Assessment of COX and platelet inhibitors
Results: COX & platelet inhibitors; 99/353 & analogs

(i). Studies on 99/353 and its analogs

I. Studies on animal models

1. Effect on collagen & epinephrine induced thrombosis and bleeding time in mice

The test compounds were screened for their antithrombotic activity against collagen and epinephrine induced thromboembolism in mice. Among the compounds evaluated S000-390 (60±0%), 99/353 (73±0%) and S002-389 (80±0%) conferred significant protection against collagen-epinephrine induced thrombosis (Table 1B). These compounds were also assessed for their effect on bleeding time.

Table 1B: Effect of test compounds on collagen & epinephrine induced thromboembolism and bleeding time

<table>
<thead>
<tr>
<th>No. of Test compounds tested</th>
<th>% Protection against collagen-epinephrine induced thrombosis</th>
<th>Tail bleeding time (%) increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-002-671</td>
<td>20.0</td>
<td>18±0</td>
</tr>
<tr>
<td>S-000-390</td>
<td>60.0</td>
<td>50±0</td>
</tr>
<tr>
<td>S-002-674</td>
<td>0.0</td>
<td>25±0</td>
</tr>
<tr>
<td>S-002-721</td>
<td>0.0</td>
<td>37.5±0</td>
</tr>
<tr>
<td>S-002-723</td>
<td>0.0</td>
<td>0±0</td>
</tr>
<tr>
<td>99/353</td>
<td>73.0</td>
<td>10±0</td>
</tr>
<tr>
<td>S-002-673</td>
<td>0.0</td>
<td>0±0</td>
</tr>
<tr>
<td>S-000-389</td>
<td>80.0</td>
<td>62.5±0</td>
</tr>
<tr>
<td>Aspirin</td>
<td>40±4</td>
<td>118±13</td>
</tr>
</tbody>
</table>

S000-390 (50±0%), S002-721 (37.5±0%), S002-389(62.5±0%) significantly increased the bleeding time at the same dose, suggesting that at the antithrombotic dose bleeding complications might be of concern and might have adverse effects as aspirin which is contraindicated in ulcer patients or during surgical interventions. Both S000-390 and S002-389 conferred protection against collagen-epinephrine induced thrombosis and also increased the bleeding time by several folds at the same
Results: COX & platelet inhibitors; 99/353 & analogs

Dose, 99/353 therefore considered for further evaluation, since 99/353 had only marginal effect on bleeding time (Table 1B).

2. Effect of 99/353 against hardened RBCs induced thrombosis

The compounds having marginal vasodilatory effects will have an advantage over just antithrombotic efficacy. Therefore, the test compounds were also assessed against hardened RBC induced thrombosis in mice which led to death in all the control animals. All the test compounds were administered 1 hour prior to the thrombotic challenge. 99/353 conferred 30% and 60% protection at 30μM/kg and 100μM/kg dose (Fig 1B), which was better than the standard drugs used in this test model, which was confirmed in by assessing its effect on isolated aortic rings (Fig 5B).
3. Effect on AV-shunt model in rats

Further studies were undertaken to evaluate the antithrombotic efficacy of 99/353 against intravascular thrombosis in arterio-venous shunt model in rats. The test compounds were administered 1 hour prior to the establishment of blood flow across the shunt (Fig 2B). 99/353 significantly reduced thrombus weight at 30μM/kg dose from that of control (12.2±0.8 vs 3.6±0.4). Moreover, significant reduction in thrombus weight was observed with the treatment of aspirin. The thrombus weights in AV shunt at 10, 30 and 100mg/kg dose of aspirin were 6.7±0.4mg, 5.8±1.4mg, and 6.67±1mg respectively (Fig 2B).

![Graph showing the effect of 99/353 and aspirin on AV-shunt model in rats](image)

**Fig 2B:** Effect of 99/353 and aspirin on AV-shunt model in rats (for **: p<0.01 and for ***: p<0.001).
II. In vitro studies


The test compound 99/353 was evaluated against collagen (5µg/ml), adenosine 5'-diphosphate (ADP 5µM), thrombin (0.64U/ml), phorbol 12-myristate 13-acetate (PMA, 1.5µM), calcium ionophore A23187 (2.5µg/ml), and arachidonic acid (AA, 0.5mM) at different concentrations in vitro in whole blood aggregometer. The IC$_{50}$ values obtained were 73.13µM, 95.81µM, 96.35µM, 255.07µM, 303.93µM, and 1214.17µM against collagen, ADP, thrombin, PMA, calcium ionophore A23187, and arachidonic acid respectively (Fig 3B). Further studies were therefore conducted to assess effect of some of the synthetic analogs on platelet aggregation.

