Evaluation of collagen, ADP & thrombin inhibitors
(i). Studies on 99/259, S002-329 and S002-333

1. Collagen-epinephrine induced pulmonary thromboembolism in mice

The test compounds were primarily screened for antithrombotic activity against collagen-epinephrine induced thrombosis in mice. The test compounds 99/259, S002-329 and S002-333 were administered through oral route 1 hour prior to thrombotic challenge at 30μM/kg dose. The standard test compound ticlopidine (200mg/kg) and clopidogrel (10mg/kg & 30mg/kg) were administered 2 hour prior to thrombotic challenge. It was found that 99/259, S002-329 and S002-333 conferred significant protection against collagen-epinephrine induced thrombosis in mice (Fig 1A). 99/259, S002-329 and S002-333 conferred 63±8%, 60±10% and 67±8% while ticlopidine clopidogrel (10mg/kg) and clopidogrel (30mg/kg) conferred only 40±0%, 60±0 and 65±5% protection respectively (Fig 1A).

![Fig 1A: Effect of 99/259, S002-329, S002-333, Ticlopidine and Clopidogrel on collagen-epinephrine induced thrombosis in mice.](image-url)
Results: Collagen, ADP & thrombin inhibitors; S002-329 & analogs

2. Effect on bleeding time

Further studies were carried out to evaluate the effect of these test compounds on bleeding time. Moreover, test compounds 99/259, S002-329 and S002-333 increased the bleeding time to 61±16, 51±15 and 33±12% respectively. The standard drug ticlopidine increased bleeding time to 9±0% from that of control. Clopidogrel increased bleeding time to 20±10% and 52±21% at 10mg/kg and 30mg/kg dose respectively (Fig 2A).

![Fig 2A: Effect of 99/259, S002-329 and S002-333 on bleeding time in mice.]

3. Effect on platelet aggregation

Since these compounds are structural analogs, we also evaluated their effect on platelet aggregation 1 hr after the administration by intra peritoneal route, 99/259 inhibited ADP (10μM) and thrombin (0.64U/ml) induced platelet aggregation while the aggregation induced by collagen (10μg/ml), PMA (1.5μM) and A23187 (2.5μg/ml) remain unaffected (Fig 3Ai).
Results: Collagen, ADP & thrombin inhibitors; S002-329 & analogs

Fig 3Ai: Effect of 99/259 on ADP, thrombin, calcium ionophore A23187, collagen and PMA induced platelet aggregation at 30µM/kg, ip (for **: p<0.01 and for ***: p<0.001).

Fig 3Aii: Ex vivo effects of S002-329 (30µM/kg, ip) against ADP (10µM), thrombin (0.64U/ml), PMA (1.5µM), A23187 (2.5µg/ml) and Collagen (2.5µg/ml) induced platelet aggregation (for *: p<0.05)
Results: Collagen, ADP & thrombin inhibitors; S002-329 & analogs

While other two compounds S002-329 and S002-333 significantly inhibited only collagen (10µg/ml) induced platelet aggregation showing specific antiplatelet activity against collagen (Fig 3Aii & 3Aiii). S002-333 inhibited only collagen (10µg/ml) induced platelet aggregation whereas the aggregation induced by other agonists remained unaffected. Furthermore, the test compounds were assessed for their possible effect on coagulation parameters by Coagulation Analyzer.

4. Effect on coagulation parameters

Test compound 99/259 and S002-333 increased thrombin time, prothrombin time and activated partial thromboplastin time marginally at 100µg/ml and 200µg/ml concentrations but the effect was not statistically significant. However, significant increase in prothrombin time was observed at 200µg/ml concentration (Table 1A).
Results: Collagen, ADP & thrombin inhibitors; S002-329 & analogs

Table 1A: *In vitro* effect of 99/259, S002-329 and S002-333 on coagulation parameters on rat PPP.

