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ZD9583, an Orally Effective Thromboxane A_2 Synthase Inhibitor and Receptor Antagonist with a Sustained Duration of Action in Rat and Dog

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Abstract

The thromboxane A_2 (TXA_2) synthase inhibitory activity and the TXA_2 receptor (TP-receptor) blocking action of ZD9583 ((4*Z*)-6-[(2*S*,4*S*,5*R*)-2-(1-[2-cyano-4-methylphenoxy]-1-methylethyl)-4-(3-pyridyl)-1,3-dioxan-5-yl]hex-4-enoic acid) has been evaluated in-vitro by use of whole blood and platelets from man, and ex-vivo by use of platelets and whole blood from rats and dogs.

ZD9583 caused concentration–dependent inhibition of human platelet microsomal TXA_2 production with an IC50 of $0{\cdot}017 \pm 0{\cdot}003$ μM; this inhibition was associated with an increase in prostaglandin E_2 (PGE_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) formation. ZD9583 also inhibited collagen-stimulated TXA_2 synthesis in whole blood from man, rat and dog giving IC50 values of $0{\cdot}027 \pm 0{\cdot}005$, $0{\cdot}02 \pm 0{\cdot}006$ and $0{\cdot}013 \pm 0{\cdot}01$ μM, respectively. The drug did not modify platelet cyclooxygenase activity as inhibition of thromboxane B_2 (TXB_2) formation was associated with a concomitant increased synthesis of prostaglandin D_2 (PGD_2), PGE_2 and $PGF_{2\alpha}$. ZD9583 had little effect on cultured human umbilical vein endothelial cell prostacyclin synthase giving an IC50 of $24{\cdot}2 \pm 4{\cdot}9$ μM. In-vitro ZD9583 caused concentration-dependent inhibition of U46619-induced aggregation responses of platelets from man, rat and dog, yielding apparent log A_2 values of $8{\cdot}7 \pm 0{\cdot}12$, $8{\cdot}8 \pm 0{\cdot}2$ and $9{\cdot}3 \pm 0{\cdot}2$, respectively. The drug was selective; at concentrations up to 100 μM it did not affect 5-hydroxytryptamine or the primary phases of adenosine diphosphate and adrenaline-induced aggregation. ZD9583 (100 μM) did not, furthermore, modify the platelet inhibitory effects of PGD_2, prostaglandin E_1 (PGE_1) and prostacyclin. Oral administration of ZD9583 (3–10 mg kg^{-1}) to both rats and dogs caused dose-dependant inhibition of collagen-stimulated TXA_2 production ex-vivo which persisted for up to 12 h. The drug also caused profound TXA_2 receptor blockade in both species for in excess of 12-h after an oral dose of 3 mg kg^{-1}. ZD9583 (3 mg kg^{-1}, p.o.), when administered to dogs over a five-day period at 12 h intervals, did not cause either tachyphylaxis or an accumulation of effect.

We conclude that ZD9583 is a potent, selective, orally active thromboxane synthase inhibitor and TXA_2 receptor antagonist.

Thromboxane A_2 (TXA_2) and prostacyclin are formed by the action of TXA_2 synthase and prostacyclin synthase, respectively, on a common substrate, prostaglandin endoperoxide H_2 (PGH_2), which in turn is the product of the action of cyclooxygenase on arachidonic acid. TXA_2 induces vasoconstriction and bronchoconstriction and plays a pivotal role in platelet activation; prostacyclin is the major vascular endothelial arachidonic acid metabolite and exerts effects opposing those of TXA_2. Localized disturbances in the natural balance of TXA_2 and prostacyclin synthesis have been identified as a major factor in the precipitation of the vascular occlusive events associated with a variety of thromboembolic disorders such as unstable angina and atherosclerosis (Majerus 1983); they might also contribute to the pathogenesis of glomerular injury and subsequent renal insufficiency (Patrono et al 1985; Pierucci et al 1989; DeRubertis & Craven 1992).

Several classes of drug are able to suppress the synthesis of TXA_2 or inhibit its action; the most notable of these are inhibitors of TXA_2 synthase and cyclooxygenase and TXA_2-receptor antagonists. Inhibition of cyclooxygenase by aspirin and other non-steroidal anti-inflammatory drugs (NSAID) reduces the formation of those eicosanoids that either initiate (TXA_2 and PGH_2) or inhibit (PGD_2 and prostacyclin) platelet aggregation. Inhibition of the synthesis of the anti-aggregatory prostanoids might reduce the therapeutic potential of this class of agent. To date, however, attempts to identify an aspirin dosage regimen to suppress platelet-derived TXA_2 selectively without compromising the synthesis of vascular endothelial prostacyclin have been unsuccessful (Patrono 1989; Chiabrando et al 1992).

