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THROMBIN RECEPTOR ANTAGONISTS

STRUCTURE–ACTIVITY RELATIONSHIPS FOR THE PLATELET THROMBIN RECEPTOR AND EFFECTS ON PROSTACYCLIN SYNTHESIS BY HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS

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Abstract—Structure–activity studies on a series of analogues of *N*-(3-methyl-*S*-(1-pyrrolidinyl carbonyl) butyl)-D-alanine ethyl ester hydrochloride (SC42619) have defined the features of this dipeptide analogue required for observation of thrombin receptor antagonist activity on the human platelet. The affinity for SC42619, and for its structural analogue SC43583 is enhanced by pretreatment of the platelets with chymotrypsin. Endothelial cell prostacyclin (PGI_2) synthesis induced by thrombin and trypsin is selectively inhibited by SC42619 provided that prolonged exposure to this antagonist is avoided. However inhibition of PGI_2 synthesis by SC42619 is not overcome by increasing the thrombin concentration. The data provide further support for identification of SC42619 and certain of its analogues as selective antagonists at the platelet thrombin receptor but suggest that these compounds may have more complex, and possibly non-selective effects on the endothelial cell.

In addition to its activity as a seryl protease involved in the promotion and control of fibrin formation [1], thrombin also induces responses in many cells by acting on specific plasma membrane receptors. For example thrombin induces platelets to aggregate, secrete and release thromboxane A_2 [2], endothelial cells to release prostacyclin (PGI_2) and to secrete von Willebrand factor [3] and fibroblasts to proliferate [4]. Most, if not all, of the cellular actions of thrombin are prevented by modification of the active site seryl residue using a reagent such as diisopropylfluorophosphate which abolishes the proteolytic activity of this enzyme [2–4]. However we have described two dipeptide analogues (SC40476 and SC42619) which selectively inhibit stimulation of platelets by thrombin but cause no reduction in the ability of this enzyme to cleave fibrinogen or the low molecular weight substrate S2238 [5]. The description of these selective thrombin receptor antagonists indicates that thrombin stimulates platelets via a classical receptor occupancy mechanism and that expression of the proteolytic activity of this protein is not an *essential* feature of the signal transduction mechanism [5].

We describe here further studies on thrombin receptor antagonists which provide insight into the structural features of the molecule required to observe such activity. We also provide additional support for their action on the platelet and characterize the activity of one such antagonist on PGI_2 synthesis by human umbilical vein endothelial cells.

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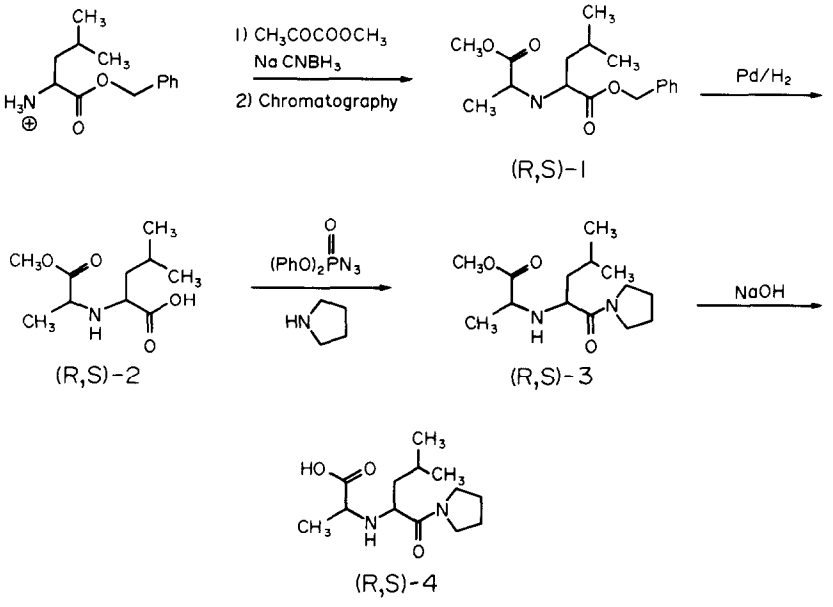
MATERIALS AND METHODS

