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Thromboxane (Tx) A_2 receptor blockade and TxA_2 synthase inhibition alone and in combination: comparison of anti-aggregatory efficacy in human platelets

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1 The present study has compared the relative anti-aggregatory effect of various compounds which interfere with thromboxane (Tx) A_2-dependent aggregation of human platelets in whole blood *in vitro*. These included the cyclo-oxygenase inhibitor aspirin, the TxA_2 synthase inhibitor dazoxiben, the TxA_2 (TP-) receptor blocking drug GR32191 and two compounds, R.68070 ((E)-5-[[[(3-pyridinyl) [3-(trifluoromethyl)phenyl]-methylen] amino]oxy] pentanoic acid) and CV-4151 ((E)-7-phenyl-7-(3-pyridyl)-6-heptenoic acid), which possess both TP-receptor blocking and TxA_2 synthase inhibitory activities in the same molecule.

2 GR32191, R.68070 and CV-4151 all antagonized aggregation to the TxA_2 mimetic U-46619, with pA_2 values of approximately 8.2, 5.4 and 4.8 respectively. This effect was specific, platelet aggregation induced by adenosine 5'-diphosphate (ADP) being unaffected by concentrations up to 10, 1000 and 300 μM respectively. In contrast, neither aspirin nor dazoxiben exhibited any measurable TP-receptor blocking activity.

3 The rank order of potency (pIC_{50}) for inhibition of TxA_2 formation in serum was R.68070 (7.4) > CV-4151 (6.9) > dazoxiben (5.7) > aspirin (5.3). In addition, all four drugs abolished collagen-induced platelet TxA_2 formation. In contrast, GR32191 produced no consistent inhibition of TxA_2 formation in either system up to concentrations of 10–30 μM.

4 The specificity of R.68070, CV-4151 and dazoxiben for TxA_2 synthase was indicated by their ability to increase serum levels of prostaglandin E_2 (PGE_2) and PGD_2 in parallel with decreases in TxA_2 formation. This profile was not observed with aspirin or GR32191. However, high concentrations of R.68070 (100 μM) and CV-4151 (1000 μM) necessary for maximum TP-receptor blocking activity, produced substantially smaller increases in PGE_2 and PGD_2, consistent with an aspirin-like effect of these compounds upon cyclo-oxygenase. With dazoxiben (1000 μM), PGE_2 and PGD_2 levels remained elevated.

5 Aspirin inhibited collagen-induced platelet aggregation, the effect correlating with inhibition of TxA_2 formation. Dazoxiben, whilst also achieving maximal inhibition of TxA_2 formation, produced significantly less inhibition of aggregation than aspirin. In contrast, GR32191 (0.1–10 μM), at concentrations specific for TP-receptor blockade, produced a significantly greater antagonism of collagen-induced platelet aggregation than aspirin. This additional effect of GR32191 was absent in platelets pretreated with aspirin, indicating the probable involvement of an endogenous anti-aggregatory cyclo-oxygenase product in response to collagen stimulation.

6 R.68070 and CV-4151 also inhibited collagen-induced aggregation, with very high concentrations of R.68070 (100 μM) producing an effect equivalent to that of GR32191.

7 In contrast, the combination of GR32191 with either dazoxiben, R.68070 or CV-4151, at concentrations specific for TxA_2 synthase, produced a synergistic inhibitory effect upon collagen-induced platelet aggregation which was greater than that achieved with either aspirin or any of the compounds used alone. Pretreatment of platelets with aspirin reversed this synergistic effect, consistent with it being dependent upon the formation and action of anti-aggregatory prostaglandins.

8 In conclusion, the present study has confirmed the superior platelet inhibitory profile of a combination of a TP-receptor blocking drug and a TxA_2 synthase inhibitor to that of either activity alone. However, the maximum inhibitory effect of the currently available compounds, R.68070 and CV-4151, which possess both activities in the same molecule, appears to be no greater *in vitro* than that obtained with the potent TP-receptor blocking drug, GR32191. This most probably reflects the inhibition by R.68070 and CV-4151 of platelet cyclo-oxygenase at the concentrations required for effective TP-receptor blockade which results in a reduction in the formation of anti-aggregatory prostanoids.

Introduction

Thromboxane A_2 (TxA_2) aggregates human platelets and constricts vascular smooth muscle (Hamberg *et al.*, 1975) through stimulation of specific thromboxane (TP-) receptors (Kennedy *et al.*, 1982). Evidence has accumulated which implicates it in the pathophysiology of thromboembolic diseases such as unstable angina, atherosclerosis and pulmonary embolism (Green & Vesterqvist, 1986; Fitzgerald *et al.*, 1987; Catella *et al.*, 1987). Thus, prevention of the actions of TxA_2 should provide an effective anti-thrombotic approach and various classes of pharmacological agent have been developed to achieve this goal.

Aspirin irreversibly acetylates cyclo-oxygenase (Roth *et al.*, 1975) and thus prevents formation of platelet-derived TxA_2. However, the beneficial inhibition of TxA_2 formation with aspirin may be compromised by the concomitant reduction in the formation of the anti-aggregatory prostaglandins (PG)D_2 and I_2, representing an important limitation of this class of anti-thrombotic drug. An alternative approach for reducing TxA_2 formation was through the development of specific inhibitors of the enzyme, thromboxane synthase (Vermylen *et*

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al., 1981). Dazoxiben (Tyler et al., 1981), an example of this class of drug, as a result of preventing TxA_2 formation, redirects PGH_2 metabolism towards increased formation of PGI_2 and PGD_2 (Defreyn et al., 1982; Orchard et al., 1985; Gresele et al., 1987). It is now recognised, however, that thromboxane synthase inhibition causes the accumulation of PGH_2, which is equipotent with TxA_2 at platelet TP-receptors (Le Breton et al., 1979; Hornberger & Patscheke, 1989) and can itself produce platelet aggregation and granule secretion (Bertele et al., 1981; Hornby & Skidmore, 1982). This effect of PGH_2 therefore counteracts the benefits arising from the redirection to anti-aggregatory prostanoids and limits the effectiveness of thromboxane synthase inhibitors (Vermylen et al., 1985). Yet a further strategy has focussed upon directly antagonizing the effects of TxA_2 by blocking its action at TP-receptors (Humphrey et al., 1990). In this respect, GR32191 (Figure 1) has been identified as a potent TP-receptor blocking drug upon human platelets and smooth muscle *in vitro* (Lumley et al., 1989) and is orally active with a long duration of action in man (Thomas & Lumley, 1990). In contrast to aspirin, which reduces PGD_2 and PGI_2 formation, GR32191 importantly preserves formation of these anti-aggregatory prostanoids (Hornby et al., 1989; Takahara et al., 1990).