Alternative approaches to inhibiting the deleterious effects of TXA_2 while conserving prostacyclin synthesis have been investigated; these include specific inhibition of TXA_2 synthase and blockade of the action of TXA_2 at its target receptor. Inhibition of TXA_2 synthase causes re-direction of PGH_2 metabolism resulting in the formation of increased levels of other prostanoids such as PGD_2, PGE_2, $PGF_{2\alpha}$ and prostacyclin. PGD_2 and prostacyclin both inhibit platelet function by stimulating adenylate cyclase and elevating intra-platelet cyclic AMP. The potential benefit derived from TXA_2 synthase inhibition is, however, negated to some extent by an attendant transient increase in levels of the unstable intermediate PGH_2, which can itself activate platelet and vascular TXA_2 receptors, thereby suppressing adenylate cyclase and causing platelet aggregation, and vasoconstriction.

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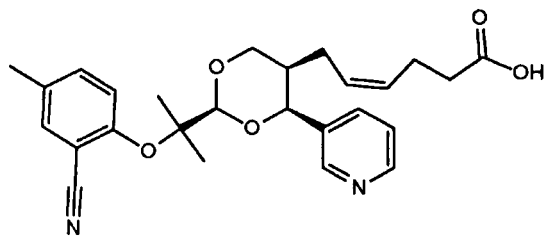

FIG. 1. Chemical structure of ZD9583 ((4*Z*)-6-[(2*S*,4*S*,5*R*)-2-(1-[2-cyano-4-methylphenoxy]-1-methylethyl)-4-(3-pyridyl)-1,3-dioxan-5-yl]hex-4-enoic acid).

TXA_2 antagonists block the activity of both TXA_2 and PGH_2 on platelet, vascular and bronchial smooth muscle receptors; they do not modulate arachidonic acid metabolism. Thus, these drugs only preserve, rather than increase, endogenous production of platelet inhibitory prostaglandins. Experimental data support the hypothesis that combination of a TXA_2 antagonist with a TXA_2 synthase inhibitor exerts antiplatelet effects superior to those of either agent alone (Bertele & de Gaetano 1982; Gresele et al 1984, 1987).

We have previously described the synthesis and in-vitro activity of a series of 2-substituted-4-(3-pyridyl)-1,3-dioxan-5-ylalkenoic acids, which have the dual properties of TXA_2 synthase inhibition and TP-receptor antagonism (Brownlie et al 1993; Faull et al 1995). The present study demonstrates that ZD9583 (Fig. 1), a leading compound from this series, when administered orally to laboratory animals is an effective inhibitor of TXA_2 synthase and also antagonizes TP-receptor-mediated responses.

Materials and Methods

ZD9583, synthesized at Zeneca Pharmaceuticals, was routinely solubilized in dimethylsulphoxide (DMSO) at 100 μM and diluted directly into assay buffer to give less than 0·1% DMSO final concentration. All reagents used were analytical grade and obtained from Sigma as were calcium ionophore (A23187), indomethacin, aspirin, arachidonic acid, TXB_2, 6-oxo$PGF_{1\alpha}$, and PGD_2, PGE_2 and $PGF_{2\alpha}$. Radioligands and RIA kits for PGD_2 and $PGF_{2\alpha}$ were purchased from Amersham. U46619((15*S*)-hydroxy-11α,9α-(epoxymethano)prosta-5*Z*,13*E*-dienoic acid; Bundy 1975) was obtained from Cayman Chemicals. Equine collagen fibrils were purchased from Hormon Chemie (Munich).

Human platelet microsomes

Preparation of human platelet microsomes and measurement of arachidonic acid metabolism was performed by use of modifications of the procedures described by Haworth et al (1982). Human venous blood was collected into 3·8% (w/v) trisodium citrate from an antecubital vein of healthy volunteers who had been drug-free for at least 7 days. Platelet-rich plasma (PRP) was prepared by differential centrifugation (200 *g*, 15 min) at room temperature. Platelets were harvested by centrifugation of PRP (600 *g*, 10 min), resuspended in Tris-HCl (50 μM, pH 8·0) containing EDTA (1 μM), disrupted by three successive cycles of freeze-thawing, and sonicated on ice by use of an ultrasonicator at maximum energy. A microsomal fraction was prepared by centrifugation of the disrupted platelet suspension (15 000 *g*, 20 min, 4°C) and centrifugation of the resultant supernatant (100 000 *g*, 100 min, 4°C). The microsomal pellet was resuspended in Tris-HCl (50 μM, pH 7·4) and stored in liquid nitrogen.

Measurement of arachidonic acid metabolism was performed by quantitative radio chromatography. [1-^{14}C]arachidonic acid (10 μM, 58·4 mCi mmol^{-1}) was incubated (30 min, 25°C) with human platelet microsomes in Tris-HCl buffer (50 μM, pH 7·4) containing haematin (10 μM) and L-tryptophan (5 μM) in the presence of vehicle or compound (final volume 0·2 mL). Reactions were terminated by the addition of ethyl acetate-1 M citric acid (0·5 mL, 19 : 1 v/v) and the extracted radioactive metabolites were separated by thin-layer chromatography (TLC; 0·2 mm silica gel). Chromatographs were developed twice in diethyl ether-acetic acid (9 : 1, v/v) and the separated metabolites were quantified by use of a Berthold TLC linear analyser. R_F values were determined by use of authentic radiolabelled eicosanoids. The potency of compounds was expressed as an IC50 value for the inhibition of TXA_2 biosynthesis.