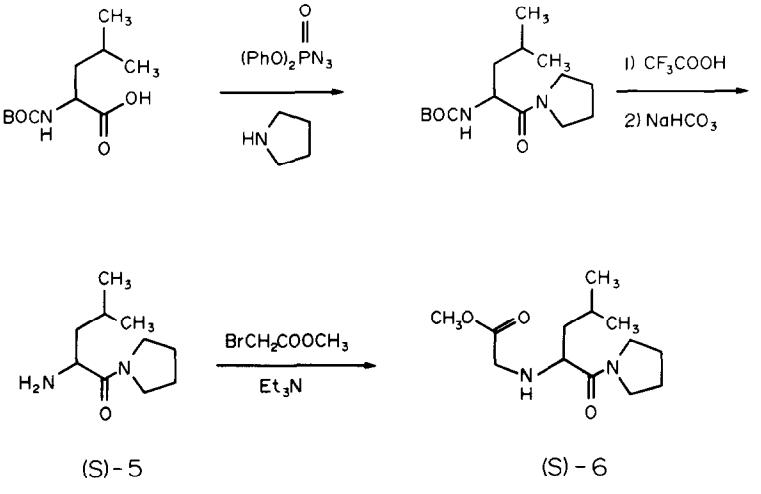
Cells. Washed human platelet suspensions in modified Tyrode's medium were prepared from blood obtained from normal drug-free human donors as described previously [5]. Chymotrypsin-treated platelets were prepared by incubating aliquots of the washed platelet suspension (10^9 cells/mL) with 5 μM chymotrypsin for 5 min at 37° in the presence of 1 μM PGI_2 and 0.05 units/mL hirudin. The suspension was then diluted with 10 vol. of modified Tyrode's buffer containing PGI_2 and hirudin as above and also 0.2% (w/v) bovine serum albumin. Platelets were collected by centrifugation at 350 *g* for 25 min at 20° and were resuspended in the modified Tyrode's buffer containing 0.2% (w/v) albumin at a concentration of 2×10^8 cells/mL.

Human umbilical vein endothelial cells were isolated essentially as described by Jaffe *et al.* [6] with modifications as described by Toothill *et al.* [7]. The cells were detached using 0.05% (w/v) trypsin + 0.02% (w/v) EDTA, reseeded on to gelatin-coated Biosilon beads and grown to confluence over approximately 6 days as described by Toothill *et al.* [7]. The cell coverage of the beads was estimated by staining with methyl violet as described by Toothill *et al.* [7]. Detached cells were seeded onto coverslips at near-confluent density and used within 24 hr as described by Hallam *et al.* [8]. These cells were loaded with Fura2 as described by Hallam *et al.* [8].

Assays. The initial rate of platelet aggregation was estimated from the increase in light transmittance of the washed platelet suspension using a Payton



Scheme 1



Scheme 2

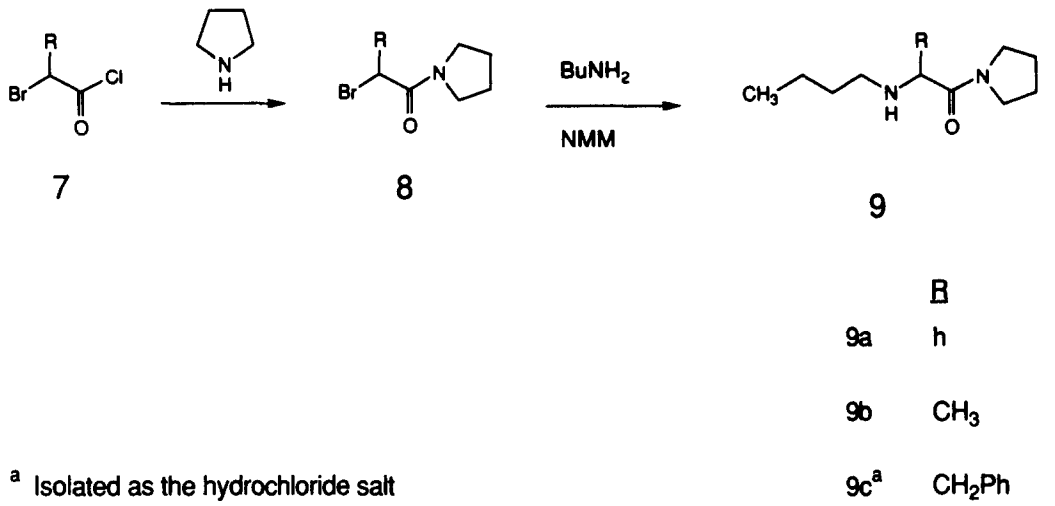
Instruments dual channel aggregrometer as described previously [5, 9]. Peptide antagonists when present were added 2 min prior to initiation of aggregation by addition of the agonist.

Fibrin formation by thrombin and the esterase activity of this enzyme were estimated as described previously [5].