Several lines of evidence now suggest that a superior anti-thrombotic profile can be achieved by combining a TP-receptor blocking drug and a thromboxane synthase inhibitor (Gresele et al., 1984). For example, in human platelet-rich plasma *in vitro*, the TP-receptor blocking drug, BM 13.177, markedly potentiates the anti-aggregatory activity of dazoxiben (Bertele & De Gaetano, 1982). Similarly, in an *in vivo* canine model of coronary thrombosis, the combination of a TP-receptor blocking drug with a thromboxane synthase inhibitor produced a substantially greater inhibition than that achieved with either compound alone, consistent with a pro-aggregatory effect of PGH_2 limiting the effectiveness of thromboxane synthase inhibitors *in vivo* (Fitzgerald et al., 1988). This latter effect has also been observed clinically, where the combined oral administration of BM 13.177 and dazoxiben to human volunteers produced inhibition of *ex vivo* collagen- and arachidonic acid-induced platelet aggregation and a prolongation of the bleeding time which were significantly greater than achieved following either treatment alone (Gresele et al., 1987). To date, the evidence for achieving a superior anti-thrombotic profile by combining a thromboxane synthase inhibitor with a TP-receptor blocking drug has been obtained by use of two separate chemical entities. Recently, two compounds, R.68070 (De Clerck et al., 1989) and CV-4151 (Imura et al., 1988) have been described which combine specific thromboxane synthase inhibitory and TP-receptor blocking activities in a single molecule (Figure 1). The aim of the present study, therefore, was to investigate whether R.68070 and CV-4151 were superior to GR32191 as inhibitors of platelet aggregation *in vitro*. The anti-aggregatory activity of dazoxiben and aspirin has also been examined for comparative purposes.

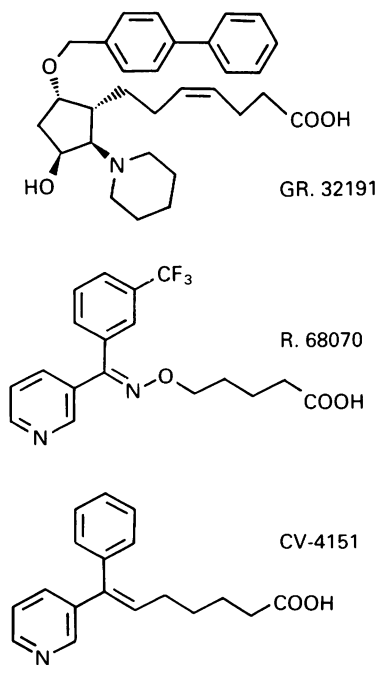

Figure 1 The chemical structures of GR32191, R.68070 and CV-4151.

Methods

Preparation of platelets

Human blood was collected from healthy male volunteers and anti-coagulated with trisodium citrate as described previously (Keery & Lumley, 1988). Citrated whole blood was centrifuged to obtain platelet-rich plasma (PRP; Lumley et al., 1989) for the preparation of human resuspended platelets in a Krebs-Henseleit buffer solution (Keery & Lumley, 1988).

Platelet aggregation

Platelet aggregation was quantified by monitoring the fall in single platelet count, produced by aggregatory agents in aliquots of whole blood or resuspended platelets, with an Ultra-Flo 100 (Becton & Dickinson) whole blood platelet counter (Lumley & Humphrey, 1981; Keery & Lumley, 1988). Unless otherwise stated, blood samples were treated with aspirin (2 mM) during the aliquoting procedure, to prevent any TxA_2 which might be formed contributing to the aggregatory response to exogenous agonists. Aliquots were placed in a shaking water bath at 37°C and incubated for 30 min before experimentation. In each sample, duplicate platelet counts were recorded before (control) and at intervals following addition of an aggregating agent until a nadir in the platelet count was achieved (Lumley & Humphrey, 1981; Keery & Lumley, 1988). The maximum fall in platelet count in each aliquot was expressed as a percentage of the control count to give percentage platelet aggregation. EC_{50} values (the concentration of agonist required to produce a 50% fall in platelet count) were determined by interpolation from semi-logarithmic plots of percentage aggregation versus concentration of agonist.

TP-receptor blocking activity on platelets

The potency of R.68070, CV-4151, dazoxiben and aspirin as TP-receptor blocking drugs was quantified in both whole blood, and in some experiments in resuspended platelets, by use of the TxA_2 (TP-receptor) agonist, U-46619 as described previously (Lumley et al., 1989). The effect of these agents was also investigated against collagen-induced aggregation. Platelet aggregation to collagen is mediated both by TxA_2 formation and adenosine diphosphate (ADP) release (Kinlough-Rathbone et al., 1977). The extent of the TxA_2 component was determined by constructing collagen concentration-effect curves (0.1–4.0 $\mu g\,ml^{-1}$) in the presence and absence of aspirin (2 mM). Further curves were then constructed in aliquots, in the presence of either GR32191, R.68070, CV-4151, dazoxiben or aspirin, or combinations of these compounds following a 10 min pre-incubation period. The inhibitory effect of each compound upon TxA_2 (measured as TxB_2) formation (see below) as well as aggregation was determined. The specificity of R.68070 (1000 μM), CV-4151 (300 μM), dazoxiben (100 μM) and aspirin (2 mM) was examined against ADP, by carrying out aggregation concentration-effect curves in the absence

(control) or presence of a single concentration of each compound.

In all experiments, inhibition of platelet aggregation, reflected as rightward displacements of agonist concentration-effect curves, was expressed in the form of concentration-ratios (CR values). These were derived by dividing the EC_{50} for an agonist in the presence of a treatment by that obtained in its absence. Interaction between U-46619 and TP-receptor blocking drugs was analysed by the method of Arunlakshana & Schild (1959) to obtain pA_2 values.

Thromboxane synthase inhibitory activity

The inhibition of human platelet TxA_2 synthase was assessed by measuring TxA_2 formation in whole blood, as its metabolically stable breakdown product TxB_2, using radioimmunoassay. The specificity of inhibitors for thromboxane synthase was assessed by determining, also by specific radioimmunoassay, the serum prostaglandin E_2 (PGE_2) and PGD_2 levels.

