂ produced only contractions of the preparation and these contractions were antagonised by GR32191 yielding comparable pA₂ values of between 7.5 and 8.0 in all cases.

The compound was specific where tested, being without significant effect upon contractions of the preparations induced by 5-hydroxytryptamine, potassium chloride or histamine at concentrations up to 10 (rat and guinea-pig) or 100 (rabbit) μ M (Table 3). GR32191, up to concentrations as high as 10 μ M, was also found to be without direct contractile effect upon any of the vascular smooth muscle preparations examined.

Airways smooth muscle

GR32191 also antagonised U-46619-induced contractions of airways smooth muscle from rat and guinea-pig (Table 3). As in vascular smooth muscle

Table 3 The potency and specificity of action of GR32191 upon isolated vascular and airways smooth muscle preparations from a range of species

Preparation	U-46619 ^a EC ₅₀ (nM)	pA ₂ ^a vs U46619	Slope ^a	(n)	5-HT	KCl	pA ₂ ^b vs Histamine
Human pulmonary artery	11.3 (8.8–14.5)	8.18 (7.81–8.55)	1.06 (0.92–1.20)	(10)	NT	NT	NT
Rat aorta	16.6 (12.7–21.7)	7.87 (7.62–8.13)	0.93 (0.83–1.04)	(8)	< 5.5	< 5.5	NT
Guinea-pig aorta	70 (32–152)	8.77 (8.10–9.44)	0.91 (0.77–1.06)	(6)	NT	< 5.0	< 5.0
Dog saphenous vein	5.2 (2.3–11.7)	8.72 (8.42–9.02)	0.90 (0.69–1.12)	(5)	NT	NT	NT
Dog coronary artery	15.9 (3.8–67)	8.26 (7.90–8.62)	1.11 (0.73–1.48)	(3)	NT	NT	NT
Rabbit aorta	14.1 (8.5–23.5)	7.21 (6.82–7.60)	0.78 (0.68–0.88)	(6)	≈ 4.0	< 4.0	NT
Rat lung	309 (246–388)	7.14 (6.85–7.44)	0.94 (0.80–1.08)	(6)	< 5.0	< 5.0	NT
Guinea-pig lung	50 (26.8–93)	8.21 (7.87–8.55)	1.05 (0.87–1.23)	(7)	NT	< 5.0	< 5.0

^a Values are the means of *n* observations with 95% confidence intervals in parentheses.^b Values represent data from 2–5 experiments. NT = not tested.

the antagonism appeared to be of a competitive, surmountable type and was specific with contractions of the preparations to either 5-hydroxytryptamine, potassium chloride or histamine being unaffected by concentrations of GR32191 up to 10 μ M (Table 3).

Actions at other prostanoid and non-prostanoid receptor types

GR32191 (10 μ M), in contrast to PGE₂ the standard agonist, did not produce a contraction of the guinea-pig isolated fundus or relaxation of the cat isolated carbachol-contracted trachea, nor did it antagonise these actions of PGE₂, ruling out agonist or antagonist actions at either EP₁- or EP₂-prostaglandin receptors. Furthermore, at this concentration the

compound neither affected contractions of the guinea-pig electrically stimulated isolated vas deferens, nor contracted the dog or cat isolated iris sphincter muscle, nor did it antagonise the actions of the standard agonist on each preparation, further ruling out actions at either EP₃ or FP-receptors, respectively. GR32191 was also tested at a single concentration of 10 μ M in a range of tissues in experiments designed to detect agonist and antagonist activity at various autonomic receptor types (see Methods). With the exception of the rat isolated spontaneously beating right atrium, in which it produced bradycardia (mean 51 (range 51–52) beats min⁻¹ (*n* = 3)), GR32191 was without direct effect upon any of the tissues studied. In addition it failed to alter significantly responses to the standard agonist on any of the test systems examined so ruling out an action at α_1 -, β_1 - and β_2 -adrenoceptors, 5-HT₁- or 5-HT₂-, dopamine D₂- or muscarinic M₁- or M₂-receptor types.