Human whole blood

TXA_2 formation in whole blood was assessed by measuring its metabolically stable degradation product, TXB_2, by means of a specific radioimmunoassay (RIA) (Bailey et al 1983). The selectivity of thromboxane synthase inhibition was assessed by measuring the immunoactivity of PGE_2, PGD_2 and $PGF_{2\alpha}$ generated in the presence of drugs. Samples of citrated human whole blood, obtained as described above, were incubated (2 h, 37°C) in the presence of equine collagen fibrils (100 μg mL^{-1}) in a shaking water bath after pre-treatment (15 min, 37°C) with either vehicle or compound. Cellular debris was sedimented by centrifugation (25 000 *g*, 2 min) and indomethacin (1 μM) was added to the resulting cell-free plasma which was stored at − 20°C until subjected to specific RIA to determine the concentration of TXB_2, PGE_2 (Haworth & Carey 1986), $PGF_{2\alpha}$ and PGD_2 present in the sample. The potency of compounds was expressed as an IC50 value for the inhibition of TXB_2 biosynthesis.

Cultured human umbilical vein endothelial cells

Human umbilical cords were obtained within 12 h of normal delivery and primary cultures of human umbilical vein endothelial cells were prepared using the methodology described by Garcia et al (1988). Cultured human umbilical vein endothelial cells (1st–4th passage) were seeded (5×10^4 cells well^{-1}) on to 24-well plates (16 mm, CoStar, Cambridge, MA, USA), maintained at 37°C in humidified air containing 5% CO_2 and left to grow to 70–80% confluence. Cell monolayers were washed twice with medium (Gibco 199), pre-incubated with vehicle or compound (37°C, 15 min), stimulated with arachidonic acid (10 μM) and incubated for a further 2 h. Incubations were terminated and cell-free medium was aspirated into indomethacin (1 μM); samples were stored at − 20°C until subjected to RIA for 6-oxo$PGF_{1\alpha}$ (Forder & Carey 1983).

Preparation of platelet-rich plasma

Venous blood was collected from an antecubital vein of healthy volunteers who had been drug-free for at least 7 days into 3·2% (w/v) trisodium citrate. Male Alderley Park Wistar rats, 250–350 g, were anaesthetized with fluothane and bled

from the abdominal aorta into silicon-coated tubes containing heparin, 50 units mL^{-1}. Alderley Park beagle dogs, 17–25 kg, were bled from a jugular vein into 3·8% (w/v) trisodium (1 part to 9 parts whole blood). PRP was prepared by centrifugation (15 min, 25°C) at 200 *g* for human and rat blood. To maximize the yield of dog PRP two separate centrifugations (15 min, 25°C) were performed at 150 *g*.

Platelet aggregation studies in-vitro

Samples (0·25 mL) of PRP in siliconized glass tubes were stirred (900 rev min^{-1}) and pre-incubated (37°C) for 60 s in a Biodata aggregometer before addition of ZD9583 or vehicle. Platelets were incubated for a further 60 s before aggregation was initiated with one of a variety of agonists. Platelet aggregation was measured as an increase in light transmission, minimum and maximum light transmission having been set previously using PRP and platelet-poor plasma, respectively. Each aggregation response was allowed to proceed to its maximum; the change in light transmission in arbitrary units at this point was expressed as a percentage of the largest change achieved in the first control curve. A complete concentration-response curve was obtained for each agonist. Antagonist activity was calculated by determining concentration ratios from agonist EC50 values derived after a 60-s preincubation of PRP with either vehicle or ZD9583. Where appropriate, antagonist activity was expressed in terms of apparent log A_2 values.

In a separate series of experiments we assessed the effect of ZD9583 on the anti-aggregatory effects of PGE_1, prostacyclin and PGD_2. A single sub-maximum (EC70) concentration of adenosine diphosphate (ADP) was used to induce platelet aggregation. Concentrations of PGE_1, prostacyclin and PGD_2 which caused 50% inhibition of the aggregation induced by ADP were determined. Samples (0·25 mL) of PRP were then pre-incubated as previously described, for 30 s, before addition of vehicle or ZD9583 and incubated for a further 15 s before addition of PGE_1 (30 nM), prostacyclin (30 nM) or PGD_2 (20 nM); aggregation was finally initiated with ADP (2 μM) 60 s later.

Ex-vivo pharmacology

Groups of conscious fasted male Alderley Park Wistar rats, 250–350 g, were dosed by oral gavage with ZD9583 or vehicle. At scheduled times after administration of the drug the animals were anaesthetized with fluothane and bled from the abdominal aorta into silicon-coated tubes containing heparin (0·1 volume, 50 units mL^{-1}).

ZD9583 was dosed either orally or intravenously to groups of conscious Alderley Park beagle dogs, 17–25 kg. The animals were then bled from a jugular vein into 3·8% (w/v) trisodium citrate (1 part to 9 parts whole blood) at various intervals after the administration of the drug.