TxB_2, PGE_2 and PGD_2 formation in clotting blood Non-anti-coagulated human blood was rapidly divided into 0.5 ml aliquots in glass tubes each containing a single concentration of either R.68070, CV-4151, dazoxiben or aspirin (1 nM–1 mM), GR32191 (30 nM–300 μM) or, the corresponding vehicle equivalent to the highest concentration of each compound tested. Tubes were vortexed for approximately 2 s to ensure adequate mixing of the compound with the blood and then incubated in a water bath at 37°C for 60 min to allow complete clot formation. Thirty minutes into the incubation period, the clot was released from the wall of the tube with a metal rod to enable clot retraction to occur. Upon completion of the incubation period, blood samples were centrifuged at 900 *g* for 15 min at 25°C (MSE Mistral 3000) to obtain serum.

Collagen-induced TxB_2 formation The effect of GR32191, R.68070, CV-4151, dazoxiben and aspirin was also determined upon TxB_2 formation induced by addition of collagen (4 $\mu g\,ml^{-1}$) to human citrated whole blood. The samples used were taken from aggregation experiments. Following measurement of peak aggregation, blood samples were treated with indomethacin (5 μM) to prevent any further TxB_2 formation and placed on ice (2–4°C). Samples were then transferred to Eppendorf tubes and centrifuged at 5600 *g* for 3 min at 25°C (Eppendorf Model No. 5414) to obtain plasma.

Preparation of serum and plasma samples for radioimmunoassay Aliquots (100 μl) of serum or plasma were transferred to Eppendorf tubes and 500 μl of absolute ethanol added to precipitate plasma proteins. The tubes were then capped, vortexed for 3 s and centrifuged at 5600 *g* for 3 min at 25°C (Eppendorf Model No. 5414) to remove the protein precipitate. Approximately 400 μl of the alcoholic supernatant was then carefully removed and transferred to a plastic container (Sarstedt) and stored at −20°C. Recovery of [^{3}H]-TxB_2, [^{3}H]-PGE_2 and [^{3}H]-PGD_2 added to serum or plasma and taken through this process was >97%.

Subsequently, TxB_2, PGE_2 and PGD_2 levels were determined by specific radioimmunoassay and expressed in $ng\,ml^{-1}$ of serum or plasma. For calculation of inhibitory potency, TxB_2 formation at each concentration of a compound was expressed as a percentage of the maximum produced in corresponding vehicle-treated control blood samples. The molar concentration of inhibitor required to produce 50% inhibition of TxB_2 formation was derived graphically by interpolation and expressed logarithmically (pIC_{50}).

Expression of results

Data are expressed as either the arithmetic or geometric mean values with 95% confidence intervals, or as the arithmetic mean value ± s.e.mean from (n) experiments. Individual data were compared by means of a paired Student's t test where $P < 0.05$ was taken to indicate a significant difference.

Drugs used

R.68070 (10 mM; (E)-5-[[[(3-pyridinyl) [3-(trifluoromethyl) phenyl]-methylen]amino]oxy]pentanoic acid; Janssen) and CV-4151 (10 mM; (E)-7-phenyl-7-(3-pyridyl)-6-heptenoic acid; Glaxo Group Research Limited) were dissolved in 10% w/v sodium bicarbonate solution and 0.9% w/v sodium chloride solution (saline) added to give a final bicarbonate concentration of 1%. Subsequent dilutions were made with saline. Dazoxiben hydrochloride (10 mM; Pfizer Central Research) was dissolved in saline and further dilutions prepared with the same vehicle. Equine collagen tendon (1 $mg\,ml^{-1}$, Hormon-Chemie) was used as a suspension and dilutions prepared in the buffer (pH 2.8) provided. Absolute ethanol (99.7% v/v; James Burrough, Essex) was used for the precipitation of proteins in human plasma and serum. Details of the preparation of U-46619 (11,9 epoxymethano-PGH_2), GR32191, ADP, indomethacin, aspirin and trisodium citrate are given elsewhere (Keery & Lumley, 1988; Lumley *et al.*, 1989). The value in parentheses after a drug name refers to the concentration of stock solution prepared. In the text, concentrations of drugs are expressed as those in whole blood or resuspended platelets. All drugs were kept on ice during an experiment.

Radioimmunoassay materials included [^{3}H]-TxB_2 (100 $Ci\,mmol^{-1}$; New England Nuclear, Southampton, Hants) and [^{3}H]-PGE_2 (184 $Ci\,mmol^{-1}$; Amersham International Plc, Amersham). Non-radioactive standard TxB_2 (1 $mg\,ml^{-1}$) and PGE_2 (1 $mg\,ml^{-1}$) were obtained from Upjohn Diagnostics, Michigan, USA. Lyophilised antisera (rabbit) to TxB_2 (Metachem Diagnostics Ltd, Northampton) and PGE_2 (ICN Biomedicals Ltd, High Wycombe, Bucks) were used. PGD_2 measurements were made with a commercially available assay kit (Amersham International Plc, Amersham).

Results

Antagonism of U-46619-induced human platelet aggregation in whole blood and resuspended platelets

In human citrated whole blood, U-46619 (0.03–1.0 μM) produced concentration-dependent platelet aggregation, as measured by the disappearance of single platelets, with a mean (95% confidence interval) EC_{50} value of 0.18 (0.12–0.25 μM; $n = 14$). GR32191 (0.01–10 μM), R.68070 (10–100 μM) and CV-4151 (10–300 μM) all antagonized U-46619-induced aggregation producing concentration-related parallel rightward displacements of the agonist concentration-effect curve. As reported previously (Lumley *et al.*, 1989), aggregation to U-46619 in the presence of GR32191 was slowed compared with controls and the maximum effect was suppressed. However, neither of these effects was observed with R.68070 or CV-4151.

