Br. J. Pharmac. (1985), **86**, 497–504

CGS 12970: a novel, long acting thromboxane synthetase inhibitor

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1 CGS 12970 is a potent selective inhibitor of human platelet thromboxane synthestase *in vitro* (IC_{50} = 12 nM). It is four orders of magnitude less potent as an inhibitor of sheep seminal vesicle cyclo-oxygenase, bovine aorta prostacyclin synthetase and human leucocyte 15-lipoxygenase.

2 The compound inhibited collagen-induced thromboxane B_2 production by human platelets *in vitro* without an effect on the accompanying platelet aggregation induced by collagen, ADP, platelet activating factor, thrombin, arachidonic acid or the prostaglandin mimetic, U 46619.

3 Administration of CGS 12970 to rats inhibited collagen-induced thromboxane B_2 production but had no effect on platelet aggregation *ex vivo*. It also had no effect on platelet aggregation induced by thrombin or on plasma clotting times.

4 A single oral dose of 1 or 3 mg kg^{-1} to rabbits inhibited thromboxane B_2 production in clotting blood *ex vivo* for 12 or 24 h respectively.

5 CGS 12970 inhibited thromboxane B_2 production *in vivo* induced by intravenous administration of collagen to rats or calcium ionophore to guinea-pigs. In both cases there was a concomitant elevation of immunoreactive 6-keto-prostaglandin $F_{1\alpha}$ but no effect on the induced thrombocytopenia.

6 As with other thromboxane synthetase inhibitors, CGS 12970 prolonged tail bleeding time in the rat. However, CGS 12970 was not as potent as other thromboxane synthetase inhibitors in this test.

7 In addition to these usual effects of thromboxane synthetase inhibitors, CGS 12970 inhibited the thrombocytopenia induced by the Forssman reaction or ADP administration. In these tests the effect of the compound was of short duration.

8 CGS 12970 had no effect on the thrombocytopenia associated with the Arthus reaction which distinguishes it from cyclo-oxygenase inhibitors. It also had no effect on thrombus formation on a cotton thread in an arteriovenous shunt in the rat.

Introduction

Thromboxane A_2 is produced in large quantities when platelets are activated by a variety of agonists. This metabolite of arachidonic acid via the cyclo-oxygenase and thromboxane synthetase pathway is a potent platelet agonist and a vasoconstrictor (Needleman *et al.*, 1976a). The physiological role of thromboxane A_2 is at present unclear since very few biological effects of selective thromboxane synthetase inhibitors have been described apart from the obvious and usually very marked inhibition of thromboxane production as adjudged by assay of the inert metabolite, thromboxane B_2 (Parry, 1983). Due to the complexity of different disease development processes there is an obvious need for thromboxane synthetase inhibitors with different pharmacological and pharmacodynamic profiles. In this paper we describe the pharmacological effects of a novel thromboxane synthetase inhibitor with a long duration of action.

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Methods

Animals

Guinea-pigs (300–500 g), Dunkin-Hartley strain, were purchased from Porcellus, Heathfield, Sussex.

Rabbits (3–4 kg) were hybrids (¼ Lop, ¾ New Zealand White) and were bred at the Horsham Research Centre. COB Wistar rats (200–500 g) were purchased from Charles River Ltd, Manston, Kent.

Enzyme assays

Human platelet thromboxane synthetase was prepared by the method of Needleman *et al.* (1976b) and assayed by a radiometric thin layer chromatographic method (Ku *et al.*, 1983). Prostaglandin H_2 (PGH_2) synthetase (i.e. cyclo-oxygenase) was prepared from sheep seminal vesicles (Takaguchi *et al.*, 1971) and assayed by a similar radiometric thin layer chromatographic method (Ku *et al.*, 1983).

Prostacyclin synthetase was prepared from bovine aorta (Gryglewski *et al.*, 1976) and assayed as described by Ku *et al.* (1983).