<table>
<thead>
<tr>
<th>Compound</th>
<th>99/259</th>
<th>S002-329</th>
<th>S002-333</th>
<th>Conc. (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Change</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thrombin time (TT)</td>
<td>10±4</td>
<td>5±5</td>
<td>9±5</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>6±1</td>
<td>9±5</td>
<td>9±4</td>
<td>200</td>
</tr>
<tr>
<td>Prothrombin time (PT)</td>
<td>-0.24±0.8</td>
<td>19±16</td>
<td>9±5</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>9±0.2</td>
<td>14±3**</td>
<td>14±7</td>
<td>200</td>
</tr>
<tr>
<td>Activated partial thromboplastin time (APTT)</td>
<td>11±4</td>
<td>7±5</td>
<td>13±7</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>10±8</td>
<td>4±3</td>
<td>11±4</td>
<td>200</td>
</tr>
</tbody>
</table>

(for **: p<0.01)

5. Effect of S002-329 on human platelet aggregation (*in vitro*)

![Figure 5A: Effect of S002-329 on collagen induced platelet aggregation in human PRP](image)

Further study was conducted to assess the *in vitro* inhibitory effect of S002-329 against collagen induced platelet aggregation. S002-329 inhibited collagen (5µg/ml)-induced platelet aggregation in human PRP in a concentration dependent manner. However, if the collagen concentration was increased to 10µg/ml, platelet aggregation was not inhibited even at 100µM concentrations. Effect of S002-329 therefore seems to be competitive in nature (Fig 5A).

60
6. **Effect of S002-333 on platelet-collagen interaction by flow cytometry**

Effect of S002-333 was assessed on collagen binding to platelets interaction by flow cytometry. It was found that S002-333 inhibited platelet-collagen interaction at 30μM concentration, however, inhibitory effect was diminished when higher concentration of collagen (20μg/ml) was used (Fig 6Ai & 6Aii).

![Graph](image)

**Fig 6Ai:** Effect of S002-333 on platelet collagen interaction at collagen (10μg/ml) (for *: p<0.05).

![Graph](image)

**Fig 6Aii:** Effect of S002-333 on platelet collagen interaction at collagen (20μg/ml).
7. **Arachidonic acid induced death in mice**

Among the three test compounds S002-329 and S002-333 were taken for further studies as they conferred better protection in primary screening models and they seem to act through collagen mediated platelet activation pathway. Therefore, S002-329 and S002-333 were also evaluated in arachidonic acid induced thrombosis model in mice at 100μM/kg dose. The animals were administered the test compounds 1 hour prior to arachidonic acid administration. S002-329 as well as S002-333 conferred 20% protection at 100μM/kg dose whereas nifedipine conferred 34-40% protection (Fig 7A).

![Graph showing percent protection](image)

**Fig 7A:** Effect of S002-329 and S002-333 on arachidonic acid induced death in mice

8. **Effect on hardened RBCs induced death**

Furthermore, test compounds S002-329 and S002-333 were evaluated against hardened RBCs induced thrombosis in mice. The animals were administered the test substances prior to thrombotic challenge. Ticlopidine (200mg/kg, po, 2h) and Clopidogrel (100mg/kg, po, 2h) conferred no protection while Nifedipine offered 28-
Results: Collagen, ADP & thrombin inhibitors; S002-329 & analogs

40% at 10mg/kg, 30mg/kg and 100mg/kg dose (Fig 8A). S002-329 conferred 13±13% protection whereas S002-333 conferred 40±31% protection at 100μM/kg dose (Fig 8A).

![Graph showing percent protection](image)

Fig 8A: Effect of S002-329 and S002-333 on hardened RBCs induced death

9. Effect on Arterio-venous shunt model in rats

S002-329 and S002-333 were further evaluated on arteriovenous shunt model in rats. The animals were pretreated 1 hour prior to the establishment of blood flow across the shunt. In the control/vehicle treated animals thrombus wt in the AV-shunt was 12.2±0.8mg after 10 min of blood flow (Fig 9A).
Results: Collagen, ADP & thrombin inhibitors; S002-329 & analogs

Fig 9A: Effect of aspirin and S002-329 on thrombus wt in AV-shunt model in rats (for **: p<0.01).

With the treatment of aspirin at 10mg, 30mg and 100mg/kg the thrombus weight was reduced to 6.7±0.4mg, 5.8±1.4mg, and 6.7±1mg respectively (Fig 9A). Significant reduction in thrombus wt was observed with the treatment of S002-329 and S002-333 at 30μM/kg dose. The thrombus wt reduced to 4.8±1.4mg and 6.93±0.9mg respectively following treatment with S002-329 and S002-333 at 30μM/kg dose (Fig 9A).