Analysis of the data yielded apparent pA_2 values which indicated the potency of GR32191 to be some 500 and 3000 times greater than R.68070 and CV-4151 respectively (Table 1). Slopes of the Schild regression not significantly different from unity indicated a surmountable, competitive interaction between U-46619 and both R.68070 and CV-4151, but not with GR32191 (Table 1). In contrast to the effects of these compounds, dazoxiben (100–300 μM) and aspirin (2 mM) were without effect upon U-46619-induced aggregation, with mean CR values of 2.0 (1.1–3.7; $n = 3$) and 0.9 (0.6–1.4; $n = 4$) respectively.

pA_2 values were also determined in resuspended platelets. Analysis of the data yielded mean pA_2 values of 8.8 (8.6–8.9) for GR32191 and 5.7 (4.9–6.6) and 5.2 (4.5–5.8) for R.68070 and CV-4151 respectively. Thus, whilst in the absence of plasma proteins the potency of each compound was increased

Table 1 A comparison of the TP-receptor blocking activity and the thromboxane A_2 synthase inhibitory activity of GR32191, R.68070, CV-4151, dazoxiben and aspirin upon human platelets in whole blood *in vitro*

	Antagonism of U-46619-induced aggregation			*Inhibition TxB_2 formation in clotting blood*	
Compound	pA_2[a]	*Slope*[a]	(n)	pIC_{50}[b]	(n)
GR32191	8.2 (7.9–8.6)	1.3 (1.1–1.5)	(6)	<3.5	(5)
R.68070	5.4 (5.2–5.6)	1.1 (0.8–1.3)	(4)	7.4 (7.2–7.6)	(9)
CV-4151	4.8 (4.3–5.3)	1.1 (0.7–1.6)	(4)	6.9 (6.7–7.1)	(9)
Dazoxiben	<3.5	—	(3)	5.7 (5.5–5.9)	(4)
Aspirin	<2.7	—	(3)	5.3 (5.1–5.4)	(5)

Values are [a] arithmetic and [b] geometric means (95% confidence interval) from (*n*) experiments.

by some 2–3 fold over that in whole blood (Table 1), the relative potencies were unaffected.

In both whole blood and in resuspended platelets, GR32191 (10 μM), R.68070 (100–1000 μM), CV-4151 (100–300 μM), dazoxiben (100 μM) and aspirin (2 mM) alone were without effect upon ADP (0.3–3.0 μM)-induced aggregation (mean CR values of <2 for each drug; $n = 4$), nor did they produce any direct effect on the baseline platelet count.

Inhibition of TxB_2 formation in human clotting whole blood

In human clotted whole blood, the mean serum TxB_2 formation was 250 ± 22 ng ml^{-1} ($n = 10$). When blood was clotted in the presence of R.68070, CV-4151, dazoxiben or aspirin (1 nM–1 mM), each compound produced a concentration-related inhibition of serum TxB_2 formation. All compounds were able to abolish TxA_2 formation with the minimum concentration required for a maximal effect (mean inhibition >95%) being obtained at 1 μM R.68070, 10 μM CV-4151, 10 μM dazoxiben and 100 μM aspirin. pIC_{50} values determined from the inhibition curves indicated that R.68070 was some three time more potent than CV-4151, which in turn was at least ten times more potent than dazoxiben in inhibiting serum TxB_2 formation (Table 1). Aspirin was approximately three times weaker than dazoxiben (Table 1). In marked contrast GR32191, up to concentrations of 30 μM, was without effect on TxB_2 formation (12 ± 6% inhibition; $n = 5$). Even at a concentration of 300 μM, a mean ($n = 5$) inhibition of TxB_2 formation of only 39 ± 7% was observed. Thus, the rank order of potency for inhibiting TxB_2 formation in human clotting blood was R.68070 > CV-4151 > dazoxiben > aspirin ≫ GR32191, with R.68070 and CV-4151 being some three to four orders of magnitude more potent than GR32191.

Since inhibition of serum TxB_2 production could be produced by inhibition of either TxA_2 synthase or cyclooxygenase, the enzymic specificity of each compound was determined by measuring both serum PGE_2 and PGD_2 in parallel with TxA_2 (TxB_2). In the absence of an inhibitor (vehicle-treated blood), mean ($\pm$s.e.mean; $n = 9$) serum levels of PGE_2 and PGD_2 were 14.2 ± 5.5 and 5.5 ± 1.4 ng ml^{-1} respectively. Over the concentration range of each compound producing inhibition of TxB_2 (Figure 2), R.68070, CV-4151 and dazoxiben produced a progressive increase in the serum levels of PGE_2 and PGD_2 which mirrored the corresponding reduction in TxB_2 formation (Figure 2). The maximum increases in PGE_2 and PGD_2 were fairly comparable between compounds, and were approximately ten and five fold greater respectively than those measured in the absence of the compounds (Figure 2). At concentrations of R.68070 and CV-4151 supramaximal for inhibition of TxB_2 formation (100 and 1000 μM), no further increase in either PGE_2 or PGD_2 formation occurred, rather the levels of PGE_2 and PGD_2 were substantially reduced (Figure 2). For example, at 100 and 1000 μM CV-4151, PGE_2 formation was reduced by 50 and 95% respectively of the mean level measured in the presence of 1 μM of the compound. A similar profile was obtained with R.68070 (Figure 2). However, in the presence of dazoxiben, even at concentrations as high as 1000 μM, PGE_2 and PGD_2 remained elevated. In contrast to the thromboxane synthase inhibitors, aspirin and GR32191 did not increase the serum concentrations of either PGE_2 or PGD_2 (Figure 2).

Effect of GR32191, R.68070, CV-4151, dazoxiben and aspirin upon collagen-induced platelet aggregation and TxB_2 formation

Collagen (0.1–40 μg ml^{-1}) added to human citrated whole blood produced concentration-related platelet aggregation (mean EC_{50} in the range 0.5–0.7 μg ml^{-1}) and increases in TxB_2 formation (Figure 3a). However, whilst near maximal aggregation to collagen was observed at 4 μg ml^{-1}, TxB_2 production continued to increase reaching 312 ± 22 ng ml^{-1} ($n = 11$) at 40 μg ml^{-1} collagen (Figure 3a). Over the concentration range 2 μM–2 mM, aspirin produced a progressive inhibition of collagen-induced TxB_2 formation with a maximum effect at 20 μM and a mean pIC_{50} of 5.5 (5.2–5.8; $n = 5$). This effect was associated with a progressive rightward displacement of the collagen aggregation concentration-effect curve, with mean CR values of 1.3 (0.7–2.4; $n = 3$) and 4.0 (2.5–6.3; $n = 3$) obtained at 2 and 20 μM aspirin. The latter effect was not significantly increased by the use of higher concentrations of aspirin (mean ($n = 3$) CR values at 200 μM and 2 mM were 4.2 (3.2–5.6) and 4.1 (2.4–7.1) respectively). Aspirin therefore fully reveals the extent of the PGH_2/TxA_2-mediated component in collagen-induced aggregation.