PGI_2 release was measured from confluent monolayers of human umbilical vein cells cultured on Biosilon beads. Columns (0.1 mL) were prepared from cell-covered beads as described by Toothill *et al.* [7]. The columns were washed for 60 min by perfusion at 0.2–0.3 mL/min with serum-free Dulbecco's minimal essential medium buffered to pH 7.2 with HEPES. PGI_2 release was then induced by a 2 min exposure to agonist dissolved in the same medium. Basal and stimulated PGI_2 release were measured in 1 min fractions taken immediately before and after agonist addition respectively. When sequential additions of different agonists were made to the same column a 15 min washout period was applied prior to addition of the second agonist. In this latter protocol the choice of the agonists applied to a single column, and the order of their addition, was made based on the data of Toothill *et al.* [7] in order to ensure that an unaltered response was obtained to the second agonist added. PGI_2 was estimated by specific radioimmunoassay of its stable hydrolysis product 6-oxo$PGF_{1\alpha}$ as described by Ager *et al.* [10].

Cytosolic Ca^{2+} concentration was measured in endothelial cells attached to glass coverslips as described by Hallam *et al.* [8].

*Chemical synthesis: analogues of SC*42619 *(Schemes* 1 *and* 2). SC42619 *N*-(3-methyl-*S*-(1-pyrrolidinyl carbonyl) butyl)-D-alanine ethyl ester hydrochloride) was synthesized as described previously [5].



Scheme 3

Reductive alkylation of (*S*)-leucine benzyl ester, *p*-toluenesulphonic acid salt, with methyl pyruvate and sodium cyanoborohydride followed by catalytic hydrogenation of the less polar (*R*,*S*)-diastereomer **1** yielded *N*-(1-(*R*)-methyloxycarbonylethyl)-(*S*)-leucine **2** [11]. The (*R*,*S*) acid **2** was then converted via the acyl azide (diphenylphosphoric acid azide) to the corresponding (*R*,*S*)-amide **3** which on alkaline hydrolysis gave the (*R*,*S*)-acidamide **4** (Scheme 1).

N-BOC-(*S*)-leucine was converted via the acyl azide followed by deprotection with trifluoroacetic acid to the (*S*)-amine **5**. Alkylation of **5** with methyl bromoacetate/triethylamine gave the (*S*)-esteramide **6** (Scheme 2).

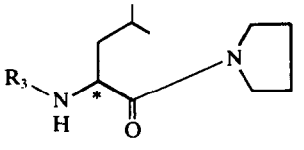
*Chemical synthesis: analogues of SC*43583. The bromoacyl halides **7** were commercially available with the exception of α-bromophenylpropionyl chloride which was prepared from oxalyl chloride and the corresponding acid. The latter compound was prepared as described by Olah *et al.* [12]. Treatment of the bromacyl halides **7** with piperidine using a procedure analogous to that described by Shi *et al.* [13] gave the bromoamides **8**. Subsequent treatment of the bromamides **8** in acetonitrile with a 10% molar excess of *n*-butylamine and *N*-methylmorpholine

Table 1. Structure–activity relationships for analogues of SC42619—inhibition of aggregation induced by thrombin

Compound	Structure[a]	IC_{50} (mM)	% Maximal inhibition[b]
SC42619	R_3 = —*CH(CH_3)$COOCH_2CH_3$	0.42 ± 0.07	100
SC43583	R_3 = —$CH_2CH_2CH_2CH_3$	0.22 ± 0.02[c]	100
3	R_3 = —*CH(CH_3)$COCH_3$	0.80 ± 0.12	85
6	R_3 = —CH_2COOCH_3	1.74 ± 0.07	70
4	R_3 = —*CH(CH_3)COOH	>2.5	40
11e	R_3 = —$CH(CH_3)_2$	>5.0	5

Dose–response curves were constructed for washed platelet suspensions using 2 nM thrombin to induce the aggregation response. IC_{50} values are expressed as means ± SD for three experiments.

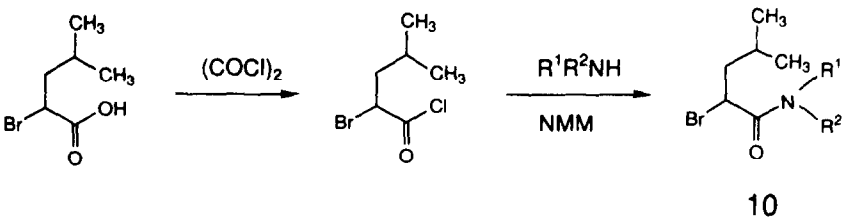
[a] The structure of the parent compound is:



with * indicating a chiral centre. In all cases the data shown are for the (*R*)isomer unless otherwise indicated.

[b] Defined as the extent of inhibition observed in the presence of a saturating concentration of the analogue, or for analogues with IC_{50} values greater than 1 mM, the extent of inhibition observed on addition of 5 mM analogue in the presence of 2 nM thrombin.

[c] The IC_{50} value given is for the racemic mixture (*R*+*S*). The IC_{50} value determined for the (*S*)isomer (0.22 ± 0.06 mM) is not significantly different from that shown for the racemic mixture.