10. Ferric chloride-induced thrombosis

Application of ferric chloride (20% on 1x2mm² piece of blotting paper) on carotid artery led to the cessation of blood flow in ferric chloride induced thrombosis model
Results: Collagen, ADP & thrombin inhibitors; S002-329 & analogs

in rats. The compounds which confer protection against intravascular thrombosis, prolongs total time to occlusion (TTO).

Fig 10A: Effect of ticlopidine and S002-329 in ferric chloride induced thrombosis in rats at various dose regimens (for *: p<0.05 and for **: p<0.01).

The animals were treated with ticlopidine (200mg/kg, po, 2h) or test compounds (30μM/kg) were given via oral route prior to the experiment. The TTO obtained in control group was 14.44±1 min. S002-329 did not prolong TTO at 30μM/kg after 1 hr of treatment. TTO in ticlopidine (200mg/kg, po, 2h) and S002-329 (30μM/kg, po, 1h) groups was 13±2 and 13±2 min respectively. However, following 3 days of treatment significant prolongation was observed in all the groups. The TTO obtained in ticlopidine (200mg/kg, po once daily, 3 days) and S002-329 (30μM/kg, po once daily, 3 days) groups were 24±3 and 21.7±2 min respectively (Fig 10A). However, with the treatment of S002-333 (30μM/kg) significant prolongation was observed at both 1hr and 3 days time point. The TTO obtained in S002-333 (30μM/kg, po, 1h) and S002-333 (30μM/kg, po, 3 days) was 27.3±7 min and 20±2 min respectively (Fig 10A). These studies suggest that S002-329 and S002-333 possess promising antithrombotic activity and can be developed into potential antithrombotic compounds.
(ii). Studies on S000-20 and their analogs

I. Studies on animal models of thrombosis

11. Collagen-epinephrine induced thrombosis

Test compound S000-20 was screened first for the anti-thrombotic activity against collagen-epinephrine induced thrombosis at 30μM/kg dose. The test compound S000-20 conferred 70±0% protection against collagen-epinephrine induced thrombosis in mice whereas ticlopidine (200mg/kg), clopidogrel (10mg/kg) and clopidogrel (30mg/kg) conferred only 40±0, 60±0 & 60±5% protection respectively (Fig 11A).

![Fig 11A: Effect of Ticlopidine, Clopidogrel and S000-20 on pulmonary thromboembolism.](image)

12. Bleeding time assessment

Test compound S000-20 increased the bleeding time 53±5% from that of the control. Moreover, increase in bleeding time following treatment with ticlopidine (200mg/kg),
Results: Collagen, ADP & thrombin inhibitors; S000-20 & analogs

clopidogrel (10mg/kg) and clopidogrel (30mg/kg) was 9±0, 20±10 and 52±21% respectively (Fig 12A). Keeping in view of the ease in chemical synthesis, S000-20 was evaluated in details in various thrombosis models.

Fig 12A: Effect of aspirin and S000-20 on bleeding time in mice (for *: p<0.05).

13. Arachidonic acid induced thrombosis in mice

S000-20 was evaluated for its protective effect against arachidonic acid induced death in mice. The test substances and standard drugs were given by oral route 1 hr prior to the thrombotic challenge. Nifedipine conferred 33±4 and 38±16 percent protection at 30mg/kg and 100mg/kg dose respectively. S000-20 also offered 30±10% protection at 30μM/kg dose against arachidonic acid induced death (Fig 13A).
**Results:** Collagen, ADP & thrombin inhibitors; S000-20 & analogs

![Graph showing percent protection](image)

**Fig 13A:** Effect of S000-20, aspirin and nifedipine against arachidonic acid induced death in mice

**14. Hardened RBCs-induced death in mice**

Further studies were carried out to evaluate the effect of S000-20 against hardened RBCs induced death in mice. Anti-thrombotic compounds having vasodilatory effects are usually found protective in this model. The test agents (S000-20, Ticlopidine, and Nifedipine) were administered orally prior to the thrombotic challenge. Clopidogrel conferred 10±10% and 0±0% protection at 30mg/kg and 100mg/kg dose respectively while ticlopidine conferred no protection. S000-20 conferred a marginal 13±7% and 7±7% protection against RBCs induced death at 30μM/kg and 100μM/kg dose respectively. The maximum protection 42±9% was offered by nifedepine at 100mg/kg dose (1h). This suggests that S000-20 (30mg/kg & 100mg/kg, 1h) possibly has no vasodilatory effect (**Fig 14A**).
15. Arterio-venous shunt model