In contrast to aspirin, GR32191 up to a concentration of 10 μM, produced no consistent inhibition of collagen (0.4–40 μg ml^{-1})-induced TxB_2 formation (Figure 3b). Despite this, GR32191 (0.01–10 μM) produced concentration-related rightward displacements of the collagen aggregation-concentration effect curve. At concentrations of 0.1–10 μM, the inhibitory effect of GR32191 was significantly greater ($P < 0.05$) than that of aspirin (Figure 3b; Table 2). This additional effect of GR32191 was absent if platelets were pre-exposed to aspirin (2 mM), the mean CR value of 5.3 (3.3–8.7; $n = 4$) for GR32191 (10 μM) in the presence of aspirin then being not significantly different from the effect obtained with aspirin alone (4.1(3.6–4.7); $n = 4$) in this sub-group of experiments.

Unlike GR32191, R.68070 (0.1 μM), CV-4151 (1 μM) and dazoxiben (1 μM) each produced substantial (>90%) inhibition of collagen-induced TxB_2 formation. Associated with this inhibition was a rightward displacement of the collagen-induced aggregation curve (Table 2). However, even at concentrations abolishing TxA_2 formation (mean inhibition >98% obtained at 1 μM R.68070, 10 μM CV-4151 and 10 μM dazoxiben) all three compounds produced maximum CR values of <3.0 which was significantly less than that produced

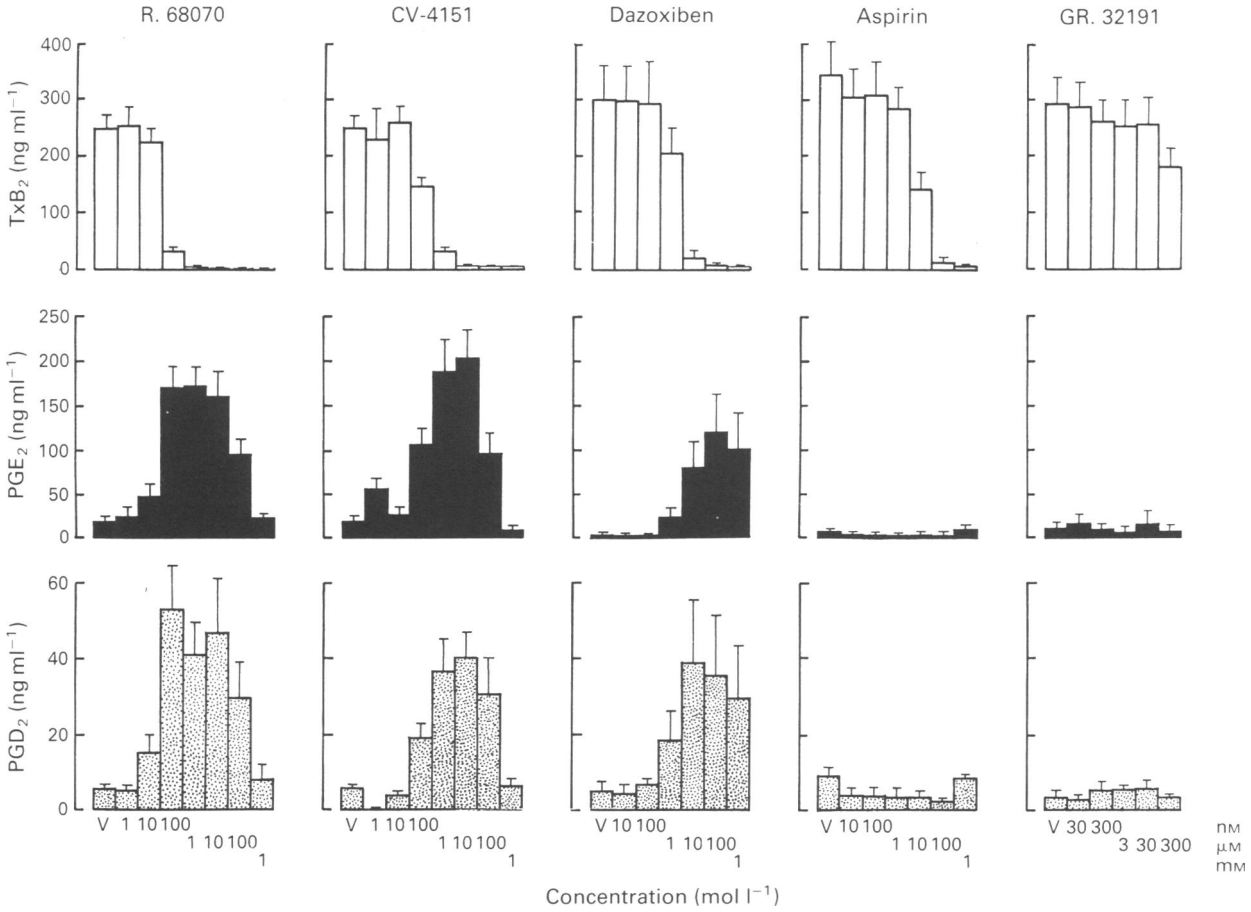

Figure 2 The effects of R.68070, CV-4151, dazoxiben, aspirin and GR32191 upon the concentration (ng ml^{-1} serum) of thromboxane B_2 (TxB_2), prostaglandin E_2 (PGE_2) and PGD_2 in human clotting whole blood. Columns show the mean from at least 3 experiments for each compound; vertical bars show s.e.mean. Responses are shown in the presence of increasing concentrations of each drug or vehicle (V) equivalent to the highest drug concentration.

by a maximally effective concentration of aspirin (Table 2). When higher concentrations (up to 100 μM) of R.68070 and CV-4151 were used, a second 'phase' of inhibition of collagen-induced aggregation was seen, the effect eventually exceeding that produced by aspirin, and in the case of R.68070, equalling that produced by GR32191 (Table 2). However, upon further increases in the concentration of R.68070 (1000 μM), the inhibitory effect upon collagen-induced aggregation was reversed towards that obtained with aspirin (Table 2).

