The effects of siguazodan, a selective phosphodiesterase inhibitor, on human platelet function

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- 1 The effects of siguazodan (SK&F 94836) a selective phosphodiesterase (PDE) inhibitor with inotropic and vasodilator activity, were studied on human platelets.
- 2 Siguazodan selectively inhibited the major cyclic AMP-hydrolysing PDE in human platelet supernatants. The inhibited enzyme has been variously termed cyclic GMP-inhibited PDE or PDE-III.
- 3 In platelet-rich plasma (PRP), siguazodan inhibited U46619-induced aggregation more potently than that induced by ADP and collagen. Treatment of the PRP with aspirin had no effect on the potency of siguazodan.
- 4 In washed platelets, siguazodan increased cyclic AMP levels and reduced cytoplasmic free calcium ($[Ca^{2+}]_i$). ADP decreased the ability of siguazodan to raise cyclic AMP and this may explain its lower potency in inhibiting responses to ADP.
- 5 Siguazodan has anti-platelet actions over the same concentration range that it is an inotrope and vasodilator.

Introduction

Siguazodan ((SK&F 94836) R,S-2-cyano-1-methyl-3-[4-(methyl-6-oxo-1,4,5,6 tetrahydropyridazine-3-yl)phenyl]-guanidine), is a potent, selective inhibitor of guanosine 3':5'-cyclic monophosphate (cyclic GMP)-inhibited cyclic nucleotide phosphodiesterase, (cGI-PDE (Beavo 1988), sometimes referred to as phosphodiesterase III), that has positive inotropic and vasodilating actions in various laboratory animals and is orally active with a long duration of action in conscious dogs (Gristwood *et al.*, 1988). These properties suggested a utility in cardiac failure and siguazodan has undergone initial evaluation in man. Inhibition of cGI-PDE is known to raise adenosine 3':5'-cyclic monophosphate (cyclic AMP) in airways smooth muscle and thus to have a potential for bronchodilatation (Torphy *et al.*, 1988), and also to increase cyclic AMP in platelets with consequent anti-thrombotic effects (Muggli *et al.*, 1985). These actions on platelets have two main consequences for clinical studies: (1) 'anti-platelet' actions might prove additional benefit in patients with ischaemic heart disease; (2) reduced responsiveness of platelets taken from subjects after administration of a compound can provide a convenient pharmacodynamic monitor of drug effect. Additionally, the availability of potent isoenzyme selective inhibitors of phosphodiesterase allows an analysis of the role of these enzymes in platelet function.

We present here the effects of siguazodan on human platelets *in vitro*, including studies of separated, partially purified sub-types of platelet PDE, measurements of cyclic AMP concentration, effects on $[Ca^{2+}]_i$ transients evoked by adenosine 5'-diphosphate (ADP), and inhibition of aggregation in both washed platelets and platelet-rich plasma evoked by ADP and the thromboxane mimetic U46619. In the functional studies, effects over and above those attributable to interference with prostaglandin pathways were assessed by working with aspirin-treated cells. A preliminary account of part of this work has been communicated to the British Pharmacological Society (Simpson *et al.*, 1988a).

Methods

Preparation of platelet-rich plasma and washed platelets and measurement of aggregation

Platelet-rich plasma (PRP) was prepared from whole blood, freshly drawn from healthy volunteers who gave informed consent, anticoagulated with 1/10 volume of acid-citrate dextrose containing: 7 mM citric acid, 93 mM sodium citrate and 139 mM dextrose. The citrated blood was centrifuged at 700g for 5 min and the PRP removed. The remaining red cells and plasma were centrifuged for a further 15 min at 900g and the platelet-poor plasma removed. This was then mixed with the PRP giving a final platelet count of $2-3 \times 10^8$ cells ml^{-1} .

Washed platelets were prepared from whole blood anti-coagulated with 1/6 volume of acid-citrate dextrose containing: 65 mM citric acid, 85 mM sodium citrate and 110 mM dextrose and centrifuged for 5 min at 700g. The PRP was removed and then centrifuged at 350g for 20 min and resuspended in N-2-hydroxyethyl piperazine-N-2 sulphonic acid (HEPES)-buffered physiological saline containing 10 mM glucose, and 0.05 $u\ ml^{-1}$ hirudin (Simpson *et al.*, 1988b).