S000-20 significantly reduced thrombus weight in the arterio-venous shunt in rats. The test substances were given by oral route 1 hr prior to establishment of blood flow in the shunt. The thrombus wt in control was 12.22±0.8mg, which was significantly reduced by aspirin at 10mg/kg (6.7±0.4mg), 30mg/kg (5.8±1.4mg), 100mg/kg (6.67±1mg) and by S000-20 (8.37±0.8mg) at 30μM/kg (Fig 15A).
Results: Collagen, ADP & thrombin inhibitors; S000-20 & analogs

16. Ferric chloride induced thrombosis

Further studies were carried out in rats to assess efficacy of S000-20 in inhibiting intravascular thrombosis induced by the topical application of ferric chloride. S000-
Results: Collagen, ADP & thrombin inhibitors; S000-20 & analogs

20 at 30μM/kg, (po) administered once daily for 3 days, significantly prolonged TTO to 26±6 min in comparison to control 14.4±1 min. Moreover ticlopidine (200mg/kg) also prolonged TTO after 3 days treatment (Fig 16A). Single dose administration of Ticlopidine (200mg/kg) or S000-20 (30μM/kg), however, had no significant effect on ferric chloride induced cessation of blood flow in carotid artery (Fig 16A).

In vitro studies

17. Effect of S000-20 on platelet aggregation

The test compound S000-20 is lipophilic in nature. Therefore, the test compound was administered to the rats 1 hour prior to the blood collection for platelet aggregation studies.

![Graph](image)

Fig 17A: Effect of S000-20 on platelet aggregation after treatment at 30μM/kg, ip (for **: $p<0.01$).
**Results:** Collagen, ADP & thrombin inhibitors; S000-20 & analogs

Compound S000-20 inhibited only Collagen (10μg/ml) and thrombin (0.64U/ml) induced platelet aggregation significantly at 30μM/kg, ip (Fig 17A).

### 18. Effect of S000-20 on coagulation parameters

Further studies were carried out to investigate the effect these test compounds on coagulation parameters such as thrombin time (TT), prothrombin time (PT) and activated partial thromboplastin time (APTT).

![Graph showing coagulation parameters](image)

Fig 18A: Effect of S000-20 on coagulation parameters.

S000-20 did not alter TT, PT and APTT at both 100μg/ml and 200μg/ml from that of control which shows specific platelet inhibitory effect of S000-20 (Fig 18A).

Studies involving S000-20 exhibited that S000-20 is having better biological activity as compared to standard antithrombotic drugs and its effect is specific on platelets. Therefore, S000-20 was evaluated in greater details *in vitro.*
19. Effect on TRAP, collagen and thrombin induced platelet aggregation in human PRP

Since S000-20 had inhibitory effect on both thrombin (0.64U/ml) and collagen (10μg/ml) induced aggregations but had no effect on TT. It was considered that S000-20 is interfering with thrombin action at the receptor level. Studies were therefore undertaken to evaluate effect of S000-20 & its structural analogs on collagen and TRAP induced platelet aggregation in human PRP.

Thrombin activates the platelets through PAR receptors and GPIb-IX-V receptor. Therefore, to confirm whether S000-20 acts on PAR or GPIb-IX-V, human specific PAR1 agonist thrombin receptor activating peptide (TRAP) SFLLRN was synthesized and the effect of S000-20 on TRAP (50μM) induced platelet aggregation was studied in human PRP.

Fig 19Ai: Effect of S000-20 on TRAP induced platelet aggregation in vitro (for *: p<0.05 and for **: p<0.01).