A portion of each blood sample was treated with collagen (100 μg mL^{-1}) for maximum stimulation of TXA_2 formation. PRP was prepared and platelet aggregation was measured using the same methods as those described above for the in-vitro studies. Changes in platelet sensitivity to U46619 resulting from the administration of ZD9583 to donor animals were expressed as concentration ratios which were obtained by comparing U46619 EC50 values in platelets from animals dosed with vehicle, to those from animals dosed with ZD9583. Platelet TXA_2 production was measured by radioimmunoassay, and values obtained from groups dosed with vehicle were compared with those from treated animals.

Expression of results

Concentration ratios were derived by comparing agonist EC50 values obtained in the presence and absence of ZD9583 and antagonist potency expressed as an apparent log A_2. Data are expressed as the arithmetic mean value ± s.e.m. from n experiments. The statistical significance of differences between the data was evaluated using Student's *t*-test. *P* values < 0.05 were considered statistically significant.

Results

Human platelet microsomes

ZD9583 (0·001–10 μM), when incubated with human platelet microsomes, caused concentration–dependent inhibition of TXB_2 formation with an IC50 of 0·017 ± 0·003 μM (n = 6). This effect was confirmed to be a result of selective inhibition of TXA_2 synthase by the observation that ZD9583, at a final concentration of 10 μM, did not cause significant inhibition of PGE_2 and $PGF_{2\alpha}$ formation. Indeed, the inhibition of TXB_2 formation by ZD9583 was associated with a concentration-dependent increase in $PGE_2/PGF_{2\alpha}$ production. Indomethacin proved to be an effective inhibitor of human platelet microsomal cyclooxygenase causing equipotent inhibition of TXB_2 and PGE_2 giving an IC50 of 0·17 ± 0·08 and 0·2 ± 0·05 μM, (n = 3), respectively (Table 1).

Whole blood

In human whole blood ZD9583 (0·01–1·0 μM) and aspirin (1·0–100 μM) caused concentration-dependent inhibition of collagen-stimulated TXA_2 production giving IC50 values of 0·027 ± 0·005 μM (n = 7) and 2·18 ± 0·68 μM (n = 4), respectively.

Inhibition of platelet cyclooxygenase by aspirin (10 μM) resulted in a reduction in TXB_2, PGE_2, $PGF_{2\alpha}$ and PGD_2 levels. In contrast, pre-incubation of human whole blood with ZD9583 (1 μM) caused profound inhibition of TXB_2 produc-

Table 1. Effect of ZD9583 and indomethacin on [1-^{14}C]-arachidonic acid metabolism by human platelet microsomes.

Drug	(M)	% Inhibition	
		Thromboxane B_2	Prostaglandin E_2/ prostaglandin $F_{2\alpha}$
ZD9583	0·001	24·4 ± 5·7	−229·7 ± 86·1
	0·01	63·8 ± 7·9	−507·2 ± 154·7
	0·1	79·1 ± 2·7	−591·6 ± 124·1
	1	79·9 ± 2·1	−616·1 ± 127·4
Indomethacin	0·001	1·1 ± 0·5	1·9 ± 0·6
	0·01	17·6 ± 6·3	7·2 ± 2·0
	0·1	44·0 ± 11·8	42·7 ± 4·1
	1	78·2 ± 3·0	68·5 ± 5·7
	10	86·6 ± 4·5	83·1 ± 3·3

Each value is the mean ± s.e.m. percentage inhibition of eicosanoid biosynthesis measured as TXB_2, PGE_2 and $PGF_{2\alpha}$ by quantitative radiochromatography in at least four individual human platelet microsome preparations. Negative values indicate an increase in eicosanoid production.

Table 2. Effects of ZD9583 and aspirin on collagen-induced eicosanoid production in human whole blood.

	Thromboxane B_2 (ng mL^{-1})	Prostaglandin E_2 (ng mL^{-1})	Prostaglandin $F_{2\alpha}$ (ng mL^{-1})	Prostaglandin D_2 (ng mL^{-1})
Vehicle	391·6 ± 51·0	6·5 ± 1·1	4·1 ± 0·6	1·2 ± 0·2
ZD9583 (1 mM)	8·3 ± 3·2***	329·2 ± 79·9**	63·5 ± 7·6*	11·2 ± 0·9***
Aspirin (10 mM)	73·8 ± 22·5***	1·7 ± 0·2**	0·4 ± 0·1*	0·5 ± 0·1*

Effect of ZD9583 and aspirin on collagen (100 $\mu g\ mL^{-1}$)-stimulated eicosanoid production in human whole blood. Data are expressed as the means ± s.e.m. values from four individuals and were determined by radioimmunoassay. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with vehicle (Student's t-test).

tion (391·6 ± 51·0 to 13·4 ± 4·8 ng mL^{-1}) and a concomitant increase in levels of PGE_2 (6·5 ± 1·1 to 329·2 ± 79·9 ng mL^{-1}), $PGF_{2\alpha}$ (4·1 ± 0·6 to 63·5 ± 7·6 ng mL^{-1}) and PGD_2 (1·15 ± 0·16 to 11·2 ± 0·85 ng mL^{-1}) (Table 2). In addition, ZD9583 inhibited collagen-stimulated TXB_2 production in rat and dog whole blood giving IC50 values of 0·02 ± 0·006 (n = 4) and 0·013 ± 0·01 μM (n = 4) respectively.