Aggregation was measured in a twin channel HU aggregometer or a 4-channel, PAP4 Biodata aggregometer. Aliquots were equilibrated to 37°C before being placed in the sample chamber, and were then incubated for 2 min with various concentrations of siguazodan. After the addition of the agonist, aggregation was determined by the change in absorbance monitored for 4 min.

Measurement of $[Ca^{2+}]_i$

This was as described by Rink *et al.* (1983). Briefly, washed platelets were prepared as described above except that the PRP was incubated with 20 μM quin 2 for 30 min at 37°C (and 100 μM aspirin, 40 $\mu g\ ml^{-1}$ apyrase for the final 5 min) before the second centrifugation step. $[Ca^{2+}]_i$ was monitored in 0.8 ml aliquots of the quin 2-loaded, washed platelets in a 37°C thermostated Aminco Bowman fluorescent spectrophotometer; excitation was at 339 nm, emission was measured at 500 nm. Ca^{2+} and fibrinogen were added to the preheated aliquots to give final concentrations of 1 mM and 1 $mg\ ml^{-1}$ respectively. Subsequently, siguazodan (or DMSO control)

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was added for 2 min after which ADP or U46619 was added for a further 2 min.

Determination of cyclic AMP content in washed platelets

Washed platelets were prepared as described above and resuspended (ca. 10^8 cells ml^{-1}) in HEPES-buffered physiological saline containing 100 μM aspirin. Aliquots (480 μl) were equilibrated at 37°C, 10 μl siguazodan (0–5 mM) was added for 2 min after which 10 μl ADP (1 mM) or U46619 (0.5 mM) was added for a further 2 min; finally the incubation was terminated by the addition of 50 μl perchloric acid (3 M). Following centrifugation, acid was removed from a 400 μl aliquot of the supernatant by extraction with 500 μl of a 50/50 (v/v) mixture of tri-*n*-octylamine and freon (Sharps & McCarl, 1982). The cyclic AMP in the upper, aqueous layer was determined by use of commercial radioimmunoassay (Amersham, product number RPA 508). The number of platelets was measured by use of a Coulter counter and the results expressed as pmol cyclic AMP per 10^8 platelets (this is approximately equivalent to μmol per litre cell volume). Addition of [^3H]-cyclic AMP to platelets showed recovery by this procedure to be >95%; therefore the results have not been corrected for recovery.

Ion-exchange chromatography of platelet PDE

Washed human platelets were obtained from Addenbrookes Hospital Blood Bank and stored at -70°C until use. Homogenisation and anion-exchange chromatography on DEAE-Sephacrose was as previously described (Simpson *et al.*, 1988b; Reeves *et al.*, 1987a). PDE activity was assayed by the method of Davis & Daly (1979) as modified by Reeves *et al.* (1987a).

Drugs

Siguazodan (Smith Kline and French Labs Ltd, Welwyn Garden City) was dissolved in DMSO to give a stock solution of 50 mM. The concentration of DMSO in platelet incubations was kept constant at 0.2% (v/v). U46619 (15S-hydroxy-11 α ,9 α -(epoxymethano)prosta-5Z,13E-dienoic acid; Upjohn, Kalamazoo, U.S.A.) was dissolved in absolute ethanol. ADP (Sigma) was dissolved in distilled H_2O . Quin 2 acetoxymethylester (Calbiochem, Cambridge U.K.) was made up as a 50 mM solution in DMSO. All other reagents were from Sigma Chemical Company Ltd, Poole.

Analysis of data

Concentration inhibition curves were fitted to the logistic equation by computer using the programme, ALLFIT (De Lean *et al.*, 1978).

Statistical analysis was by Student's *t* test. All results are shown as mean $\pm$ s.e.mean.

Results

Effects of siguazodan on platelet PDE activity

Figure 1 shows the profile of human platelet PDE activities following chromatography on DEAE-Sephacrose. It can be seen that when assayed with 1 μM cyclic AMP there is one major cyclic AMP-PDE in human platelet supernatant. This activity has been referred to as the cyclic GMP-inhibited PDE (cGI-PDE) (Beavo, 1988) or PDE III (Weishaar *et al.*, 1986). This activity is inhibited by 100 μM siguazodan, whereas the earlier minor peak is unaffected. We have previously obtained similar results with another specific cGI-PDE inhibitor, SK&F 94120 (Simpson *et al.*, 1988b). The dose-inhibition curve showed that siguazodan produced a concentration-dependent inhibition of platelet cGI-PDE with an IC_{50} of 0.8 μM . For comparison, siguazodan inhibited cGI-PDE from human heart with the same IC_{50} (Table 1).