![IC$_{50}$ values of 99/353 against various inducers of platelet aggregation.](image-url)
5. **Effect of 99/353 analogs on ADP, AA and PMA induced platelet aggregation.**

The compound 99/353 was studied along with some analogs against ADP (5μM)-induced platelet aggregation. The compounds S-002-671, S-000-390, S-002-674, S-002-721 and S-002-723 were found to be potent inhibitor of ADP (5μM)-induced platelet aggregation than 99/353. However 99/353 was considered better as it offered maximum protection against pulmonary thromboembolism in mice (Table 2B).

<table>
<thead>
<tr>
<th>COMPOUNDS</th>
<th>IC₅₀ (μM) (95% lower limit – 95% upper limit)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-002-671</td>
<td>8.00 (6.22-10.30)</td>
</tr>
<tr>
<td>S-000-390</td>
<td>20.00 (16.60-25.93)</td>
</tr>
<tr>
<td>S-002-674</td>
<td>28.05 (23.79-33.08)</td>
</tr>
<tr>
<td>S-002-721</td>
<td>78.80 (51.86-119.78)</td>
</tr>
<tr>
<td>S-002-723</td>
<td>82.32 (66.67-101.66)</td>
</tr>
<tr>
<td>99/353</td>
<td>95.81 (75.78-121.16)</td>
</tr>
<tr>
<td>S-002-673</td>
<td>124.65 (98.29-158.08)</td>
</tr>
<tr>
<td>S-000-389</td>
<td>216.41 (183.38-255.40)</td>
</tr>
<tr>
<td>Ticlopidine</td>
<td>360.94 (291.60-446.76)</td>
</tr>
</tbody>
</table>

Data represents the mean IC₅₀ of at least 3 independent experiments

6. **Effect of 99/353 on coagulation parameters.**

The probable effect 99/353 on coagulation parameters thrombin time (TT), prothrombin time (PT), and activated partial thromboplastin time (APTT) was assessed by semi-automated Coagulation Analyzer. It was found that 99/353 did not affect TT, PT and APTT at 100μg/ml and 200μg/ml suggesting that it doesn’t have any adverse effect on coagulation factors (Fig 4Bi & ii).
Results: COX & platelet inhibitors; 99/353 & analogs

Fig 4Bi: Effect of 99/353 on thrombin time (TT), prothrombin time (PT), and activated partial thromboplastin time (APTT) at 100μg/ml concentration in vitro.

Fig 4Bii: Effect of 99/353 on thrombin time (TT), prothrombin time (PT), and activated partial thromboplastin time (APTT) at 200μg/ml concentration.
7. **Effect of 99/353 on vascular relaxation**

Furthermore, studies were carried out to confirm the vasorelaxant properties of 99/353 by isolated organ bath studies. The aortic rings from the rat aorta were prepared and vessel contraction was induced by phenyl epinephrine. Thereafter, vessel relaxation was induced by different log concentration of 99/353. It was found that 99/353 induced vessel relaxation significantly. It caused vasorelaxation by 13.74±4% and 22±5% at 100μM and 300μM respectively (**Fig 5B**).

![Graphical representation of the vasorelaxant effect of 99/353 and Acetyl Choline (ACh) on PE pre-contracted aortic rings vascular relaxation at various concentrations](image)

**Fig 5B:** Graphical representation of the vasorelaxant effect of 99/353 and Acetyl Choline (ACh) on PE pre-contracted aortic rings vascular relaxation at various concentrations

These shows that 99/353 has potent vasodilator effect in addition to its antiplatelet effects.
8. Effect of 99/353 on thrombin-induced platelet activation on flow cytometry

Thrombin is one of the potent activator of platelets. Therefore, platelets were isolated from human blood and the level of platelet activation was observed in the presence and absence of 99/353. The platelet activation was probed by PAC-1 FITC antibody. Significant reduction in thrombin (1U/ml)-induced platelet activation was observed at 30μM concentration (Fig 6B). 99/353 caused 39% reduction in platelet activation at 30μM concentrations. Therefore, it is now confirmed that 99/353 might be acting through common receptor GPIIb/IIIa which is unique to the platelets and play key role in the final event of platelet aggregation.