Human umbilical vein endothelial cells

Primary cultures of human umbilical vein endothelial cell monolayers spontaneously produced prostacyclin (9 ± 2·2 pg/10^3 cells, n = 5). Because the capacity of the human umbilical vein endothelial cells to synthesize prostacyclin diminished with progressive passage, only cultures which had undergone fewer than 5 passages were used in this study. Stimulation of human umbilical vein endothelial cells with arachidonic acid (10 μM) caused a marked increase in the amount of prostacyclin released (280 ± 52 pg/10^3 cells, n = 7). Pretreatment of human umbilical vein endothelial cells with indomethacin (0·01–10 μM) resulted in concentration-dependent inhibition of arachidonic acid-stimulated release of prostacyclin giving an IC50 value of 0·08 ± 0·02 μM. Unlike indomethacin, however, ZD9583 did not potently inhibit the arachidonic acid-stimulated release of prostacyclin from endothelial cells; the IC50 value was 24·2 ± 4·9 μM (n = 6).

Platelet aggregation

In-vitro, ZD9583 (100 μM) did not induce shape change or aggregation of platelets from man, rat or dog. ZD9583 (0·005–0·05 μM) caused concentration-dependent parallel shifts to the right of U46619 aggregation curves for PRP from man. Evaluation of these data gave an apparent log A_2 value of 8·7 ± 0·12 (n = 4). ZD9583, when added to human PRP in-vitro at a concentration greater than 0·1 μM, caused insurmountable blockade of U46619 responses (Fig. 2). In addition, ZD9583 also inhibited U46619-induced aggregation of rat and dog PRP giving apparent log A_2 values of 8·8 ± 0·2 (n = 6) and 9·3 ± 0·2 (n = 4), respectively.

ZD9583 (0·01–1 μM) caused slight inhibition of collagen-induced aggregation of human platelets (Table 3); the effect was, however, evident only at low concentrations of collagen, the response of which is known to be mediated via cyclooxygenase product release. The drug also inhibited the secondary, but not the primary phase of platelet aggregation induced by both adrenaline and ADP (Fig. 3). In contrast, ZD9583 (100 μM) did not modify 5-hydroxytryptamine (5-HT)-induced aggregation of human platelets (Table 3).

In-vitro, prostacyclin (30 nM), PGE_1 (30 nM), and PGD_2 (20 nM) caused 51·7 ± 1·7, 60·3 ± 1·6 and 46·7 ± 3·9% inhibition, respectively, of human platelet aggregation induced by a single sub-maximum concentration of ADP (2 μM). ZD9583 (100 μM) did not profoundly ($P > 0.05$, Student's t-test, paired data) affect PGE_1, prostacyclin or PGD_2 inhibition of the primary phase of ADP-induced human platelet aggregation (Table 4).

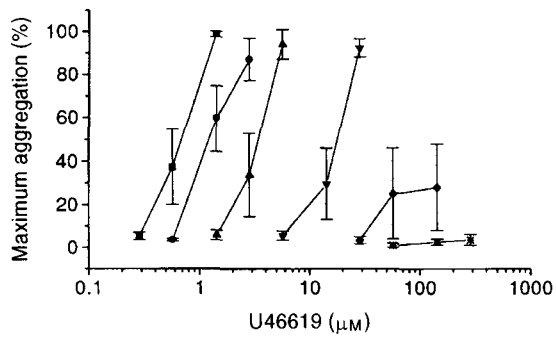

FIG. 2. Effect of vehicle (■) or ZD9583 0·005 μM (●), 0·01 μM (▲), 0·02 μM (▼), 0·05 μM (◆), 0·1 μM (*) on U46619-induced aggregation of human platelets in-vitro. Data are mean ± s.e.m. responses from at least four individuals.

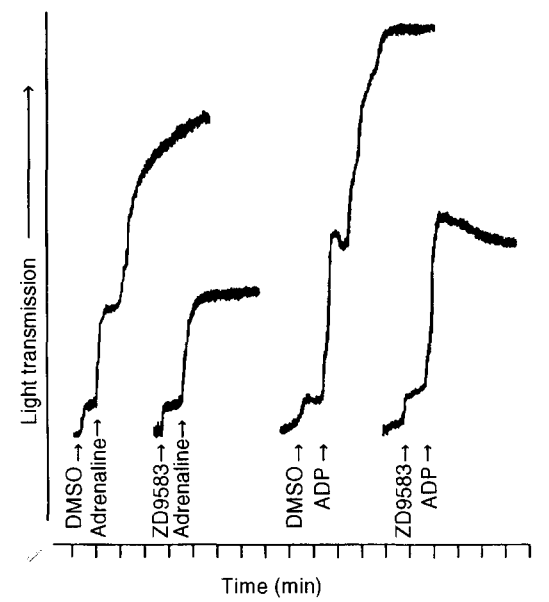

FIG. 3. Typical traces showing the effect of vehicle or ZD9583 (0·1 μM) on the biphasic aggregation of human platelets induced by ADP (2·0 μM) and adrenaline (0·1 μM).