Figure 1 Separation of phosphodiesterase activities from human platelets on DEAE-Sephacrose. A supernatant fraction was prepared from human platelets and applied to a DEAE-Sephacrose column as described under 'Methods'. The column was eluted with a sodium acetate gradient (indicated by the unbroken line) and the fractions assayed with 1 μM cyclic AMP (■) or 1 μM cyclic AMP + 100 μM siguazodan (□).

Effect of siguazodan on cyclic AMP content in human platelets

Table 2 shows that siguazodan increased the measured content of cyclic AMP in intact human platelets resuspended in HEPES-buffered physiological saline. The first column shows the responses of platelets unstimulated by added agonist; statistically significant increases in cyclic AMP are apparent at concentrations of siguazodan above 0.3 μM . The increases in cyclic AMP at concentrations up to 3 μM are modest, around 20%, and are not dose-related. However, increasing concentrations of siguazodan produced further increments in cyclic AMP to more than double the basal level at the highest concentrations tested. Although a close quantitative comparison is clearly inappropriate, these results show that siguazodan does elevate cyclic AMP in intact platelets in a similar concentration range to that which inhibits the isolated cGI-PDE.

Effect of siguazodan on agonist-evoked increases in $[\text{Ca}^{2+}]_i$

Among the ways in which cyclic AMP inhibits platelet function is a reduction in signal transduction and in Ca^{2+} mobilization. We therefore examined the effect of siguazodan on stimulated increases in $[\text{Ca}^{2+}]_i$ in quin 2-loaded platelets resuspended in HEPES-buffered saline (Figure 2). Siguazodan, was able to inhibit almost completely the $[\text{Ca}^{2+}]_i$ elevation evoked by 20 μM ADP. The calculated IC_{50} for this effect was 1.5 μM . With 10 μM U46619 (a thromboxane mimetic) as the stimulus, the IC_{50} for the $[\text{Ca}^{2+}]_i$ response was 0.5 μM .

Table 1 Inhibition of human platelet and heart cGI-PDE by siguazodan

[Siguazodan] (μM)	pmol AMP	
	Platelet	Heart
0	2.08	4.16
0.1	2.29	3.71
0.3	1.33	2.97
1	0.95	2.06
3.2	0.55	1.30
10	0.14	0.61

Cyclic GMP-inhibited cyclic nucleotide phosphodiesterase (cGI-PDE) was prepared from human platelets as described under 'Methods' and from human ventricle as described by Reeves *et al.* (1987a). The results show pmol AMP formed per assay tube for each concentration of siguazodan and are the mean of two experiments; the concentration of cyclic AMP was 1 μM . Calculated IC_{50} values were 0.84 μM (platelet), 0.81 μM (heart).

Table 2 The effect of siguazodan on cyclic AMP in intact, washed human platelets

Siguazodan μM	(pmol cyclic AMP per 10^8 cells)		
	Control	U46619 ($10\ \mu\text{M}$)	ADP ($20\ \mu\text{M}$)
0	1.10 ± 0.09	1.07 ± 0.07	1.25 ± 0.15
0.3	$1.31 \pm 0.20^*$	$1.21 \pm 0.09^*$	1.13 ± 0.09
1	$1.31 \pm 0.16^*$	$1.19 \pm 0.09^*$	1.38 ± 0.16
3	$1.29 \pm 0.15^*$	$1.33 \pm 0.12^{**}$	1.43 ± 0.27
10	$1.71 \pm 0.33^*$	$1.52 \pm 0.14^{**}$	1.33 ± 0.12
30	$1.66 \pm 0.17^{**}$	$1.76 \pm 0.11^{***}$	$1.46 \pm 0.10^*$
100	$2.19 \pm 0.26^{**}$	$2.34 \pm 0.28^{**}$	$1.91 \pm 0.17^{**}$

Washed human platelets were incubated with siguazodan for 2 min and variously with U46619 or ADP for a further 2 min. The reaction was terminated by the addition of perchloric acid and cyclic AMP determined by radioimmunoassay. For full details see 'Methods'. Results are shown as pmol cyclic AMP per 10^8 platelets and are expressed as means $\pm$ s.e.mean ($n = 5$ for all observations). Results were analysed by Student's *t* test vs. control (no siguazodan) $*0.05 > P > 0.01$, $**0.01 > P > 0.001$. Platelets treated with ADP and $30\ \mu\text{M}$ and $100\ \mu\text{M}$ siguazodan had significantly lower cyclic AMP levels than cells treated with siguazodan alone ($P < 0.05$).