Effect of combining GR32191 with either dazoxiben, R.68070 or CV-4151 upon collagen-induced platelet aggregation

The combination of TP-receptor blocking and thromboxane synthase inhibitory activities in both R.68070 and CV-4151 failed to improve upon the inhibitory action of a TP-receptor blocking drug alone (Table 2). It was therefore decided to test the 'combination' hypothesis by using separate drug entities to

Table 2 A comparison of the effect of GR32191, R.68070, CV-4151 and dazoxiben with that of aspirin upon collagen-induced platelet aggregation in human whole blood

	Collagen concentration-ratio[a] (CR) *Drug concentration* (μM)						
Drug	*Aspirin*[b] (2 mM)	0.01	0.1	1.0	10	100	1000
GR32191	4.1 (3.7–4.6)	1.8[c] (1.3–2.5)	4.9* (4.3–5.6)	7.2* (5.7–9.1)	6.0* (5.1–7.0)	NT	NT
R.68070	4.0 (3.2–5.0)	NT	2.4[c] (2.0–2.9)	2.5 (2.0–3.0)	4.8 (3.6–6.6)	7.3* (5.1–10.4)	4.7[c] (1.9–11.6)
CV-4151	4.3 (3.2–5.7)	1.4 (0.9–2.0)	1.9 (1.5–2.3)	2.1 (1.6–2.8)	2.7 (2.3–3.1)	5.4 (3.9–7.4)	NT
Dazoxiben	3.8 (3.2–4.6)	1.1 (0.8–1.6)	1.0[c] (0.9–1.04)	1.8 (1.5–2.3)	2.1 (1.8–2.4)	2.9[c] (1.8–4.8)	NT

[a] Collagen CR values are expressed as the geometric mean (95% confidence intervals) where values represent data from at least 10 experiments unless indicated otherwise ([c] = 3–6 experiments). [b] Data for aspirin are shown for each separate batch of experiments. NT = not tested.

* Significantly greater than aspirin ($P < 0.05$).

For each drug all values are compared with the corresponding aspirin value by means of Student's paired t test.

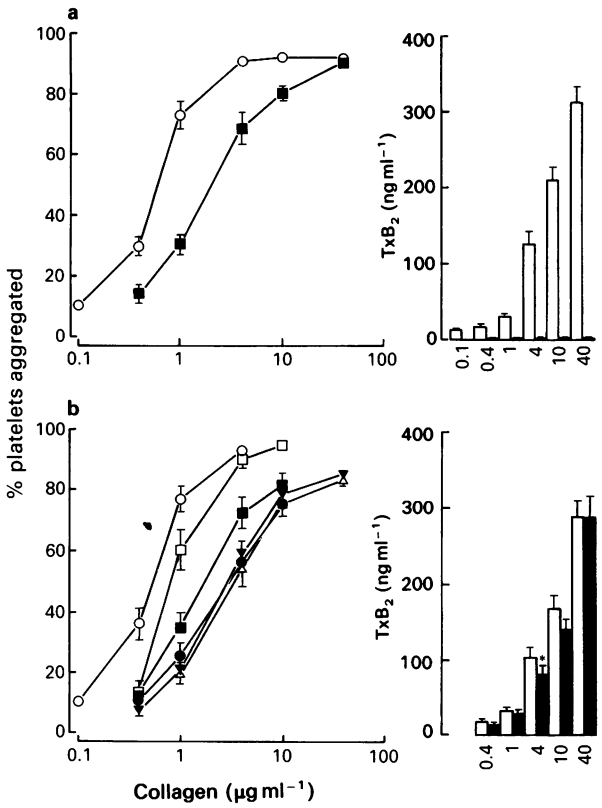

Figure 3 The effect of (a) aspirin and (b) GR32191 upon collagen-induced platelet aggregation and thromboxane B_2 (TxB_2) formation in human whole blood. In (a), the control concentration-effect curve (○) and that in the presence of aspirin (2 mM; ■) are the mean from 11 experiments with s.e.mean shown by vertical bars; the effect of aspirin (filled columns) upon control collagen-induced TxB_2 production (open columns) is taken from the same experiments. In (b), the effect of GR32191 (0.01, □; 0.1, ●; 1.0, ▼ and 10 μM, △) upon collagen-induced platelet aggregation are the mean from at least 6 experiments. The control and aspirin symbols are as shown in panel (a). The effect of GR32191 (10 μM; filled columns) upon collagen-induced TxB_2 production (open columns) are taken from a separate ($n = 11$) series of experiments where vertical bars represent s.e.mean. * Significantly reduced compared to control ($P < 0.05$).

optimise the required level of each activity. Thus, a maximally effective, but specific, TP-receptor blocking concentration of GR32191 (10 μM) was combined with a maximally effective, but specific (see Figure 2), TxA_2 synthase inhibitory concentration of either dazoxiben (10 μM), R.68070 (1 μM) or CV-4151 (10 μM). The combination of GR32191 and dazoxiben resulted in a superior effect to aspirin or to either compound alone (Figure 4), a mean collagen CR value of 13.4 being obtained (Table 3). Similar effects were seen with both R.68070 and CV-4151 in combination with GR32191 (Table 3). The same

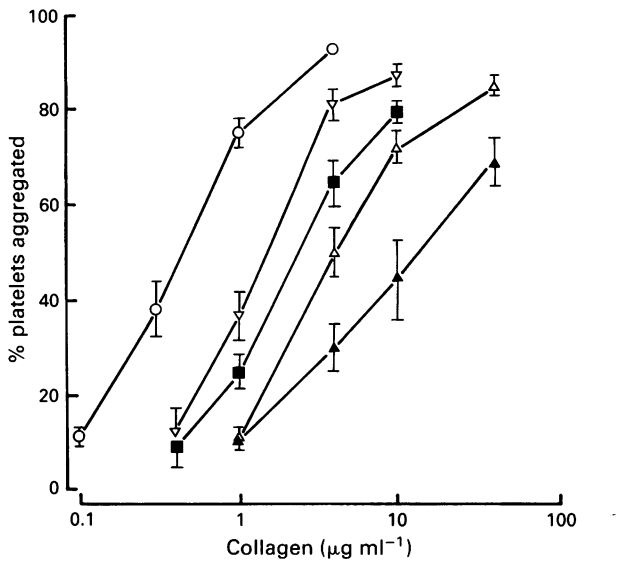

Figure 4 The effect of a combination of dazoxiben (10 μM) and GR32191 (10 μM) on collagen-induced platelet aggregation in human whole blood. Responses are shown in the absence (○) and presence of aspirin (■, 2 mM), dazoxiben (▽), GR32191 (△) and a combination of dazoxiben and GR32191 (▲). Each point is the mean of 4–6 determinations where vertical bars represent s.e.mean.

magnitude of inhibitory effect was also obtained even when a 100 fold lower concentration of GR32191 (0.1 μM) was combined with dazoxiben (10 μM) (mean collagen CR value 13.6 (7.9–23.2; $n = 8$)). The 'combination' CR values were not significantly different from each other, but significantly different ($P < 0.05$) from the CR value produced either by each compound alone, or that produced by aspirin (Table 3). In platelets pre-incubated with aspirin (2 mM), the superior effect of a combination of GR32191 with either R.68070 or dazoxiben was absent, the collagen concentration-ratios for the combination then being similar to those obtained with aspirin alone.