Table 3. The potency and selectivity of ZD9583 on human platelet aggregation induced by various agents.

Agonist	Agonist EC50	ZD9583 (μM)	Concentration ratio
U46619	0.72 ± 0.1 μM	0.005	2.1 ± 0.4
		0.01	5.0 ± 0.1*
		0.02	24.1 ± 0.8*
		0.05	> 250†
ADP (primary phase)	1.8 ± 0.3 μM	100	1.6 ± 0.3
Adrenaline (primary phase)	2.8 ± 0.8 μM	10	1.2 ± 0.4
5-HT	0.5 ± 0.09 μM	100	1.7 ± 0.1
Collagen	0.7 ± 0.2 μg mL^{-1}	0.01	3.0 ± 0.4
		0.1	6.0 ± 1.3
		1.0	6.8 ± 1.7

Data are means ± s.e.m. from at least four individuals. Concentration ratios were determined by comparing agonist EC50 values in the presence of vehicle or ZD9583. *$P < 0.01$ compared with control (paired t-test). †Greater than value represents insurmountable receptor blockade.

Table 4. Selectivity of ZD9583 at human platelet eicosanoid receptors.

Treatment	% Inhibition of ADP-induced aggregation
ZD9583	4.8 ± 1.5
Prostacyclin (30 nM)	51.7 ± 1.7
Prostacyclin (30 nM) + ZD9583	60.0 ± 3.5
Prostaglandin E_1 (30 nM)	60.3 ± 1.6
Prostaglandin E_1 (30 nM) + ZD9583	61.9 ± 1.2
Prostaglandin D_2 (20 nM)	46.7 ± 3.9
Prostaglandin D_2 (20 nM) + ZD9583	51.6 ± 4.7

Effect of ZD9583 (100 μM) on eicosanoid-induced inhibition of ADP (2 μM)-stimulated human platelet aggregation. Each value represents the mean ± s.e.m. of observations from at least four individuals.

Table 5. The effect of orally dosed ZD9583 on collagen-induced TXA_2 production in rat whole blood ex-vivo.

ZD9583 (mg kg^{-1} p.o.)	% Inhibition of thromboxane B_2 production at (h post dose)				
	1	3	5	8	12
1	38 ± 13	17 ± 11	9.2 ± 10	–	–
3	92 ± 2.5	80 ± 8.2	60 ± 4.4	87 ± 1.4	66 ± 7.4
5	97 ± 0.8	94 ± 1.4	85 ± 3.3	84 ± 2.3	77 ± 6.6
10	98 ± 0.5	91 ± 4.4	84 ± 3.9	83 ± 1.4	80 ± 3.5

The TXB_2 concentration in collagen (100 μg mL^{-1})-stimulated whole blood from vehicle- and ZD9583-treated animals was determined by radioimmunoassay. Values are expressed as percent inhibition of TXB_2 formation compared with the vehicle-dosed group and represent the mean ± s.e.m. of measurements from six animals.

Platelet aggregation and TXA_2 formation ex-vivo

Incubation of equine collagen fibrils (100 μg mL^{-1}) with samples of whole blood taken from rats and dogs dosed with vehicle resulted in the formation of 264.6 ± 41.9 (n = 31) and 694.2 ± 98.0 ng mL^{-1} (n = 32) of TXB_2, respectively, as determined by radioimmunoassay. Addition of U46619 to PRP prepared from the blood of the same control rats and dogs caused dose-related platelet aggregation with an EC50 of 0.54 ± 0.13 (n = 9) and 0.85 ± 0.15 μM (n = 15), respectively.

When administered orally to rats, ZD9583 (1, 3, 5 and 10 mg kg^{-1}) caused dose-dependent inhibition of collagen-stimulated TXB_2 production ex-vivo which persisted for at least 12 h at doses in excess of 1 mg kg^{-1} (Table 5). In addition, platelets from animals dosed with ZD9583 (3–10 mg kg^{-1}, p.o.) remained refractory to U46619 for several hours. Addition of the TXA_2 mimetic (100 μM) to PRP from dosed animals failed to induce significant aggregation. This insurmountable TP-receptor blockade persisted for at least 12 h after the administration of doses of ZD9583 in excess of 1 mg kg^{-1}.

After intravenous administration of ZD9583 (0.01–0.3 mg kg^{-1}) to dogs, collagen-induced TXA_2 synthesis in whole blood ex-vivo and platelet sensitivity to U46619 were both significantly reduced. Doses in excess of 0.1 mg kg^{-1} suppressed TXA_2 synthesis for more than 5 h (Table 6). Similarly, addition of U46619 (100 μM) to PRP derived from animals which had been dosed intravenously 5 h previously with 0.01 mg kg^{-1} ZD9583 was insufficient to elicit a significant platelet aggregation response ex-vivo.

When dosed orally to dogs ZD9583 (1 and 3 mg kg^{-1}) caused blockade of platelet TP-receptors which could not be reversed by the addition of up to 100 μM U46619; this activity persisted for more than 24 h. In addition to insurmountable receptor blockade the compound also caused dose-dependent inhibition of collagen-stimulated TXA_2 production (Table 7).