Effects of siguazodan on platelet aggregation

Siguazodan inhibited aggregation in response to U46619, ADP and collagen in washed platelet suspensions, PRP and aspirin-treated PRP with IC_{50} s in the range $1\text{--}10\ \mu\text{M}$ (Table 3). The similarity of the values in washed platelets and PRP confirm the low binding of siguazodan to serum proteins ($\approx 30\%$ over the range $0.2\text{--}60\ \mu\text{M}$; SK&F file data). Siguazodan was most potent when U46619 was the agonist; the similarity of inhibition in PRP and aspirin-treated PRP suggest that this is not due to endogenous thromboxane A_2 formation.

Figure 2 Inhibition by siguazodan of ADP- and U46619-stimulated increases in cytoplasmic free calcium. Platelets were loaded with quin-2 and $[\text{Ca}^{2+}]_i$ calculated as described under 'Methods'. Following a 2 min incubation with various concentrations of siguazodan the platelets were challenged with either $10\ \mu\text{M}$ U46619 (●) or $20\ \mu\text{M}$ ADP (■) and the $[\text{Ca}^{2+}]_i$ recorded after a further 2 min. The results are the % of the $[\text{Ca}^{2+}]_i$ recorded in the absence of siguazodan and are shown as mean ($n = 3$). Error bars for s.e.mean are only shown when they are significantly larger than the symbol.

Table 3 Inhibition of human platelet aggregation by siguazodan

	U46619	Stimulus ADP IC_{50} (μM)	Collagen
Washed platelets	1.4 ± 0.03 (3)	ND	ND
PRP	1.8 ± 0.07 (3)	4.0 ± 0.35 (3)	6.9 ± 0.70 (4)
PRP + $100\ \mu\text{M}$ aspirin	1.8 ± 0.03 (3)	5.2 ± 0.28 (3)	10.9 ± 2.8 (4)

PRP or washed platelets were incubated with various concentrations of siguazodan for 2 min before the addition of $10\ \mu\text{M}$ U46619, $20\ \mu\text{M}$ ADP or $5\ \mu\text{g ml}^{-1}$ collagen, aggregation was monitored for the subsequent 4 min. The values shown are the calculated IC_{50} (in μM) for the inhibition of aggregation. ND = not determined.

Figure 3 Effect of siguazodan on ADP-induced platelet aggregation. Platelet-rich plasma was prepared and incubated for 2 min with vehicle control (■), $2.5\ \mu\text{M}$ (◆), $5\ \mu\text{M}$ (●) or $10\ \mu\text{M}$ siguazodan (▲). The platelets were challenged with the concentrations of ADP shown and the aggregation recorded after a further 2 min. Results are shown as mean ($n = 3$); error bars representing s.e.mean are shown only when they are significantly larger than the symbol.

Therefore, the ability of U46619 and ADP to modulate the siguazodan-stimulated increases in cyclic AMP were studied. Table 2 shows that neither of the agonists affected basal content, but that ADP decreased the ability of siguazodan to increase cyclic AMP content. This effect was most marked at the higher concentrations of siguazodan and may provide an explanation for the decreased potency when ADP is the agonist.

The proposed mechanisms of action of siguazodan would generate a functional inhibition that was not competitive with the agonist, and it seemed important to verify that this was the case. Figure 3 demonstrates clearly that the inhibitory effect of siguazodan on ADP-evoked aggregation of platelet plasma was not competitive. The dose-response curve for ADP showed a decrease in maximum effect with increasing concentrations of siguazodan without any detectable shift to the right.

Discussion

These results show that siguazodan inhibits the cGI-PDE separated from human platelets with a potency similar to that which it inhibits this PDE isoenzyme from human cardiac ventricles. In intact platelets, siguazodan increased cyclic AMP over a concentration range compatible with the observed effect on the separated enzyme. Siguazodan inhibited $[\text{Ca}^{2+}]_i$ and aggregation in washed platelets over a similar concentration range to that which produces positive inotropic effects in guinea-pig isolated ventricle strips (Reeves *et al.*, 1987b). The potency of the compound in inhibiting aggregation in platelet-rich plasma suggests that the presence of plasma protein does not influence the access to cell and tissues.