It was found that S000-20 inhibited TRAP (50μM) induced aggregation at 30-300μM concentration thus suggesting its role in modulating PAR1 mediated thrombin signaling in platelets (Fig 19Ai). Moreover, it was found that S000-20 inhibited collagen (10μg/ml) and thrombin (0.64U/ml) induced aggregation at 100μM and 1mM respectively in vitro (Fig 19Ai & 19Aii).
Results: Collagen, ADP & thrombin inhibitors; S000-20 & analogs

Fig 19Aii: Effect of S000-20 on collagen induced platelet aggregation in vitro (for *: p<0.05 and for **: p<0.01).

These studies revealed that S000-20 was more potent in inhibiting collagen and TRAP induced platelet aggregation in human PRP (Fig 19Ai, ii & iii).
Results: Collagen, ADP & thrombin inhibitors; S000-20 & analogs

20. Effect of S000-20 and its analogs on TRAP and Collagen induced platelet aggregation in human PRP

![Graph showing inhibitory effect of S000-20 and its analogs against collagen and TRAP induced platelet aggregation.](image)

**Fig 20Ai:** Inhibitory effect of S000-20 and its analogs against collagen and TRAP induced human platelet aggregation (for *: p<0.05, for **: p<0.01 and for ***: p<0.001).

Furthermore, comparative studies were carried out with analogs of S000-20 against collagen and TRAP induced platelet aggregation at 300µM concentration (Fig 20Ai).

It was found that S000-20 and its analogs significantly reduced collagen (5µg/ml) and TRAP (50µM/L) induced platelet aggregation at 300µM *in vitro* among them S004-83, S004-90, & S004-91 seems to improve on collagen specificity (Fig 20Ai, ii & iii) while S004-83 exhibited inhibition against TRAP as well as collagen.
Results: Collagen, ADP & thrombin inhibitors; S000-20 & analogs

Fig 20Aii: Inhibitory effect of S000-20 and its analogs against collagen and TRAP induced human platelet aggregation (for *: p<0.05, for **: p<0.01 and for ***: p<0.001).

Fig 20Aiii: Inhibitory effect of S000-20 and its analogs against collagen and TRAP induced human platelet aggregation (for *: p<0.05, for **: p<0.01 and for ***: p<0.001).
21. Effect on Thrombin generation

Thrombin generation plays an important role in the progression of thrombosis. The platelet active compounds which inhibit platelet mediated thrombin generation seem to be better in anti-thrombotic therapy. S000-20 was therefore investigated \textit{ex vivo} for thrombin generation inhibiting capability.

![Graph showing thrombin generation](image)

\textbf{Fig 21Ai: Ex vivo Effect of S000-20 on thrombin generation induced by various inducers in rat PRP (for *: p<0.05).}
Results: Collagen, ADP & thrombin inhibitors; S000-20 & analogs

Fig 21Aii: Effect of S000-20 on thrombin generation induced by various inducers in rat PRP ex vivo.

Fig 21Aiii: In vitro effect of S000-20 on thrombin generation induced by various inducers in rat PRP (for **: p<0.01).
Results: Collagen, ADP & thrombin inhibitors; S000-20 & analogs

The test compound S000-20 at 30μM/kg, was administered 1 hr prior to the collection of blood for thrombin generation. The thrombin generation was induced by CaCl₂ (16.6mM), collagen (0.3 and 1mg), ristocetin (1mg/ml), ADP (2.5μM and 5μM), recombinant tissue factor, rTF (1 and 5pM), thrombin (1 and 3U/ml), A23187 (2.5μg/ml), PMA (1.5μM) or arachidonic acid (0.5mM). S000-20 significantly reduced CaCl₂, ADP (2.5μM & 5μM) induced thrombin generation at 30μM/kg dose (Fig 21Ai & ii). Further study was therefore carried out to confirm the inhibitory effect of S000-20 against ADP and collagen induced thrombin generation in vitro. It was found that S000-20 inhibited ADP (5μM) and collagen (2.5μg/ml) induced thrombin generation in vitro at 100μM concentration significantly (Fig 21Aiii).

22. Effect on platelet-collagen interaction by flow cytometry.

Further studies were done to evaluate the effects of S000-20 on platelet-collagen interactions. Platelets were incubated with collagen labeled with fluorescent dye Oregon green 488 (10μg/ml and 20μg/ml).