Lipoxygenase was obtained from human leucocytes by a modification of the method of Hulliger & Blazkovec (1967) and assayed by the method of Ku *et al.* (1983).

Preparation of platelet-rich plasma

Human blood was obtained from the antecubital vein of healthy volunteers who denied taking any medication within two weeks of venepuncture. It was anticoagulated with 0.1 volumes acid-citrate dextrose (Aster & Jandl, 1964) and centrifuged at 250 *g* for 20 min at room temperature to prepare platelet-rich plasma.

Guinea-pig or rat blood was obtained by cardiac puncture under ether anaesthesia. It was anticoagulated with 0.1 volumes 3.8% w/v trisodium citrate and platelet-rich plasma was prepared (Butler *et al.*, 1979a).

Preparation of serum samples

Blood was withdrawn from the marginal ear vein of rabbits and allowed to clot at 37°C for 60 min before being centrifuged at 10,000 *g* for 2 min. The serum was frozen on dry-ice and stored at −20° until the radioimmunoassay of thromboxane B_2 was carried out.

Platelet aggregation and clotting times

Platelet aggregation was measured in a Payton dual channel aggregation module (Butler *et al.*, 1979a). It was quantified by measuring the rate of aggregation at a minimum of six different concentrations of each agonist.

When samples were required for thromboxane B_2 determination, the reaction was stopped by mixing the contents of the cuvette (0.4 ml) with 10 mg ml^{-1} indomethacin in methanol (20 µl) and centrifuging to remove the platelets at 10,000 *g* for 2 min (Butler *et al.*, 1982).

Clotting times were assessed by the reagents and methods of Dade Diagnostics Inc. (Miami, U.S.A.) using a Burkhard Coagulometer (Uxbridge).

Radioimmunoassay of arachidonic metabolites

Radioimmunoassays of thromboxane B_2 and 6-keto-prostaglandin $F_{1\alpha}$ were performed as described by Butler *et al.* (1982). The lower limits of detection were 50 pg ml^{-1} in both cases.

Animal models

Collagen-induced thrombocytopenia, with concomitant measurement of thromboxane B_2 and of 6-keto-prostaglandin $F_{1\alpha}$ was assessed in rats by the method of Maguire & Wallis (1983).

Thromboxane B_2 and 6-keto-prostaglandin $F_{1\alpha}$ production induced by the calcium ionophore, A 23187 (Calbiochem) in guinea-pigs were assessed by the method of Ku *et al.* 1983.

Rat tail-bleeding time was measured by a modification of the transection method of Dejana *et al.* (1979) (Butler *et al.*, 1982). The methods of assessing effects on the thrombocytopenia induced by the Forssman reaction (Butler & White, 1980a), the Arthus reaction (Butler *et al.*, 1979b) and intravenous administration of adenosine 5′-pyrophosphate (ADP; Butler & White, 1980b) in guinea-pigs have been described previously.

Thrombus formation on a cotton thread in an arteriovenous shunt in the rat *in vivo* was measured as the wet weight of the thrombus after 15 min of blood flow (Smith & White, 1982).

Statistical analysis

Statistical analysis was carried out by Student's *t* test.

Materials

CGS 12970, 3-methyl-2 (3-pyridyl)-1-indoleoctanoic acid, was synthesized by Dr B. Renfroe, CIBA-GEIGY Corporation, Ardsley, N.Y. and dissolved in polyethylene glycol 400 (PEG 400) for oral administration to laboratory animals. Platelet activating factor was synthetic 1-0-hexadecyl-2-0-acetyl-sn-glyceryl-3-phosphorylcholine from CIBA-GEIGY Ltd, Basle, Switzerland and was prepared for use as previously described (Ambler & Wallis, 1983). The following drugs were also used: ADP, arachidonic acid, 5-hydroxytryptamine (5-HT), thrombin (all obtained from Sigma) and U 46619 (9,11-dideoxy,9α-11α-methanoepoxy-$PGF_{2\alpha}$; Upjohn Diagnostics).