	NR^1R^2	R^3
11a	N	Bu[a]
11b[a]	N	Bu[a]
11c[a]	$NHBu^a$	Bu[a]
11d[a]	$N(CH_3)Bu^a$	Bu[a]
11e	N	$CH(CH_3)_2$

[a] Isolated as the hydrochloride salt

Scheme 4

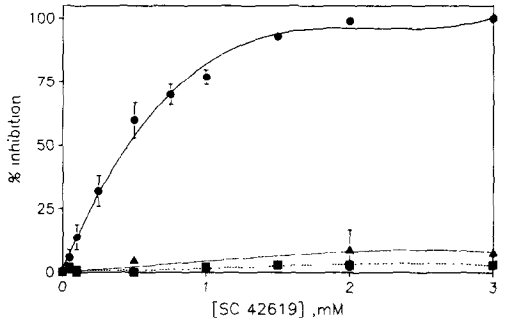


Fig. 1. Effect of SC42619 on the amidolytic (▲), clotting (■) and platelet aggregating (●) activities of thrombin. For measurement of amidolytic and clotting activity thrombin (2 nM) was incubated with SC42619 at the indicated concentration for 2 min at 37° before initiation of the reaction by addition of 50 μM S-2238 (amidolysis) or 0.625 μg/mL fibrinogen (clotting). For measurement of platelet aggregation the washed platelet suspension was incubated with the indicated concentration of SC42619 before addition of thrombin (2 nM). In all cases the data are expressed as percent inhibition of the response observed to thrombin in the absence of the peptide. The data shown are the means ± SE from three experiments using triplicate determinations.

gave after 18 hr at 20° the racemic α-aminoamides **9a-c** (Scheme 3).

Treatment of 2-bromoisocaproic acid dichloromethane with oxalyl chloride and a catalytic amount of dimethylformamide gave the acid chloride. This compound was treated for 2 hr at 20° with equimolar amounts of the relevant amine and *N*-methylmorpholine to give the corresponding bromoamides **10**. Subsequent treatment of the bromoamides **10** with the relevant amine in acetonitrile as described above gave the racemic leucine amide derivatives **11a–e** (Scheme 4). Compound **11a** was also prepared as the (*S*) enantiomer from the (*S*)-amine **5** and butyl bromide.

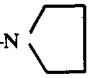
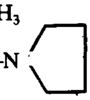
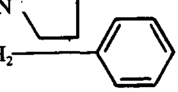
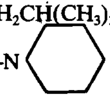
Materials. ADP, ATP, bradykinin, fibrinogen, human thrombin, trypsin (type III-S), mellitin, vasopressin and histamine chloride were obtained from the Sigma Chemical Co. (Poole, U.K.); and S-2238 from KABI Diagnostica (Sweden).

RESULTS AND DISCUSSION

Structure–activity relationships for analogues of SC42619

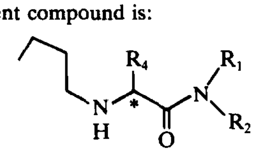
Structural analogues of SC42619 and the derivative

Table 2. Structure–activity relationships for analogues of SC43583—inhibition of aggregation induced by thrombin

Compound	Structure[a]	IC$_{50}$ (mM)[b]	% Maximal inhibition
SC43583	R$_4$ = —CH$_2$CH(CH$_3$)$_2$		
A	R$_1$R$_2$ = —N 	0.24 ± 0.02	100
9a	R$_4$ = —H		
9b	R$_1$R$_2$ = —N 	>5.0	30
	R$_4$ = —CH$_3$		
9c	R$_1$R$_2$ = —N 	0.91 ± 0.31	80
	R$_4$ = —CH$_2$— 		
B	R$_1$R$_2$ = —N	0.14 ± 0.09	100
11b	R$_4$ = —CH$_2$CH(CH$_3$)$_2$		
11d	R$_1$R$_2$ = —N 	0.47 ± 0.05	100
	R$_4$ = —CH$_2$CH(CH$_3$)$_2$		
	R$_1$R$_2$ = —N(CH$_2$)$_3$CH$_3$ (N–CH$_3$)	0.37 ± 0.02	100
11c	R$_4$ = —CH$_2$CH(CH$_3$)$_2$		
	R$_1$R$_2$ = —NH.(CH$_2$)$_3$CH$_3$	0.15 ± 0.05	100

Dose–response curves were constructed for washed platelet suspensions using 2 nM thrombin to induce aggregation. The IC$_{50}$ values are expressed as means ± SD for three experiments.