Fig 22Ai: Effect of S000-20 on platelet-collagen binding by flow cytometry analysis (for *: p<0.05; for **: p<0.01).
Results: Collagen, ADP & thrombin inhibitors; S000-20 & analogs

It was found that S000-20 inhibited platelet-collagen interaction in a concentration dependent manner when platelets were incubated with 10μg/ml of collagen. Maximal reduction in collagen-platelet interaction was obtained at 100μM of S000-20 (Fig 22Ai).

![Graph showing effect of S000-20 on platelet-collagen binding by flow cytometry analysis.](image)

Fig 22Aii: Effect of S000-20 on platelet-collagen binding by flow cytometry analysis.

However, no reduction in platelet-collagen interaction was observed when collagen concentration was increased to 20μg/ml (Fig 22Aii). Therefore, S000-20 seems to be a competitive antagonist and inhibited collagen-platelet interactions.
(iii). Discussion

Platelet, clotting factors and endothelium dysfunction play key role in the pathogenesis of thrombosis. It is desired to study each components of thrombosis so as to develop potential antithrombotic compounds. Collagen is one of the potent activator of platelets and it is present in vascular sub endothelium. During vascular injury it gets exposed and thus activates platelets.

There are various signaling pathways, each initiated by different platelet agonists. Platelet activators, signaling pathways and intracellular signaling molecules seem to be important target for the development of antiplatelet drugs. ADP, collagen, thrombin, epinephrine, TxA₂ and PAF are important platelet agonists.

Ticlopidine and Clopidogrel are the widely used ADP receptor antagonists used in various clinical conditions. Ticlopidine is associated with neutropenia and thrombotic thrombocytopenia that has restricted its use. Moreover, it is has delayed onset of actions (Hollopeter et al., 2001). Therefore, clopidogrel is being preferred over ticlopidine as ADP receptor antagonist. However, it has weak antithrombotic effect and is associated with bleeding complications. It has been found to reduce about 9% serious thrombotic complications in high risk patient populations (Antithrombotic-Trialists-Collaboration, 2002; CAPRIE Steering Committee 1996). Clopidogrel is highly effective in the animal models of thrombosis, however in most clinical conditions; it has been found to inhibit ADP induced platelet aggregation by 40-50% (Thebault et al., 1999).

Phosphodiesterase inhibitors like cilostazol, dipyridamole have also been widely used for antithrombotic therapy and have lesser effect on bleeding (Goto, 2005). However, they are associated with hypotension and gastrointestinal irritability (Goto, 2005; Schaefer et al., 2006; ESPRIT Study Group, 2006; Douglas et al., 2005).

COX is one of the major targets of antithrombotic drugs. COX inhibitors aspirin have been used as an antithrombotic drug for several decades. Extensive clinical studies
have shown that aspirin reduces the incidence of acute myocardial infarction and stroke by 34% and 25%, respectively. It also prevented vascular death in ~15% of patients (Antithrombotic-Trialists-Collaboration, 2002). Collagen mediated platelet adhesion and activation becomes more important in diseased conditions like atherosclerosis where rupture of atherosclerotic plaques leads to exposure of collagen and subsequent platelet activation and platelet aggregation (Penz et al., 2005).

However, no appropriate drug is available in market to prevent specifically collagen associated signaling or activation of platelets in clinical condition. DZ-697b a product from Daiichi Pharmaceuticals Co., Japan is under pre-clinical development. DZ-697 has been found to inhibit ristocetin and collagen-induced platelet aggregation specifically (Ogihara et al., 2005). Moreover, it has been found to confer more anti-thrombotic effect in comparison to aspirin at much lower dose (Shibutani et al., 2005). In the present study, it was found that some compounds significantly inhibited collagen induced platelet aggregation.

The test compounds were thus initially screened for their antithrombotic activity against collagen-epinephrine induced pulmonary embolism in mice. Collagen is a potent insoluble activator of platelets whereas epinephrine is both platelet activator and vasoconstrictor (Baumgartner, 1977; Spalding et al., 1998). Intravenous injection of collagen-epinephrine solution leads to adhesion of platelets, and their activation and aggregation. These events lead to the formation of microaggregates which get entrapped into lung microcirculation. It subsequently causes hypoxia and death of the animal. The compounds having vasodilatory or platelet inhibitory effects confer protection in this model of thrombosis (DiMinno et al., 1983). This model has pathophysiological implications as in atherosclerotic conditions, rupture of atherosclerotic plaques leads to the formation of thrombus which get easily dislodged due to high shear rate in the blood circulations. The platelet micro-aggregates can get their way through coronary artery and cerebral arteries which results in myocardial infarction or cerebral stroke respectively (DiMinno et al., 1983).
Discussion: Collagen, ADP & thrombin inhibitors