Table 1 Effect of CGS 12970 on arachidonate metabolizing enzymes *in vitro*

Enzyme	IC_{50} (μM)
Platelet thromboxane synthetase	0.012
Seminal vesicle cyclo-oxygenase	310
Aorta prostacyclin synthetase	100
Leucocyte 15-lipoxygenase	450

Results

Arachidonic acid metabolizing enzymes in vitro

CGS 12970 potently inhibited human platelet thromboxane synthetase. Concentrations some 4 orders of magnitude higher were needed to inhibit seminal vesicle cyclo-oxygenase, bovine aorta prostacyclin synthetase or human leucocyte lipoxygenase (Table 1).

Thromboxane B_2 formation and platelet aggregation in vitro *(human)*

CGS 12970 inhibited thromboxane B_2 biosynthesis in human platelets when endogenous arachidonate was mobilized by the addition of collagen (Horm) *in vitro* (Figure 1). The effect was both to shift the collagen concentration-response curve to the right and to depress the maximum thromboxane B_2 production achievable at high collagen concentration. In these experiments there was no effect on the platelet aggregation at any of the collagen concentrations used (Figure 1). Similarly there was no effect on human platelet aggregation induced by the other agonists: ADP, arachidonic acid, platelet activating factor, 5-HT, thrombin or the prostaglandin mimetic, U 46619, at concentrations up to 140 μM, when these were each measured as full agonist concentration-response curves.

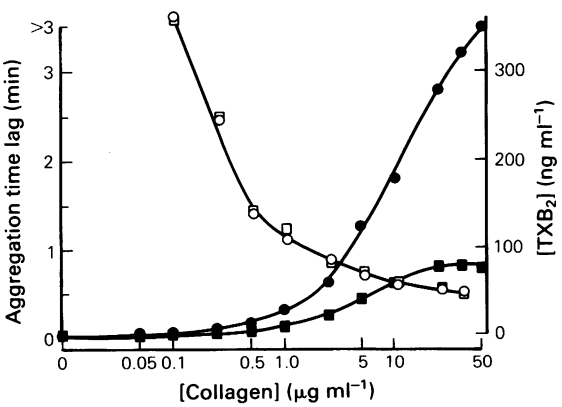

Figure 1 The effect of CGS 12970 on collagen-induced thromboxane B_2 (TXB_2) formation and platelet aggregation induced by collagen in human platelets *in vitro*. CGS 12970 (14 μM) or saline, was incubated with the platelet-rich plasma for 1 min before the addition of collagen at various concentrations. After measurement of the platelet aggregation, the same samples were used for thromboxane B_2 radioimmunoassay. (○) Platelet aggregation in control samples, (□) platelet aggregation in the presence of CGS 12970, (●) thromboxane B_2 concentration in controls, (■) thromboxane B_2 concentration in the presence of CGS 12970.

Thromboxane B_2 formation, platelet aggregation and coagulation times ex vivo *(rat)*

A single oral dose of CGS 12970 1 mg kg^{-1} to rats inhibited the thromboxane B_2 formation but had no effect on the platelet aggregation induced by collagen (Figure 2). The effect was characterized by almost complete suppression of thromboxane B_2 production at all collagen concentrations used.

There was also no effect at 3 mg kg^{-1} p.o. on platelet aggregation induced by thrombin, when this was measured in full agonist concentration-response studies.