Discussion

The present study has compared the relative effectiveness of various drugs upon TxA_2-induced human platelet aggregation *in vitro*. The drugs studied, which interfere by different mechanisms with the action of TxA_2, were the TP-receptor blocking drug GR32191, the TxA_2 synthase inhibitor dazoxiben, the cyclo-oxygenase inhibitor aspirin and two compounds with both TP-receptor blocking and TxA_2 synthase inhibitory activity in the same molecule, namely R.68070 and CV-4151. Examination of the ability of each drug to block platelet TP-receptors and inhibit platelet TxA_2 synthase revealed a relatively high degree of specificity. Thus, GR32191 antagonized platelet aggregation induced by U-46619, but not that induced

Table 3 A comparison of the effect of GR32191 alone and in combination with either dazoxiben, R.68070 or CV-4151 upon collagen-induced platelet aggregation in human whole blood

TxSI (μM) used in combination with GR32191	*Collagen concentration-ratio (CR)* *Aspirin (2 mM)*	*GR32191 (10 μM)*	*TxSI alone*	*TxSI + GR32191*
Dazoxiben (10)	4.2 (3.8–4.7)	6.0 (5.1–7.0)	2.1 (1.8–2.4)	13.4* (11.1–16.0)
R.68070 (1.0)	4.5 (3.5–5.7)	6.2 (3.6–10.4)	2.4 (1.8–3.1)	16.1* (9.9–26.0)
CV-4151 (10)	5.3 (3.4–8.4)	6.7 (4.1–10.8)	2.5 (2.1–2.9)	14.3* (7.2–28.3)

Collagen CR values shown are geometric mean values (95% confidence interval) from at least 8 experiments. TxSI = TxA_2 synthase inhibitor.
* Significantly greater than either aspirin or each drug alone ($P < 0.05$).

by ADP. GR32191 was the most potent TP-receptor blocking drug tested ($pA_2 = 8.2$), being some 500 and 3000 times more potent than R.68070 and CV-4151 respectively in human whole blood. Despite their low potency, R.68070 and CV-4151 were specific platelet TP-receptor antagonists. In contrast, dazoxiben displayed no antagonist effect against U-46619. In terms of TxA_2 synthase inhibitory activity, R.68070 was the most potent compound tested ($IC_{50} = 40$ nM), being 3 times more active than CV-4151 and 30 times more active than dazoxiben. Both the TxA_2 synthase inhibitory and TP-receptor blocking potencies of these drugs agree well with the published data (Terashita *et al.*, 1986; Imura *et al.*, 1988; De Clerck *et al.*, 1989). All three compounds were specific for thromboxane synthase, since concentrations of PGE_2 and PGD_2 increased as TxB_2 levels decreased. However, at high concentrations of both R.68070 and CV-4151 (100 μM and above), PGE_2 and PGD_2 formation were also inhibited indicating a possible action of these two compounds at the cyclo-oxygenase enzyme. In contrast, GR32191 displayed negligible TxA_2 synthase inhibitory activity whilst aspirin produced an effect upon TxA_2 production consistent with cyclo-oxygenase inhibitory activity. Thus, the initial part of the study confirmed the pharmacological mode of action of each drug and we therefore went on to examine their ability to inhibit collagen-induced platelet aggregation.

Collagen is a major component of the sub-endothelium of blood vessels and is considered to play a physiologically important role in platelet activation (Baumgartner, 1977; Nievelstein & de Groot, 1988). When collagen is added to blood, platelets adhere to collagen fibrils and this contact phase is associated with activation of phospholipases C and A_2 and the release from membrane phospholipids of arachidonic acid (Blackwell *et al.*, 1977; Sano *et al.*, 1983). This is then converted to TxA_2 by the sequential action of the cyclo-oxygenase and TxA_2 synthase enzymes. However, aggregation to collagen is also mediated by platelet-derived adenosine diphosphate (ADP) (Kinlough-Rathbone *et al.*, 1977). The extent of the TxA_2-mediated component in the response to collagen can be revealed by using a maximally effective concentration of aspirin. Thus, aspirin produces an approximately 4 fold rightward displacement of the collagen aggregation concentration-effect curve. The remaining aggregation can be shown to be mediated predominantly by ADP (Kinlough-Rathbone *et al.*, 1977). In the present study GR32191, over the concentration-range 0.01–10 μM, resulted in a progressive rightward displacement of the collagen concentration-effect curve. At 10, 1 and even 0.1 μM GR32191 antagonism of collagen-induced aggregation was significantly greater than that of aspirin (2 mM). For this to occur, GR32191 must be affecting the ADP-induced component of collagen-induced aggregation, either directly or indirectly. However, GR32191 (10 μM) has been shown to be specific having no effect upon aggregation induced by ADP (Lumley *et al.*, 1989). It is most likely, therefore, that this additional inhibition seen with GR32191 is indirect, being induced by an endogenous anti-aggregatory agent or agents. Since the superior effect of GR32191 could be prevented by pretreating platelets with aspirin, this suggests the involvement of an anti-aggregatory cyclo-oxygenase product such as PGI_2 or PGD_2. Indeed, both prostaglandins can be detected following collagen-induced aggregation of human platelets (Orchard *et al.*, 1985). Whereas aspirin will inhibit the production of these anti-aggregatory prostanoids, GR32191 has no such effect and therefore will allow their anti-aggregatory effects to be expressed. If PGI_2 and PGD_2 have anti-aggregatory activity *in vivo*, then by preserving their action, GR32191 would be predicted to have an important clinical advantage over aspirin (Lumley *et al.*, 1990).