Test compounds 99/259, S000-20, S002-329 and S002-333 conferred significant protection against collagen and epinephrine induced pulmonary thromboembolism (Fig 1A & 11A). Moreover, 99/259, S000-20, S002-329 and S002-333 did not increase the bleeding time as was observed in aspirin treated mice (Fig 2A & 12A). The bleeding complications associated with antithrombotic drugs plagues their extensive use in clinical conditions. Therefore, bleeding complication associated with these test agents was assessed by tail bleeding time assay in mice. Platelet inhibition leads to impaired haemostasis and increases the bleeding time. Test compounds also increased the bleeding time but it was less than the standard drugs such as aspirin that increases the bleeding time up to 118±13% (Table 1B). It is desired that antithrombotic compounds should not alter the bleeding time to a large extent (Dejana et al., 1979).

The test agents 99/259, S000-20, S002-329 and S002-333 were evaluated in detail for antithrombotic activity along with standard drugs clopidogrel, aspirin, ticlopidine and heparin at various doses. Compound 99/259 inhibited ADP and thrombin induced platelet aggregation significantly, suggesting that it might be acting through ADP receptors. Inhibition of platelet ADP receptors might contribute to the reduction of thrombin mediated platelet activation (Fig 3Ai). S000-20, S002-329 and S002-333 were found to act through collagen signaling pathways in platelets as significant inhibition of collagen mediated platelet aggregation was observed in the presence of both the test agents (Fig 3Aii, 3Aiii & 17A). Moreover, S002-329 reduced collagen-induced platelet aggregation in competitive manner suggesting its competitive nature of inhibition and prominent action against collagen mediated activation of platelets (Fig 5A). Since, there are many signaling proteins associated with collagen mediated platelet activation and aggregation, it is desired to identify those signaling molecules. Collagen antagonists have been proposed to be one of the major strategies for successful antithrombotic therapy (Vanhoorelbeke et al., 2003).

Moreover, knock out studies have also confirmed the importance of collagen associated signaling in platelets (Kato et al., 2003). Massberg et al., 2003 have found that inhibition or deletion of GPVI by JAQ1 antibody abrogates stable platelet
Discussion: Collagen, ADP & thrombin inhibitors

Adhesion and aggregation on denuded carotid artery of mice while in α2-null platelets, collagen induced platelet aggregation was delayed but not reduced. Collagen induced platelet aggregation was inhibited in SLP-76 deficient platelets (Clements et al., 1999) while in PLCγ-null mice, collagen induced platelet aggregation was only diminished but was not inhibited completely (Mangin et al., 2003). Reduction in collagen-GPVI interaction and thrombus formation with moderate increase in BT has been observed in FcRγ knock out mice (Konishi et al., 2002). Recent studies on collagen VIII knock out mice revealed the importance of collagen in cell migration, atherosclerosis and restenosis (Adiguzel et al., 2006).

99/259 and S002-333 did not alter coagulation parameters thrombin time, prothrombin time and activated partial thromboplastin time (Table 1A). However, significant alteration was observed in prothrombin time in the presence of S002-329 at 200μg/ml concentrations (Table 1A).

Tests compounds S002-329 and S002-333 were also studied against arachidonic acid induced death in mice. Intravenous injection of arachidonic acid builds up arachidonic acid concentrations in the blood which is taken up by platelets. These in turn get metabolized in platelets to form TxA2, which is potent activator of platelets and vasoconstrictor. The compounds which inhibit cyclooxygenase or thromboxane synthetase confer protections in this model. Moreover, compounds acting as TxA2 receptor antagonists are also effective in this model (Nabata et al., 1987; Sakai et al., 1985; Liu et al., 2004). Marginal protection was obtained with S002-329 and S002-333 suggesting possibility of their interference directly or indirectly with COX mediated platelet activation (Fig 7A).