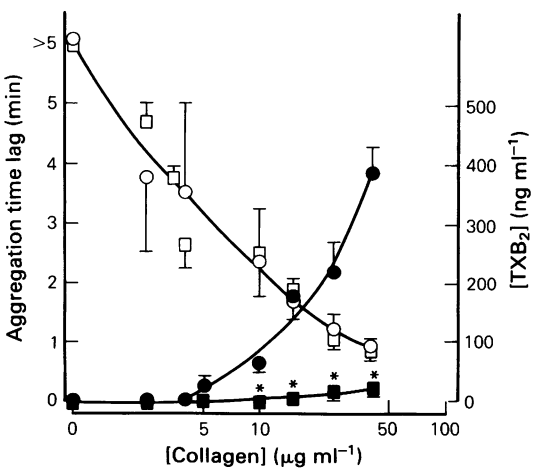

Figure 2 The effect of CGS 12970 (1 mg kg^{-1} p.o) on collagen-induced thromboxane B_2 (TXB_2) formation and platelet aggregation induced by collagen in rat platelets *ex vivo*. Platelet-rich plasma was prepared from rats treated with either polyethylene glycol 400 (PEG 400) alone or CGS 12970 in PEG 400 (1 mg kg^{-1} p.o.). Collagen-induced platelet aggregation was measured and thromboxane B_2 concentration was determined in the same samples. (○) Platelet aggregation in control samples, (□) platelet aggregation in treated animals, (●) thromboxane B_2 concentration in control samples, (■) thromboxane B_2 concentration in treated animals. Results are mean with vertical lines showing s.e.mean ($n = 5$); $^*P < 0.05$.

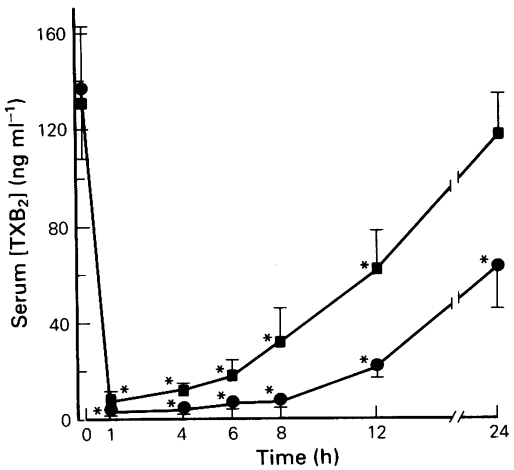

Figure 3 Time course of the effect of CGS 12970 on thromboxane B_2 (TXB_2) production in clotting blood in rabbits *ex vivo*. CGS 12970 was administered orally immediately after the first blood sample was taken, at 1 mg kg^{-1} (■) or 3 mg kg^{-1} (●). The blood was allowed to clot for 60 min at 37°C before the serum was removed for assay. Thromboxane B_2 concentrations were measured by radioimmunoassay. Results are mean and vertical lines show s.e.mean ($n = 5$); *$P < 0.05$.

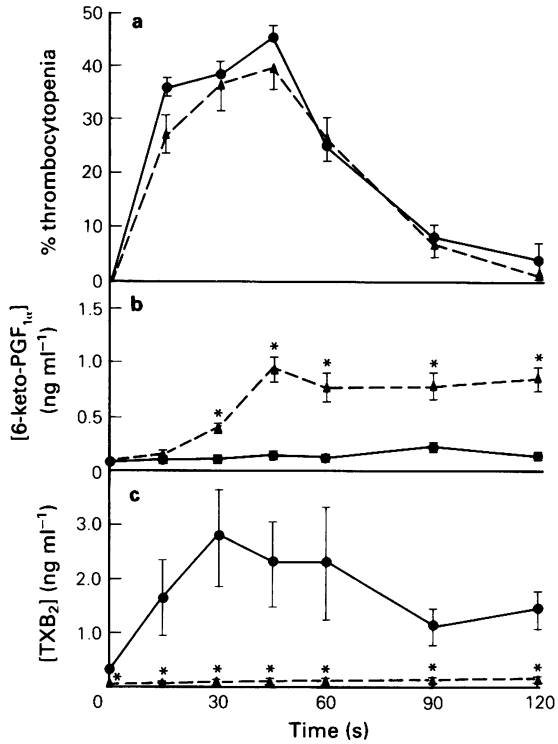

Figure 4 The effect of CGS 12970 (3 mg kg^{-1} p.o.) on collagen-induced thrombocytopenia (a) and plasma thromboxane B_2 (TXB_2) (b) and 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) (c) concentrations *in vivo*. Two samples were taken for measurement of each parameter before collagen (100 μg kg^{-1} i.v.) was administered at time 0. (●) Control animals, (▲) animals treated with CGS 12970 (3 mg kg^{-1} p.o.) 1 h before the experiment. Results are mean and vertical lines show s.e.mean ($n = 5$). *$P < 0.05$.