Table 4 The potency of GR32191 against contractions of the rat isolated aorta induced by various natural and synthetic prostanoids

Agonist	pA ₂	Slope	(n)
U-46619	7.9 (7.6–8.1)	0.9 (0.8–1.0)	(8)
EP-171	7.6 (7.2–7.9)	0.9 (0.7–1.1)	(6)
SQ26655	8.0 (7.8–8.2)	1.0 (0.9–1.1)	(6)
PGD ₂	7.8 (7.5–8.1)	1.1 (0.9–1.4)	(6)
PGE ₂	7.9 (7.7–8.1)	1.1 (1.0–1.2)	(6)
PGF _{2a}	8.1 (7.7–8.4)	1.0 (0.8–1.1)	(6)
PGI ₂	7.9 (7.6–8.2)	1.1 (0.8–1.3)	(6)

Values are arithmetic means (95% confidence interval) from *n* experiments.

Discussion

The present study has shown GR32191 to be a potent and specific TP-receptor blocking drug *in vitro* on platelets and vascular as well as airways smooth muscle preparations from a variety of species including man. The drug, at concentrations up to at least three orders of magnitude greater than those which produce a threshold blockade of the TP-receptor, does not interact with any other receptor types examined, prostanoid or non-prostanoid, nor

does it have any action upon cyclo-oxygenase, thromboxane synthase or prostacyclin synthase (this study; Hornby *et al.*, 1989). The compound is orally active and well tolerated in man, producing a long lasting antagonism of U-46619-induced platelet aggregation *ex vivo* (Thomas *et al.*, 1987). This profile of high potency, specificity and duration of action make GR32191 an ideal drug tool to elucidate the pathophysiological role of TXA₂ in human thromboembolic disease.

The profile of action of GR32191 is consistent with a blocking action at TP-receptors. Thus the drug antagonises platelet aggregation and vascular and airways smooth muscle contraction induced by agents such as U-46619, SQ26655 and EP171, which have been characterised as TP-receptor agonists (Kattleman *et al.*, 1986; Harris *et al.*, 1987; Lawrence *et al.*, 1988). In addition GR32191 antagonises platelet aggregation to agents which act indirectly via endogenously formed TXA₂ such as collagen and arachidonic acid (Hornby *et al.*, 1989). The drug also antagonises platelet aggregation induced by the prostaglandin endoperoxide PGH₂ (Hornby *et al.*, 1989), implying, as others have suggested (Armstrong *et al.*, 1983) that PGH₂ and TXA₂ interact with the same receptor to induce platelet aggregation. However, in addition to this indirect evidence, recent radioligand binding studies have directly shown that GR32191 interacts with the platelet TP-receptor (Lawrence *et al.*, 1988). Thus, GR32191 was found to displace the TP-receptor ligand [¹²⁵I]-PTA-OH (Narumiya *et al.*, 1986) from human platelets with an estimated mean ($\pm$ s.e.mean) K_D value of 4.6 ± 0.7 nM. This value compares closely with the K_D value of 1.7 ± 0.2 nM for GR32191, derived from the pA₂ for U-46619 in human resuspended platelets (present study).

The profile of action of GR32191 upon vascular and airways smooth muscle appears to be of a competitive, surmountable type. Thus the lack of any depression of the maximum response to TP-receptor agonists and slopes of the Schild regression not significantly different from unity (the only exception being the rabbit aorta) are both observations which support this conclusion. In contrast, on human platelets the drug slows the rate of response to U-46619, whether it is aggregation or shape change, and can lead to a suppression of the agonist maximum response. This is particularly noticeable when using the turbidometric technique for assessing platelet aggregation (this study; Hornby *et al.*, 1989). In addition, slopes of the Schild regression significantly greater than unity for antagonism of platelet aggregation were observed whether using U-46619 or other TP-receptor agonists such as SQ26655 and EP171. It may be, however, that the slowing of the aggregatory response can be separated from the mechanism producing the high slope of the Schild