[a] The structure of the parent compound is:



[b] The IC$_{50}$ values given are for the racemic mixture (R+S) for all compounds except **9a**. For SC43583 comparison of IC$_{50}$ values for the racemic mixture and the (S)isomer does not indicate significant stereoselectivity.

[c] Defined as the extent of inhibition observed in the presence of a saturating concentration of analogue or, for the less potent analogues (IC$_{50}$ > 1 mM) the extent of inhibition observed on addition of 5 mM analogue in the presence of 2 nM thrombin.

compound **11a** have been synthesized to probe the structural features of these compounds which are responsible for their activity as thrombin receptor antagonists. Some of the data obtained in these studies are summarized in Tables 1 and 2 which show both the IC$_{50}$ values determined at a fixed near-saturating concentration of thrombin (2 nM) [5] and also the percentage maximal inhibition observed on addition of a saturating concentration of the analogue. None of the compounds listed in Tables 1 and 2 caused significant inhibition of esterolysis or fibrin formation catalysed by thrombin when added at concentrations up to 5 mM. This finding is illustrated for SC42619 in Fig. 1. At this concentration these compounds also failed to inhibit platelet aggregation induced by agonists other than thrombin (data not shown). Hence they resemble SC40476 in behaving as selective thrombin receptor antagonists. For some of the compounds e.g. SC43583 **11a** further studies confirmed that their addition caused a parallel shift to the right in the dose–response curve describing aggregation induced by thrombin (Fig. 2a). It was therefore reasonable to conclude that the compounds caused their effects by occupancy of the platelet thrombin receptor although we cannot totally exclude the possibility that they may act at some site in the signal transduction pathway distal to the receptor. Insight into the structural features required for presumptive action as a selective thrombin receptor antagonist could then be obtained from a quantitative comparison based on their effects on aggregation induced by thrombin.

The data contained in Table 1 indicate that the length of the carbon chain in the alanyl ester moiety at R$_3$ is a critical factor in maintenance of antagonist potency. For example substitution of the ethyl ester

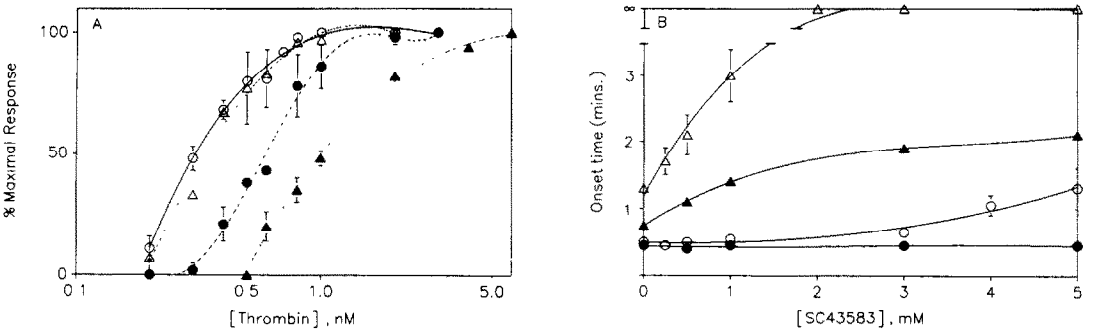


Fig. 2. Effect of SC43583 on the dose–response curve describing aggregation induced by thrombin for control (○, ●) and chymotrypsin-treated (▲, △) platelets (A) and on the onset time observed for control (○, ●) and chymotrypsin-treated (▲, △) platelets in the presence of a non-saturating (0.8 nM) and a saturating (2 nM) concentration of thrombin (B). In (A) the open symbols indicate data obtained in the absence of SC43583 and the closed symbols those obtained in the presence of 1 mM SC43583. In (B) the open and closed symbols indicate data obtained in the presence of 0.8 and 2 nM thrombin, respectively. The data shown are the means ± SE from three experiments using triplicate determinations.

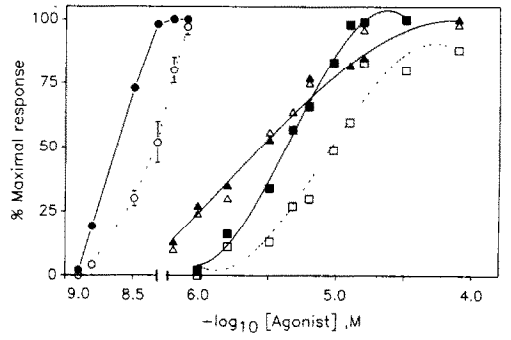


Fig. 3. Effect of *p*-nitrobenzyl-(*R*,*S*)-leucyl-*N*-pyrrolidine amide on aggregation induced by thrombin (●, ○), ADP (▲, △) and 11,9-epoxymethanoPGH$_2$ (■, □). Platelet-rich plasma was incubated in the absence (closed symbols) or in the presence (open symbols) of 2 mM *p*-nitrobenzyl-(*R*,*S*)-leucyl-*N*-pyrrolidine amide for 2 min at 37° before addition of the agonists at the concentrations indicated in the figure. The results are expressed as a percentage of the maximal response induced by that agonist in the absence of the antagonist. The data shown are from a single experiment but are typical of three similar experiments.