There was no effect on prothrombin time or activated partial thromboplastin time when rats received 30 mg kg^{-1} p.o. 1 h before the blood sample was taken.

Thromboxane B_2 formation ex vivo *(rabbit)*

A single oral dose of CGS 12970 (1 or 3 mg kg^{-1}) to rabbits inhibited thromboxane B_2 formation in clotting blood *ex vivo* by 94% or 98% respectively, when this was measured after 1 h. At a dose of 1 mg kg^{-1}, thromboxane B_2 synthesis was significantly inhibited for 12 h but not for 24 h, whereas at a dose of 3 mg kg^{-1} synthesis was inhibited for 24 h (Figure 3).

Table 2 Effect of oral administration of CGS 12970 on thromboxane B_2 and 6-keto-prostaglandin $F_{1\alpha}$ concentrations after i.v. administration of calcium ionophore, A 23187 (0.5 mg kg^{-1}) to rats

Experiment no.	*Treatment* (mg kg^{-1} p.o.)	*Thromboxane B_2* (ng ml^{-1})	*6-keto-Prostaglandin $F_{1\alpha}$* (ng ml^{-1})
1	Vehicle alone	44.7 ± 12.2	1.10 ± 0.74
	0.3	6.81 ± 3.15*	15.9 ± 5.61*
	1.0	5.61 ± 3.79*	19.1 ± 5.45*
	2.0	0.71 ± 0.51*	6.18 ± 1.81*
2	Vehicle alone	60.0 ± 5.16	1.34 ± 0.23
	0.10	4.51 ± 1.11*	3.63 ± 0.88*
	0.20	1.52 ± 0.91*	4.85 ± 1.68*
	0.40	0.60 ± 0.28*	6.36 ± 1.61*

*$P < 0.05$ compared to vehicle alone; $n = 5$ per group.

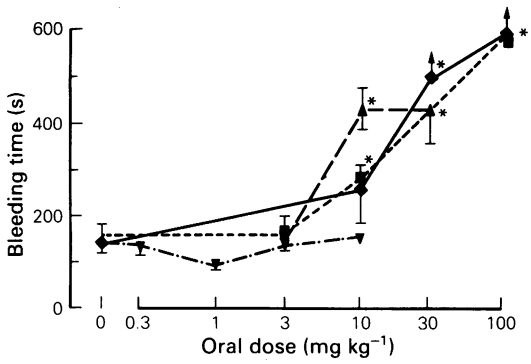

Figure 5 The effect of CGS 12970 on rat tail bleeding time. Comparison with reference compounds. Tail bleeding time was measured in groups of 5 rats after oral treatment for 1 h with either CGS 12970 (–◆–) N(7-carboxyheptyl) imidazole (--■--), OKY 1555 (–▲–), or indomethacin (- –▼- –). Arrows indicate groups containing a result where bleeding had not ceased at the end of the experiment (600 s). Results are mean and vertical lines show s.e.mean ($n = 5$). $*P < 0.05$.

Thrombocytopenia and arachidonate metabolism induced by collagen in vivo *(rat)*

Intravenous administration of collagen to rats causes a rapidly reversible loss of platelets from the circulation, as monitored in serial blood samples (Figure 4). Oral administration of CGS 12970 (3 mg kg^{-1}) to the animals 1 h before the experiment had no effect on the thrombocytopenia but reduced the immunoreactive thromboxane B_2 in the same plasma samples to undetectable concentrations. Concomitantly there was an increase in immunoreactive 6-keto-prostaglandin $F_{1\alpha}$ in the samples.