regression, since with the TP-receptor agonist EP-171 only the latter effect was observed with GR32191. The slowing of the aggregatory response and suppression of the maximum response have also been observed with other TP-receptor blocking drugs of similar structure to GR32191 (e.g. AH23848, Brittain *et al.*, 1985) as well as with several other antagonists of widely differing chemical structure such as EP092 (Armstrong *et al.*, 1985), SQ29548 (Ogletree *et al.*, 1985) and ICI 192,605 (Jessup *et al.*, 1988). One common feature of most of these studies is the use of the TP-receptor agonist U-46619 to induce platelet aggregation. It has been shown that at high concentrations this agonist can elevate intra-platelet cyclic AMP (Best *et al.*, 1979), possibly by stimulation of the platelet IP-receptor (Stratton & Hornby, 1987). Therefore at the high concentrations of U-46619 needed to induce aggregation in the presence of TP-receptor blockade, this effect may influence the profile of antagonism of platelet aggregation observed. However, this is unlikely to be the sole explanation for the effects seen with drugs like GR32191, since experiments with other TP-receptor blocking drugs such as EP045 (Armstrong *et al.*, 1985), BM 13.177 (Patscheke & Stegmeier, 1985) and BM 13.505 (Patscheke *et al.*, 1987), in which profiles consistent with surmountable competitive antagonism were obtained, also utilised U-46619 as the agonist.

An alternative explanation for the antagonist profile observed with GR32191 on platelets may be related to its rate of dissociation from the platelet TP-receptor. Preliminary experiments with [³H]-GR32191 have shown that it exhibits a relatively slow dissociation rate ($t_{1/2}$ of approximately 60 min) from TP-receptors on human platelets (Armstrong, personal communication). Slow dissociation of an antagonist coupled with the use of a weak agonist would be expected to produce a hemi-equilibrium state, such that full equilibrium between agonist and receptors in the presence of antagonist does not occur (see Kenakin, 1984). Since U-46619 appears to be a low efficacy agonist, which needs to occupy about 40% of the receptor population to elicit full aggregation (Armstrong *et al.*, 1985), this would predictably lead to the reduced maximal response observed to U-46619 in the presence of high concentrations of GR32191 and/or slopes of Schild regressions greater than unity. Interestingly, the receptor occupancy required by U-46619 to induce shape change is much less than for aggregation (approximately 5%). It is perhaps not surprising therefore that, under experimental conditions (turbidometric measurement) in which the maximum aggregatory response to U-46619 was markedly suppressed in the presence of GR32191, a similar suppression of the shape change response maximum was not observed.

A further important feature of GR32191 is its ability to block TP-receptors in human platelets and vascular smooth muscle over a similar concentration range. This profile of blockade of TP-receptors in both tissue types is desirable in a drug of this type since it is likely that, in addition to its platelet aggregating activity, TXA₂-induced vasoconstriction may also play a key role in the clinical pathology of ischaemic vascular disease. GR32191 also antagonised U-46619-induced contraction in preparations of vascular and airways smooth muscle from other species. However, differences in the potency of the drug were apparent. One of the preparations where the drug appeared to be weak (low pA₂ and low slope) was the rabbit aorta. We have previously observed this profile with AH23848 (unpublished data) and it has been found for other TP-receptor blocking drugs (Jones *et al.*, 1982; 1987; Narumiya *et al.*, 1986). Another preparation in which GR32191 appeared to be weak (low pA₂) was the rat lung. The data in rat lung contrasts with that in the rat aorta which in our experience has been predictive for the potency of compounds such as GR32191 and AH23848 (Brittain *et al.*, 1985) upon human vascular smooth muscle. This preparation is a useful one for studying the effects of TXA₂ and its mimetics since, from the evidence presented in the present study, it appears that the only prostanoid receptors it contains are of the TP-type (Table 4).