at R_3 in SC42619 by a methyl ester **3** led to a two-fold decrease in potency while further shortening to either a methyl **11e** or a carboxyl **4** group gave compounds which caused minimal inhibition in this system (Table 1). However neither the side chain methyl nor the carboxyl groups of SC42619 appeared to be required since their replacement by an *n*-butyl moiety as in SC43583 **11a** gave an approximately two-fold increase in potency as compared with SC42619 (Table 1). The size of the hydrophobic group at R_3 also determined specificity for action at the thrombin receptor. If the *n*-butyl group present at R_3 in SC43583 **11a** was replaced by a benzyl, or substituted benzyl, group such compounds were no longer selective and inhibited aggregation induced by vasopressin, PAF and 11,9-epoxymethanoPGH$_2$ as well as causing a shift to the right in the dose–response curve describing aggregation induced by thrombin. These benzyl analogues of SC42619 however had no effect on the rate or extent of aggregation induced by ADP. The effect of the *p*-nitrobenzyl analogue of SC42619 (*p*-nitrobenzyl-(*R*+*S*)-leucyl-*N*-pyrrolidine amide) on aggregation induced by thrombin, 11,9-epoxymethanoPGH$_2$ and ADP is shown in Fig. 3.

Further studies to examine the effect of substitution at R_1R_2 and R_4 were performed using analogues of SC43583 **11a**. The results of these studies are summarized in Table 2. The presence of a bulky hydrophobic group at R_4 is critical since marked loss of antagonist potency occurred when the isobutyl group was replaced by a methyl group **9b** or a hydrogen atom **9a**. A significant increase in potency was observed when a phenyl group was introduced at this position **9c** (Table 2A). In contrast to the effect of such substitution at R_3 selectivity for inhibition of aggregation induced by thrombin was not lost when a benzyl group was inserted at R_4. At R_1R_2 the presence of the ring structure is not essential since a significant increase in potency was achieved when the pyrrolidine ring was replaced by a simple alkyl chain (*N*-butylamine) **11c**. Bulky substituents are not favoured at R_1R_2 since antagonist potency was decreased when the pyrrolidine ring was replaced by piperidine **11b** or when *N*-butylamine was substituted by *N*-methyl-*N*-butylamine **11d**. None of these structural modifications caused loss of selectivity for action at the thrombin receptor.

The structure–activity analysis therefore indicated that optimal antagonist activity in these dipeptide analogues is obtained in a compound which has relatively bulky hydrophobic groups in all of the three sites investigated. Removal of any one of these groups caused loss of potency as a thrombin receptor antagonist. The most potent compounds had IC_{50} values in the range 0.1 to 0.2 mM when assayed in the presence of 2 nM thrombin (Table 2). Further studies have indicated that no marked enhancement of inhibitory potency occurred when all the desirable structural features identified at R_1R_2, R_3 and R_4 were incorporated into a single molecule [14].

Effect of SC43583 on aggregation of chymotrypsin-treated platelets induced by thrombin

McGowan *et al.* [15] have shown that prior treatment of platelets with chymotrypsin under controlled