Arachidonate metabolism induced by A 23187 in vivo *(rat)*

Intravenous administration of the calcium ionophore, A 23187 induces thromboxane synthesis in the blood. This can be assessed by taking blood samples 2 min after the administration.

Oral doses of CGS 12970 given 2 h before the calcium ionophore inhibited thromboxane B_2 formation and again caused an increase in immunoreactive 6-keto-prostaglandin $F_{1\alpha}$. In these experiments CGS 12970 significantly inhibited thromboxane formation at doses as low as 0.1 mg kg^{-1} p.o. (Table 2).

Tail bleeding time in vivo *(rat)*

CGS 12970 prolonged tail bleeding time in the rat at oral doses of 30 mg kg^{-1} or greater. Higher doses caused bleeding to be prolonged beyond the predetermined time limit for the measurement (10 min). Other thromboxane synthetase inhibitors showed a similar effect (Figure 5).

Thrombocytopenia induced by the Forssman reaction in vivo *(guinea-pig)*

Unlike other inhibitors of arachidonate metabolism, CGS 12970 inhibited the thrombocytopenia induced by the administration of Forssman antiserum to guinea-pigs (Table 3). A dose of 30 or 100 mg kg^{-1} p.o. was required to produce the effect. The inhibitory effect of CGS 12970 was detectable 1 h after administration, but not at 2 h or longer times. Other thromboxane synthetase inhibitors and indomethacin did not show this effect.

Thrombocytopenia induced by ADP in vivo *(guinea-pig)*

CGS 12970 also inhibited the thrombocytopenia induced by the intravenous administration of ADP to

Table 3 A comparison of CGS 12970 with reference compounds as inhibitors of thrombocytopenia induced by the Forssman reaction in guinea-pigs

	Oral dose (mg kg^{-1})			
	10	30	100	300
Treatment		*% inhibition of thrombocytopenia*		
CGS 12970	28	61*	55*	30
CGS 13080	–	–	0	0
N (7-Carboxyheptylimidazole)	–	–	2	32
OKY 1555	–	37	39*	12
Dazoxiben	–	–	33	8
Indomethacin	–	30	–	–

(–): Not determined; $*P < 0.05$; $n = 5$ per group.

Table 4 Inhibition of ADP-induced thrombocytopenia in guinea-pigs by CGS 12970 and other inhibitors of the prostaglandin pathway

	Oral dose ($mg\,kg^{-1}$)		
	30	100	300
Treatment	*% inhibition of thrombocytopenia*		
CGS 12970	7	41*	11
CGS 13080	–	0	1
N (7-Carboxyheptyl) imidazole	–	9	19
OKY 1555	–	17	7
Dazoxiben	–	0	0
Indomethacin	0	–	–

(–): not determined; $*P < 0.05$; $n = 5$ per group.

guinea-pigs (Table 4). The effect occurred at a comparatively high oral dose ($100\,mg\,kg^{-1}$) but neither other thromboxane synthetase inhibitors nor indomethacin shared this effect. The inhibitory effect of CGS 12970 was only observed 1 h after administration and not at longer times.

Thrombocytopenia induced by the Arthus reaction in vivo *(guinea-pigs)*

At doses up to $300\,mg\,kg^{-1}$ p.o. CGS 12970 had no effect on the thrombocytopenia induced by the Arthus reaction *in vivo* in the guinea-pig, when this was tested 1 h after administration of the compound.

Thrombus formation in vivo *(rat)*

CGS 12970 had no significant effect on thrombus formation on a cotton thread in an arteriovenous shunt at $100\,mg\,kg^{-1}$ p.o.

Discussion

CGS 12970 is a potent orally active thromboxane synthetase inhibitor with a long duration of action in experimental animals. *In vitro* it exhibits considerable selectivity for the thromboxane synthetase compared to other enzymes of eicosanoid metabolism. In whole human platelets it inhibits thromboxane B_2 production induced by collagen and when examined *ex vivo* in rabbits, oral doses of the compound inhibit thromboxane B_2 (TXB_2) production in clotting blood for several hours. *In vivo* CGS 12970 shares many pharmacological properties seen with other thromboxane synthetase inhibitors in experimental animal models.