The differences in the antagonist potency of GR32191 and other TP-receptor blocking drugs in various tissues discussed above could be taken as evidence for TP-receptor heterogeneity. However, there appears to be no obvious pattern to the differences. It is therefore conceivable that drugs such as GR32191, which are structurally quite different from the natural ligand TXA₂, may not be the ideal drug tools to investigate the possible sub-division of receptors. For example, such drugs may interact with binding sites which do not form part of the receptor itself and it is these exoreceptor sites which are poorly conserved between species. An analogous situation has been claimed for antagonists of the 5-HT₂ receptor in smooth muscle (Leff & Martin, 1986).

GR32191 is one of several currently available and structurally diverse TP-receptor blocking drugs. The drug represents a progression, in terms of potency and profile, from our previously published results with compounds AH19437 and AH23848 (see Introduction). Thus the compound is more potent than AH23848 upon human platelets and vascular smooth muscle *in vitro* but more importantly lacks partial agonist activity at any TP-receptor examined to date (this study; Lumley *et al.*, 1988). Other TP-receptor blocking drugs of note include SQ29548 (Ogletree *et al.*, 1985), BM 13.177 and BM 13.505

Table 5 A comparison of the TP-receptor blocking activity of a range of compounds against U-46619-induced aggregation of human platelets and contraction of rat isolated aortic strip

Compound	pA ₂		
	Human platelets Whole blood	Resuspended	Rat aorta
AH19437	6.1 (5.8–6.3)	NT	5.9 (5.2–6.6)
AH23848	7.8 (7.6–8.0)	8.3 (8.0–8.7)	7.9 (7.5–8.4)
GR32191	8.2 (7.9–8.6)	8.8 (8.6–8.9)	7.9 (7.6–8.1)
SQ29548	7.8 (7.6–8.1)	8.5–8.9 ^c	8.9 (8.4–9.3)
BM 13.177	6.0 (5.6–6.4)	5.4–6.2 ^c	6.2 (5.4–7.0)
BM 13.505	6.7 (6.5–6.9)	7.6–8.1 ^c	7.9 (7.4–8.4)
SQ28668	5.5 (5.0–6.0)	5.7–5.8 ^c	6.0 (5.6–6.3)
EP092	7.2 (6.8–7.6)	NT	8.0–8.1 ^c
13-APA	5.3 (5.1–5.4)	NT	4.8 (4.5–5.1)

Values are arithmetic means (95% confidence intervals) from at least 4 experiments.

^c Range, *n* = 2–3.

NT = not tested.

(Patscheke & Stegmeier, 1985; Patscheke *et al.*, 1987), ICI 192,605 (Jessup *et al.*, 1988), EP092 (Armstrong *et al.*, 1985) and ONO 3708 (Kondo *et al.*, 1987). With the possible exception of the Boehringer Mannheim compounds, these TP-receptor blocking drugs are highly potent *in vitro*. We have determined the potencies of several of these compounds, under identical conditions to those described in this study for GR32191 and the results are compared in Table 5. As can be seen GR32191 represents one of the most potent TP-receptor blocking drugs available. The other compounds which are potent *in vitro* and, in some cases *in vivo* in experimental animals are SQ29548, EP092 and ICI 192,605. However, none of these compounds appears yet to have been tested in man. Of those which have, namely BM 13.177, BM 13.505 and SQ28668 (Patscheke *et al.*, 1986; 1987; Friedhoff *et al.*, 1986), all are, in contrast to GR32191, relatively weak and of short duration of action.

In summary, GR32191 possesses a profile of action consistent with it being a TP-receptor blocking drug on platelets and vascular and airways smooth muscle from man and a range of other species. The high degree of specificity of GR32191 for TP-receptors over both prostanoid and non-prostanoid receptors, its lack of effect upon a range of enzyme systems and lack of direct effects upon platelets or smooth muscle demonstrate its value in defining the actions of drugs at TP-receptors. In addition, this profile of action of GR32191, coupled with its high oral potency and duration of action in man, make it an exciting drug for the clinical elucidation of the pathophysiological role of TXA₂.

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