When administered orally to dogs at 12-h intervals for 5 days, ZD9583 (3 mg kg^{-1}) caused profound, insurmountable TP-receptor blockade in platelets from all animals 1 and 12 h after the first dose; this blockade persisted for 24 h after the final dose. Persistent enzyme inhibition was observed for up to 12 h after each drug administration; significant inhibition was still evident 24 h after the final dose (Table 8). When dosing was terminated no evidence of platelet hyper-reactivity was observed.

Table 6. The effect of intravenously dosed ZD9583 on collagen-induced TXA_2 production in dog whole blood ex-vivo.

ZD9583 (mg kg^{-1} i.v.)	% Inhibition of thromboxane A_2 production at (time post dose)					
	2 min	5 min	30 min	1 h	3 h	5 h
0·01	54 ± 19	72 ± 9	17 ± 11	–	–	–
0·03	86 ± 1·7	86 ± 1·8	46 ± 11	40 ± 12	27 ± 14	–
0·1	96 ± 2	83 ± 9	83 ± 3·6	42 ± 13	57 ± 19	54 ± 20
0·3	96 ± 1·1	96 ± 1·2	89 ± 1·1	70 ± 1·2	57 ± 3·3	40 ± 6·2

The TXB_2 concentration in collagen (100 $\mu g\ mL^{-1}$)-stimulated whole blood obtained before and at selected times after ZD9583 administration to dogs was determined by radioimmunoassay. Data are expressed as percent inhibition of TXB_2 formation compared with the pre-dose blood sample from the same animal. Data represent means ± s.e.m. of measurements from four animals.

Table 7. The effect of orally dosed ZD9583 on collagen-induced TXA_2 production in dog whole blood ex-vivo.

ZD9583 (mg kg^{-1} p.o.)	% Inhibition TXA_2 production at (h post dose)			
	2	5	8	24
1	74 ± 6·0	65 ± 5·0	59 ± 3·5	51 ± 11·0
3	87 ± 4·3	76 ± 11	64 ± 16	49 ± 7·0

The TXB_2 concentration in collagen (100 $\mu g\ mL^{-1}$)-stimulated whole blood obtained before and at selected times after ZD9583 administration to dogs was determined by radioimmunoassay. Data are expressed as percent inhibition of TXB_2 formation compared with the pre-dose blood sample from the same animal. Each value represents the mean ± s.e.m. of measurements from four animals.

Table 8. The effect of repeated oral dosing of ZD9583 to dogs.

Dose number	Time after dosing (h)	% Inhibition of TXB_2 formation
1	1	98·9 ± 0·4
	3	89·1 ± 4·5
	5	87·0 ± 7·7
	8	76·4 ± 12·4
	12	80·9 ± 8·1
2	12	80·8 ± 10
3	12	75·6 ± 5·9
4	12	85·2 ± 3·0
5	12	77·1 ± 20·1
6	12	81·5 ± 10·6
8	12	86·3 ± 5·0
9	12	86·6 ± 7·3
10	12	73·2 ± 13·0
	24	52·7 ± 25·7

The TXB_2 concentration in collagen (100 $\mu g\ mL^{-1}$)-stimulated whole blood obtained before and at selected times after administration of ZD9583 (3 mg kg^{-1}, p.o.) to dogs was determined by radioimmunoassay. Data are expressed as percent inhibition of TXB_2 formation compared with the pre-dose blood sample from the same animal. Each value represents the mean ± s.e.m. of measurements from four animals.

Discussion

This study has demonstrated that ZD9583 potently inhibits TXA_2 synthase and also acts as a TP-receptor antagonist. These two activities were expressed over a similar concentration range in-vitro, the IC50 for TXA_2 synthase inhibition and TP-receptor blockade in human blood being 17 nM and 2 nM, respectively. ZD9583 was highly selective because concentration-related inhibition of human platelet microsome-mediated TXA_2 biosynthesis was accompanied by a profound increase in the production of PGE_2 and $PGF_{2\alpha}$, indicating that the compound did not inhibit human platelet cyclooxygenase. In addition, when pre-incubated with collagen-stimulated human whole blood ZD9583 reduced the synthesis of TXA_2 and caused a concomitant increase in the quantities of immunoreactive PGD_2, PGE_2 and $PGF_{2\alpha}$ produced. Inhibition of plasma TXA_2 formation can also be achieved by blockade of cyclooxygenase using aspirin or indomethacin; in these instances, however, parallel inhibition of the stable prostanoids is also observed.

Primary cultures of human umbilical vein endothelial cells are rich in cyclooxygenase and prostacyclin synthase and as such have the capacity to synthesize significant quantities of prostacyclin, especially when stimulated with arachidonic acid. ZD9583 caused weak inhibition of arachidonic acid-induced prostacyclin production (IC50 24 μM) but only at concentrations three orders of magnitude greater than those required to inhibit TXA_2 synthesis.