The present study has also demonstrated a superior antagonist effect of a TP-receptor blocking drug upon collagen-induced aggregation when compared with that of a maximally effective concentration of a specific TxA_2 synthase inhibitor. The inability of this latter class of drugs to antagonize fully the TxA_2-induced component of collagen- or arachidonic acid-induced platelet aggregation has been reported by others (Bertele *et al.*, 1981; 1982; Fitzgerald *et al.*, 1985; Gresele *et al.*, 1987). The reason for such a weak inhibitory profile has been suggested to be due to accumulating PGH_2, which occurs in the presence of TxA_2 synthase inhibition. PGH_2 is itself a potent stimulator of the platelet TP-receptor, and can substitute for TxA_2 at TP-receptors to cause aggregation (Bertele *et al.*, 1981; Hornby & Skidmore, 1982). It has, however, been argued that the accumulating PGH_2 would be redirected to anti-aggregatory PGI_2 and PGD_2 which would augment the effect of reduced TxA_2 formation with TxA_2 synthase inhibitors (Vermylen *et al.*, 1981; Defreyn *et al.*, 1982). Evidence for a rediversion of prostaglandin endoperoxides to PGI_2 has been demonstrated following administration of TxA_2 synthase inhibitors to man (Fitzgerald *et al.*, 1983). Despite this effect, the clinical studies performed to date with this class of drugs have failed to demonstrate any marked anti-thrombotic effect (Fiddler & Lumley, 1990). The efficacy of the TxA_2 synthase inhibitors would therefore appear to depend upon the balance between the pro-aggregatory effect of PGH_2 and the anti-aggregatory effect of PGI_2 and PGD_2.

Evidence consistent with such a pro-aggregatory effect of PGH_2 reducing the full anti-aggregatory potential of a TxA_2 synthase inhibitor has been obtained in the present study. Thus, when GR32191 was combined with a maximally effective concentration of dazoxiben, a significant potentiation of the inhibitory effect of the latter compound was observed. The collagen curve was displaced markedly to the right of that in the presence of either aspirin or GR32191. As discussed above in terms of the enhanced platelet inhibitory effect of GR32191, this effect of the combination of drugs involves inhibition of the non-TxA_2-mediated component of collagen-induced aggregation. Since the concentrations of dazoxiben and GR32191 used were without effect themselves upon ADP-induced aggregation, and since the enhanced effect could be largely prevented by aspirin treatment of platelets, these findings are again consistent with the action of PGI_2 and/or PGD_2. It has in fact been reported (Orchard *et al.*, 1985) that concentrations of both PGI_2 and PGD_2 are enhanced when platelet aggregation is induced with collagen in the presence of dazoxiben *in vitro*.

Both R.68070 and CV-4151, up to concentrations of 1 and 10 μM respectively, produced rightward displacements of the collagen concentration-effect curve which were less than that achieved with aspirin. At these concentrations, the compounds produced a maximum inhibition of TxB_2 production. Interestingly, the magnitude of the inhibitory effect against collagen-induced aggregation of the compounds was very similar to that produced by maximally effective concentrations of dazoxiben (10–100 μM). Thus, it is suggested that up to concentrations of 1 and 10 μM R.68070 and CV-4151 respectively, these drugs produce their effects against collagen-induced aggregation solely through TxA_2 synthase inhibition. When 10 and 100 μM R.68070 were employed, an effect against collagen-induced aggregation equivalent to aspirin and GR32191 was obtained with the two concentrations respectively. Since inhibition of TxA_2 synthesis was already maximal, the effect of R.68070 was most probably due to TP-receptor blocking activity, which would be present at these concentrations. In the case of CV-4151, a weaker effect than that achieved with R.68070 was always observed, probably reflecting the weaker TP-receptor blocking potency of the compound.

R.68070 and CV-4151 possess both TxA_2 synthase inhibitory and TP-receptor blocking activity. Whilst this latter action is relatively weak for both compounds, it would have been anticipated that if a sufficiently high concentration of each drug were used, a superior effect to GR32191 alone and one approaching that of GR32191 and dazoxiben would have been achieved. This was not the case. In fact, a high concentration of R.68070 produced smaller inhibition of collagen-induced aggregation. The most likely explanation for this

effect is the lack of specificity of high concentrations of R.68070 for TxA_2 synthase. This was indicated by the reduced formation of both PGE_2 and PGD_2 in human clotting blood with 100 and 1000 μM of R.68070 and CV-4151. Thus, as the concentrations of R.68070 and CV-4151 are increased, so cyclo-oxygenase becomes inhibited and the peak concentrations of PGI_2 and/or PGD_2 may actually fall, resulting in a reduced anti-aggregatory effect. In keeping with this hypothesis, when a lower, but still maximal and specific, TxA_2 synthase inhibitory concentration of each drug was combined with a maximal TP-receptor blocking concentration of GR32191, an inhibition of collagen-induced aggregation equivalent to that of the combination of GR32191 and dazoxiben was seen. The effect, where examined, was also greatly reduced by aspirin pretreatment. Thus, in the present *in vitro* study, the non-specific effect of both R.68070 and CV-4151 meant that their true anti-aggregatory potential could not be realised. In the presence of TxA_2 synthase inhibition, the redirection of PGH_2 to anti-aggregatory prostanoids in whole blood *in vitro* is limited by the absence of a major source of prostacyclin synthase. However, *in vivo* it is conceivable that more of the accumulating PGH_2 would be redirected to PGI_2, thus enhancing the anti-aggregatory potential of the combined TP-receptor blocker/TxA_2 synthase inhibitors.

In summary, the effectiveness of various classes of compounds which interfere with the formation and action of TxA_2 have been examined upon collagen-induced human platelet aggregation. At maximal, but specific concentrations, the order of effectiveness was GR32191 combined with either dazoxiben, R.68070 or CV-4151, > GR32191 = R.68070 > CV-4151 > aspirin > dazoxiben. Thus, the concept of a combination of TxA_2 synthase inhibitory and TP-receptor blocking activity being superior to either agent alone has been substantiated. However, R.68070 and CV-4151, compounds which possess both activities, were not able to achieve the maximum possible effect, most probably due to a non-specific action on cyclo-oxygenase when used at the high concentrations necessary to achieve TP-receptor blockade. It remains to be seen in clinical studies whether either drug can produce a superior anti-thrombotic effect to a specific TP-receptor blocking drug such as GR32191.

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