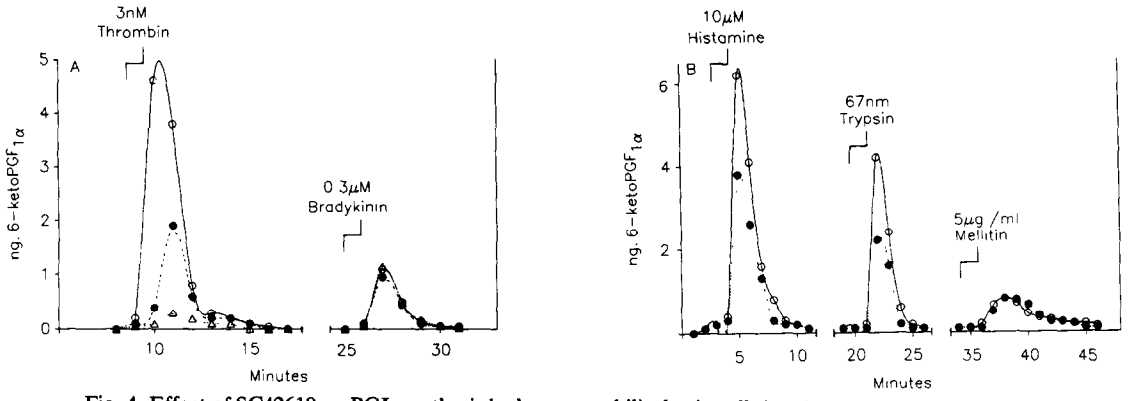


Fig. 4. Effect of SC42619 on PGI_2 synthesis by human umbilical vein cells in microcarrier bead columns. In (A) 0 (○), 0.5 (●), or 2.5 (△) mM SC42619 was added with the indicated agonist. In (B) the columns were perfused continuously in the absence (○) or in the presence (●) of 1 mM SC42619 and the agonists were added as indicated. The data shown are from a single experiment but are typical of three similar experiments.

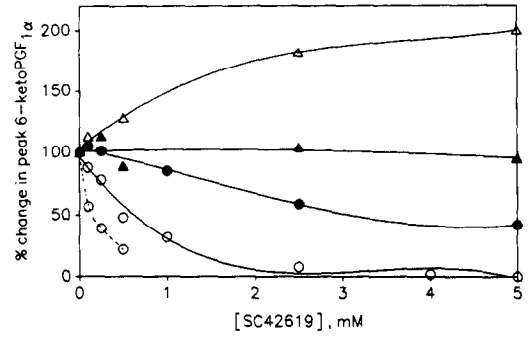


Fig. 5. The extent of PGI_2 synthesis by human umbilical vein endothelial cells induced by 3 nM thrombin (○), 67 nM trypsin (●), 10 μM histamine (△) or 0.3 μM bradykinin (▲) as a function of the concentration of SC42619. The data were obtained from studies similar to those shown in Fig. 3A (solid lines) or 3B (dashed line) and are expressed relative to the extent of PGI_2 synthesis induced by each agonist in the absence of SC42619 as 100%. The data points shown are the means of duplicate determinations from a single experiment but are typical of data obtained in two similar experiments. The mean absolute extents of PGI_2 release observed in the absence of SC42619 were 4.6 ng (3 nM thrombin), 3.2 ng (67 nM trypsin), 1.0 ng. (10 μM histamine) or 1.2 ng (0.3 μM bradykinin).

Table 3. Effect of an increase in thrombin concentration on the extent of inhibition by a fixed concentration of SC42619

[Thrombin] (nM)	Peak 6-keto $PGF_{1\alpha}$ (ng)	% Inhibition by 5 mM SC42619
1	0.8 ± 0.1	88 ± 5
2	1.6 ± 0.2	94 ± 5
5	4.0 ± 0.2	98 ± 2
20	9.4 ± 0.5	94 ± 4

The data shown are means ± SE from a single experiment but are typical of those obtained in three similar experiments. The studies were performed using the protocol described for Fig. 4A in which the cells are perfused with a mixture of thrombin + SC42619 at the concentrations indicated.

conditions caused a delay in initiation of aggregation and secretion induced by α-thrombin such that the characteristics of these responses resemble those resulting from stimulation by α-thrombin. Inhibition of adenylate cyclase by thrombin is also abolished by pretreatment of platelets with chymotrypsin but the ability of this agonist to increase cytosolic Ca^{2+} concentration as well as responses to other agonists are not altered [16]. On the basis of these data McGowan and Detwiler [17] have proposed that the platelet thrombin receptor has a complex structure in which separate regions are differentially coupled respectively to adenylate cyclase via G_i, and to phospholipase C via G_P. Inhibitory coupling to adenylate cyclase is not required to induce aggregation or secretion but does facilitate these responses thus indicating some role for reduced adenylate cyclase activity in the signal transduction sequence used by thrombin.

Studies using SC42619 and SC43583 have provided further support for this postutulate. Pretreatment of the platelets with chymotrypsin had no significant effect on the relationship between the rate of aggregation and thrombin concentration (Fig. 2a) although the time to attain the maximal rate of aggregation (onset time) was significantly increased as shown previously by McGowan and Detwiler [17] (Fig. 2b). Addition of SC43583 caused a significantly greater shift to the right in the dose–response curve relating rate of aggregation to thrombin concentration in chymotrypsin-treated platelets as compared to that observed in control platelets (Fig. 2a). The difference was significant ($P < 0.01$) for all data points except for those at saturating thrombin concentration. The EC_{50} value for thrombin was increased from 0.50 ± 0.07 nM in the absence of SC43583 (control or chymotrypsin-treated) to 0.91 ± 0.10 nM (control) or 1.14 ± 0.08 nM (chymotrypsin-treated) in the presence of 1 mM SC43583. Addition of SC43583 also caused a much more marked increase in the onset time for aggregation in chymotrypsin-treated than in control platelets (Fig. 2b). For example in chymotrypsin-treated platelets addition of 1 mM SC43583 caused a 2.3-fold increase in the onset time for aggregation induced by 0.8 nM