In addition to its selective action on TXA_2 synthase ZD9583 is also a specific TP-receptor antagonist on platelets from man, rat and dog, competitively inhibiting U46619-induced aggregation of human platelets. Adrenaline- and ADP-induced human platelet aggregation is characterized by a biphasic response; the initial phase is known to be unaffected by inhibitors of cyclooxygenase whereas the secondary, irreversible phase is associated with, and dependent upon, cyclooxygenase product release. ZD9583 blocked the TXA_2-dependent component of ADP, adrenaline and collagen-induced platelet aggregation but did not affect the direct receptor-mediated aggregation induced by 5-HT or the primary phase of the ADP or adrenaline responses. Prostacyclin, PGE_1 and PGD_2 exert potent anti-platelet activity by specific receptor-mediated stimulation of adenylate cyclase resulting in elevation of intra-platelet cyclic AMP. ZD9583, at concentrations in excess of three orders of magnitude greater than those required for significant TP-receptor blockade, did not modify the anti-platelet activity of prostacyclin, PGE_1 or PGD_2.

The potent TXA_2 synthase inhibitory activity of ZD9583 and its TP-receptor antagonist activity were evident after oral administration of the drug to rats and dogs. Oral doses of 3 mg kg^{-1} and greater resulted both in anti-platelet activity and in inhibition of TXA_2 synthase that was demonstrable ex-vivo for periods in excess of 12 h. On repeated oral administration of ZD9583 to dogs over five days there was no evidence

of cumulation of effect, or of platelet hyper-reactivity on cessation of dosing.

The widespread use of aspirin is an important advance in anti-thrombotic therapy (Antiplatelet Therapy Trialists Collaborators 1994a). Aspirin is currently indicated for the treatment of acute myocardial infarction, unstable angina and the prevention of thrombotic complications after coronary angioplasty. Although aspirin is the most widely used anti-platelet therapy, it has several clinically important limitations (Antiplatelet Therapy Trialists Collaborators 1994b). Failure of anti-thrombotic therapy contributes to the relatively high incidence of reocclusion after successful thrombolysis, abrupt thrombotic closure of coronary vessels after angioplasty and the development of unstable angina and myocardial infarction. McAuliffe et al (1993) and others (Watts et al 1991; Menys 1994) have previously demonstrated that pharmacological manipulation of arachidonic acid metabolism with a TXA_2 synthase inhibitor results in the generation of platelet-inhibitory prostanoids which, in the presence of TP-receptor blockade, combine to produce a more efficacious anti-thrombotic effect than that achieved by inhibition of cyclooxygenase. ZD9583, as we have demonstrated in this study, has both these activities and thus has the potential to offer superior anti-platelet efficacy in comparison with agents currently available in the clinic.

In addition to its role in the precipitation of the thrombotic events associated with coronary artery disease there is evidence which suggests that elevated glomerular TXA_2 synthesis might contribute to the progression of renal failure (Patrono et al 1985; Niwa et al 1987). In a model of streptozotocin-induced diabetic nephropathy Hora et al (1990) demonstrated that elevated glomerular TXA_2 synthesis is accompanied by increased proteinurea and subsequent thickening of the glomerular basement membrane. In patients with lupus nephritis, the rate of urinary excretion of TXB_2 is two to five times greater than in healthy controls (Patrono et al 1985). The rate of TXB_2 excretion in these subjects showed positive correlation with the prevalence of renal lesions and deteriorating renal function. Cyclooxygenase inhibitors have been used to normalize urinary TXA_2 excretion in patients with systemic lupus erythematosus; conventional anti-inflammatory doses of aspirin, in common with a variety of NSAID, further reduce renal function, however. It is postulated that this exacerbation of the condition occurs because NSAID inhibit the production of both vasodilator prostanoids and the vasoconstrictor TXA_2. In a separate study intravenous administration of the TXA_2 receptor antagonist BM13,177 to patients with lupus nephritis caused an increase in renal blood flow and glomerular filtration rate suggesting that impairment of renal function is, in part, haemodynamically mediated and that the haemodynamics of the glomerulus are influenced by the local balance of arachidonic acid metabolites. Thus, an agent such as ZD9583 which not only inhibits the formation of TXA_2 but also redirects arachidonic acid metabolism resulting in local elevation of vasodilatory prostanoids might exert beneficial haemodynamic effects in the kidney and hence prove of therapeutic benefit in chronic renal disorders which are accompanied by an elevation in TXA_2 biosynthesis.

TXA_2 synthase inhibitors and TXA_2 receptor antagonists are currently undergoing clinical evaluation. Early data indicate disappointing efficacy with TXA_2 synthase inhibitors highlighting the potential of transiently elevated PGH_2 to act as a TP-receptor agonist, suppressing adenylate cyclase, thereby negating the anti-aggregatory potential of prostacyclin and PGD_2 (Fiddler & Lumley 1990). TP-receptor antagonists preserve rather than increase the endogenous production of platelet-inhibitory prostaglandins and hence have a limited potential to exhibit greater efficacy than that of cyclooxygenase inhibition. ZD9583 is a combined TXA_2 synthase inhibitor/antagonist and should overcome the potential limitations of either drug alone.

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