The finding that CGS 12970 effectively inhibited collagen-induced TXB_2 formation *in vitro* and yet had no effect on platelet aggregation induced by any agonist is consistent with the previous demonstration that PGG_2 and PGH_2 are also agonists of platelet aggregation (Needleman *et al.*, 1976a). CGS 12970 also has no significant effect on the thrombocytopenia induced by collagen in rats *in vivo*. In this regard, it behaves as another thromboxane synthetase inhibitor, dazoxiben, but unlike CGS 13080 which was shown to possess antithrombocytopenic activity (Maguire & Wallis, 1983). Although the effects of thromboxane synthetase inhibitors on the thrombocytopenia in this model are frequently small and statistically insignificant, there are indications of a more pronounced effect during the recovery phase of the reaction which suggests a possible dependence on the circulating prostacyclin level which is slow to reach maximum (Maguire & Wallis, 1983) (Figure 4).

The property of all thromboxane synthetase inhibitors to prolong the tail arteriolar bleeding time (transection method) in rats (Butler *et al.*, 1982) is also shared by CGS 12970, although weaker in extent. In clinical studies, however, most thromboxane synthetase inhibitors were not shown to affect the capillary bleeding time in man (Tyler *et al.*, 1981; MacNab *et al.*, 1984). In so much that the effect is demonstrable in rats, the drug action of CGS 12970 must be mediated through the changes in relative concentrations of TXA_2 and PGI_2. The bleeding time prolongation is most likely a consequence of the effect exerted on the diameter of the cut vessels (vasodilation) rather than through an action on platelet aggregation itself (Butler *et al.*, 1982).

CGS 12970 has two properties that are not shared by other thromboxane synthetase inhibitors: its ability to inhibit thrombocytopenia induced by either intravenous administration of ADP or by the Forssman reaction in the guinea-pig. Although these effects occur at relatively high doses ($100\,mg\,kg^{-1}$ p.o.) of CGS 12970, it should be noted that the guinea-pig is a species that inherently requires much higher doses of

thromboxane synthetase inhibitors to produce an inhibitory effect on TXB_2 production (K.D. Butler & R.A. Shand, unpublished observations). The ability of CGS 12970 to inhibit platelet activation in such models is clearly not related to inhibition of thromboxane A_2 formation but the mechanism remains unknown at present.

Cyclo-oxygenase inhibitors are known to protect against thrombocytopenia associated with the Arthus reaction in guinea-pigs (Butler *et al.*, 1979b) whereas thromboxane synthetase inhibitors are rarely shown to be effective (K.D. Butler, 1983, unpublished observations). This activity difference is suggestive of PGG_2 and PGH_2 being the dominant mediators of thrombocytopenia rather than TXA_2.

Contrary to the hope that thromboxane synthetase inhibitors would be anti-thrombotics when the first lead compound was described (Moncada *et al.*, 1977) and in common with dazoxiben in a similar model in man (Dodd *et al.*, 1983), CGS 12970 has no effect in the extracorporeal shunt thrombosis model used in this study. In this extracorporeal shunt thrombosis model, cyclo-oxygenase inhibitors are marginally effective in that they inhibit thrombus formation by about 20 to 30% (Smith & White, 1982); CGS 12970 had no such effect.

The unique ability of CGS 12970 to inhibit ADP- and Forssman reaction-induced thrombocytopenia, coupled with its long duration of action (12–24 h) indicates the suitability of this compound for a wide range of acute and chronic pharmacological studies. While the effect of CGS 12970 in prevention of thrombosis remains to be demonstrated, it may be effective in conditions where vasoconstriction or vasospasm are caused by trauma, particularly where platelet activation is a contributing factor.

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(*Received May 3, 1985.*
Accepted July 8, 1985.)