The profile of activity against different PDE sub-types, shown in Figure 1, indicates that cGI-PDE is the dominant cyclic AMP hydrolysing enzyme in human platelet supernatants. In other tissues, e.g. heart, cyclic AMP hydrolysis is more evenly balanced between various PDE isoenzymes (Reeves *et al.*, 1987a). These biochemical findings are reflected

in the ability of siguazodan to elevate cyclic AMP in intact stimulated platelets, a finding which suggests that in the resting condition the steady-state cyclic AMP concentration is mainly determined by the relative activities of adenyl cyclase and cGI-PDE. The fact that 100 μ M siguazodan only raised cyclic AMP 2 fold above the resting level suggests that another cyclic AMP hydrolysing enzyme is available. Possibly the modest cyclic AMP hydrolysing activity of one or more PDE 1 isoenzymes is sufficient to serve this purpose. The possibility of hydrolysis by PDEs associated with the particulate fraction of platelets must also be considered, as must activation of the supernatant cGI-PDE itself (Macphee *et al.*, 1988).

Modest increases in cyclic AMP levels appear to be a characteristic of cGI-PDE inhibitor action in a number of other tissues. In heart, increases in cyclic AMP of less than 50% are associated with significant increases in contractility (Evans, 1986; Murray *et al.*, 1987). Relaxation of vascular smooth muscle by cGI-PDE inhibitors results in a doubling of cyclic AMP levels (Kauffman *et al.*, 1987). In the heart (Murray *et al.*, 1987), smooth muscle (Silver *et al.*, 1988) and platelets (Seiler *et al.*, 1987), it has been shown that these small increases in cyclic AMP are associated with activation of cyclic AMP-dependent protein kinase. These results are in keeping with the known properties of isolated cyclic AMP-dependent protein kinase, which shows a high degree of positive cooperativity in its activation (Robinson-Steiner & Corbin, 1982).

The data clearly show that the inhibition of platelet function exhibited by siguazodan is not agonist specific and is, as expected, non-competitive with the agonist. There is a clearly reproducible distinction between the dose-inhibition curves for ADP and U46619, and the data in Table 2 suggest a partial explanation for this difference in terms of a smaller elevation of cyclic AMP in ADP-stimulated platelets. This might reflect some reduction of cyclase activity by ADP via its known action in activating G_i , the GTP binding protein associated with inhibition of adenyl cyclase (Tremblay & Hamet, 1987). Another possibility is that, because ADP and

U46619 appear to use somewhat different signal transduction processes to cause aggregation, the stimulus-response coupling with U46619 is more susceptible to these modest elevations of cyclic AMP. Thromboxane receptor antagonism may be excluded, as 100 μ M siguazodan had no effect on [125 I]-PTA-OH binding to washed human platelets (measured by the method of Mais *et al.*, 1985) (unpublished observations). The somewhat greater sensitivity of U46619-evoked responses, compared with the ADP or collagen responses, suggests that U46619 may be the ligand of choice for *ex vivo* studies of siguazodan in human volunteers or patients. Indeed a detectable loss of responsiveness to U46619 was seen in *ex vivo* measurements on human volunteers, with doses of siguazodan giving plasma levels in excess of 0.1 μ M (SK&F file data). From the *in vitro* work described here and previous studies on cardiac preparations there is no basis for expecting a dose separation for cardiac and platelet effects of this compound.

In conclusion, siguazodan has the general effects anticipated for a potent, selective cGI-PDE inhibitor in biochemical and functional studies of human platelets. All its actions can, in principle, be accounted for by inhibition of this enzyme and a consequent increase in cyclic AMP. Points of interest in terms of mechanisms of signal transduction are the apparent dominance by cGI-PDE of cyclic AMP hydrolysis in human platelets, the only modest increase in measured cyclic AMP that is associated with substantial functional antagonism, and the different dose-inhibition relations for different ligands. From a clinical perspective, the results show that *ex vivo* monitoring of platelet function should be a valuable measure of pharmacodynamics, and that an anti-platelet action is to be expected at doses that exhibit the inotropic and vasodilator action aimed at ameliorating the symptoms and signs of cardiac failure. Whether this anti-platelet action translates to clinical benefit can be determined only in controlled trials designed to answer this question.

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