thrombin but did not significantly increase this parameter in control platelets. Similar results to those shown in Fig. 2 for SC43583 were obtained using SC42619 (data not shown). Modification of the thrombin receptor with loss of coupling to G_i [17] therefore enhances sensitivity to selective antagonists at this receptor thus providing further support for the postulate that inhibition of adenylate cyclase facilitates aggregation induced by thrombin even in the absence of an inhibitory agonist. The data of Fig. 2 also support the postulate that the dipeptide analogues interact with the platelet rather than with thrombin [5] since their efficacy as antagonists is enhanced by pretreatment of the platelet with chymotrypsin.

Effect of thrombin receptor antagonists on agonist-induced PGI_2 synthesis in human umbilical vein endothelial cells

Rapid, transient PGI_2 synthesis is observed on stimulation of human umbilical vein endothelial cells with agonists such as thrombin, histamine, ATP and bradykinin as well as other agents such as divalent cation ionophores and mellitin [6, 7, 18–20]. Desensitization studies have indicated that trypsin induces PGI_2 synthesis as a consequence of interaction at the endothelial cell thrombin receptor [7]. We have used this system to probe the cellular selectivity of compounds identified as thrombin receptor antagonists on the basis of their effects on platelet aggregation and secretion.

Figure 4a shows the effect of SC42619 on PGI_2 synthesis when columns of human umbilical vein endothelial cells on microcarrier beads were challenged with 3 nM thrombin and 0.3 μM bradykinin in the presence and absence of this antagonist. In this experiment SC42619 was presented to the cells over the same time period as the agonists. Figure 5 summarizes data obtained under similar conditions but over a wider range of SC42619 concentrations and using histamine, bradykinin, thrombin and trypsin as agonists. In both cases addition of SC42619 caused dose-dependent inhibition of PGI_2 synthesis induced by thrombin and trypsin but had no such effect on that induced by histamine or bradykinin. In fact addition of SC42619 enhanced PGI_2 synthesis induced by histamine (Fig. 5). Addition of SC42619 to this system in the absence of an agonist gave no increase in PGI_2 synthesis (data not shown). The IC_{50} for inhibition by SC42619 of PGI_2 synthesis induced by a non-saturating dose of thrombin (3 nM) is obtained from Fig. 5 as 0.5 mM. Addition of a high concentration (5 mM) of SC42619 caused complete inhibition of PGI_2 synthesis induced by 3 nM thrombin but gave only 50% inhibition of this response induced by a saturating dose of trypsin (67 nM) (Fig. 5).

In contrast when the columns were perfused continuously with SC42619 and then challenged with various agonists selective inhibition of PGI_2 synthesis induced by thrombin and trypsin was no longer observed. Under these latter conditions the presence of SC42619 caused significant reduction in the extent of PGI_2 synthesis induced by both trypsin and histamine (Fig. 4b). Comparable results have been obtained when PGI_2 synthesis was stimulated by bradykinin, ATP and thrombin (data not shown). PGI_2 synthesis induced by receptor-independent stimuli e.g. mellitin (Fig. 4b) or divalent cation ionophores (data not shown) was not affected by continuous perfusion with SC42619. The agonist non-selective effect of SC42619 did not appear to result from irreversible damage to the cells. Inhibition by this compound could be reversed after continuous perfusion if a wash-out period was introduced between perfusion with SC42619 and addition of the agonist (data not shown). However when the columns were perfused continuously with SC42619 a significant decrease was observed in the concentration range over which PGI_2 synthesis induced by 3 nM thrombin was inhibited (Fig. 5, dashed line).

The data of Figs 4 and 5 suggest that under suitable conditions SC42619 can act as a selective antagonist at the endothelial cell thrombin receptor with an affinity comparable to that for this receptor on the platelet (Table 1) [5]. Other evidence is not simply compatible with this postulate. Thus the extent of inhibition of PGI_2 synthesis by a saturating concentration (5 mM) of SC42619 was not reduced by a 20-fold increase in thrombin concentration (Table 3). The failure to observe any reduction in the extent of inhibition of PGI_2 synthesis suggested that exposure of endothelial cells to SC42619 in the presence of thrombin did not simply shift the dose–response curve for this agonist to the right as described previously for platelet aggregation and secretion induced by this agonist [5]. Hence a more complex mechanism of inhibition may be involved which may relate to the ability of SC42619 to block Ca^{2+} influx [5]. Preliminary studies have indicated that at high concentration (5 mM) SC42619 reduces the increase in cytosolic Ca^{2+} concentration induced by bradykinin as well as that induced by thrombin [20].

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