NO-donating aspirin (NCX-1046) exhibit better antithrombotic properties along with inhibition of associated thrombosis. NO releasing capability enhances its antithrombotic effects as well as it provides gastro-protective actions. Based on this, we evaluated the test compounds for their possible vasodilatory effects (Napoli et al., 2001; Napoli et al., 2002a; Napoli et al., 2002b). Marginal protection against
hardened RBCs induced thrombotic challenge was however observed in the presence of S002-329 and S002-333 suggesting that these compounds do not possess significant vasodilatory effect (Fig 8A).

Antithrombotic drugs that can reduce intravascular thrombosis events are better suited to be developed for clinical conditions. Arteriovenous thrombosis model mimics in vivo formation of intravascular thrombus. S002-329 and S002-333 significantly reduced the thrombus weight. Their effect was comparable to that of aspirin. Hence, S002-329 and S002-333 seems to be effective against intravascular thrombosis (Fig 9A). Moreover, ferric chloride induced thrombosis in rats is the model where intravascular thrombosis initiated by tissue factor and collagen plays important role in thrombosis (Furie and Furie, 2006). It was found that S002-329 and S002-333 did not prolong TTO at 1 hour time point. However, significant increase in TTO was noted after 3 days of treatment (Fig 10A).

Studies on S000-20 showed significant antiplatelet effect against collagen induced platelet aggregation. The S000-20 and its synthetic analogs showed inhibitory activity against collagen and thrombin (Fig 20Ai, ii & iii). Collagen and thrombin are the two potent activators of platelets. In atherosclerotic condition, both collagen and thrombin are the major activator of platelets. Release of tissue factor from the ruptured cells and exposure of collagenous content of the unstable plaque lead to intravascular thrombosis (Nakagawa et al., 2007; Penz et al., 2005).

Moreover, the effect of S000-20 was specific on platelets, as it did not alter coagulation parameters such as thrombin time, prothrombin time and activated partial thromboplastin time (Fig 17 & Fig 18A). Inhibition of platelets leads to reduction in thrombin generation from platelets (Razmara et al., 2007). Moreover, PAR4 also mediate platelet dependent thrombin generation (Hemker et al., 2006; Vretenbrant et al., 2007). Collagen mediated platelet activation is brought about by two receptors present on platelets; GPIa/IIa and GPVI (Siljander et al., 2004). Therefore, S000-20 might be inhibiting any of these two receptors and thereby inhibiting thrombin
formation. Siljander et al., (2004) have shown in their studies that inhibition of GPVI inhibited the exposure of PS on platelet membrane which is a prerequisite for activation of platelets and generation of prothrombinase complex (Altman et al., 2006; Siljander et al., 2004). In these studies, it has been have found that inhibition of GPVI receptor by antibodies inhibited thrombin generation from platelets. Platelets provide the potential surface for thrombin generation. Activation of platelets leads to changes in membrane conformations leading exposure of PS and other negatively charged phospholipids. This further engages clotting factors and calcium which forms the prothrombinase complex on platelet membrane leading to thrombin generation. Therefore, inhibition of platelets either by ADP receptor antagonists or COX inhibitors can reduce the level of platelet mediated thrombin generation. S000-20 mediated action subsequently realized to be mediated by PAR1 receptors. However, it could also be down stream to collagen mediated actions. Collagen activation of platelets leads to thrombin generation which acts on platelets through PAR1 receptors (Fig 19Ai, ii & iii) (Kahn et al., 1999).

S000-20 therefore seems to act through both collagen and thrombin receptors, suggesting dual mode of action of S000-20. But, the exact receptors being affected by S000-20 remain to be identified. Moreover, efficacy profile studies shows that it is effective against intravascular thrombosis (Fig 15A & Fig 16A). It however did not possess vasodilatory effects (Fig 14A), and had no interference with cyclooxygenase pathway (Fig 13A).

The results of present study thus suggest that S000-20 has promising antithrombotic effect, which is possibly being mediated by platelet collagen and thrombin receptors. S000-20 did not affect coagulation parameters but prevented thrombin induced platelet activation (Hemker et al., 2006).

Thus S000-20, S002-329 and S002-333 seem to act primarily against collagen mediated signaling in platelets and offered protection against intravascular thrombosis in various animal models.