![Bar Graph]

*Fig 6B: In vitro effect of 99/353 on thrombin (1U/ml) induced platelet activation on flow cytometry (for **: p<0.01).*
Studies on S001-556

I. Studies on animal models

9. Collagen-epinephrine induced pulmonary thromboembolism in mice

The test compound S001-556 had been found to possess significant antioxidant property as well as inhibition of COX-1 in vitro. This compound was administered by oral route 1 hour prior to thrombotic challenge. S001-556 conferred 76±4% protection at 30µM/kg dose while aspirin conferred only 38±4% protection at 30mg/kg dose (Fig 7B).

10. Effect on bleeding time

Fig 8B: Effect of S001-556 on bleeding time in mice (for ***: p<0.001)
Results: COX & platelet inhibitors; S001-556

Studies were also conducted to evaluate its effect on bleeding time in mice. The test compound S001-556 or standard drug aspirin were administered 1 hour prior to the clipping of tip of tail of mice. The test compound did not have adverse effect on bleeding time in mice while aspirin increased the bleeding time by 118±13% at 30mg/kg dose which offered lesser protection against thrombosis (Fig 8B).

11. Arachidonic acid induced death in mice

![Graph showing effect of test compounds against arachidonic acid induced death in mice.]

Fig 9B: Effect of S001-556 against arachidonic acid induced death in mice (for *: \( p<0.05 \)).

Furthermore studies were performed to evaluate the effect of this compound against arachidonic acid induced death in mice. It was found that S001-556 conferred 20-30% protection against arachidonic acid induced thrombotic challenge (Fig 9B), while aspirin which is a COX-1 inhibitor conferred no protection at 30mg/kg and 100mg/kg dose.
12. Hardened RBCs induced death in mice

![Graph showing effects of different compounds on hardened RBCs death in mice.](image)

**Fig 10B:** Effect of S001-556 on hardened RBCs induced death in mice

Further studies were done to evaluate the effects of S001-556 on hardened RBCs induced thrombotic challenge in mice. S001-556 conferred 40±0 and 40±20% protection at 30μM/kg and 100μM/kg dose respectively (**Fig 10B**). Aspirin conferred 20-28% protection against hardened RBCs induced death.

13. Arterio-venous shunt model

Significant inhibition in thrombus weight was observed with the treatment of S001-556 at 30μM/kg dose, 1 hour prior to the establishment of AV-shunt and blood flow across the shunt (12.22±0.8 mg vs 8.07±0.2mg). Standard drug aspirin also reduced thrombus weight around 6mg (**Fig 11B**). This suggests that S002-556 is effective against intravascular thrombosis.
Results: COX & platelet inhibitors; S001-556

**Fig 11B:** Thrombus weight in AV-shunt in rats following treatment with aspirin or S001-556 (for *: p< 0.05 and for **: p<0.01).

14. Ferric chloride induced thrombosis

Effect of S001-556 was also evaluated against ferric chloride induced thrombosis in rats. Treatment with antiplatelet compounds like aspirin (30mg/kg, po, 1h), ticlopidine (200mg/kg, po, 2h) did not prolong the TTO. Moreover, following 3 days of treatment (once daily) prolonged TTO significantly (14.44±1 vs 25.67±4.6 min for aspirin and 14.44±1 vs 24±3 min for ticlopidine). The TTO in S001-556 at 30μM/kg and 30μM/kg, for 3 days, once a daily pretreated rat was 11±1 and 20±6 min respectively (Fig 12B), which was not significantly more than the control. However, anticoagulants like heparin increased the TTO dose dependently (Fig 12B).
Results: COX & platelet inhibitors; S001-556

![Graph](image)

Fig 12B: Effect of S001-556 on ferric chloride induced thrombosis (for *: p<0.05 and for **: p<0.01)

II. In vitro studies

15. Platelet aggregation.

S001-556 was administered 1 hr prior to blood collection and platelet rich plasma was isolated from the blood. It was observed that S001-556 inhibited arachidonic acid (0.5mM) and collagen (10μg/ml) induced platelet aggregation significantly. No significant effect was inhibition was observed, against thrombin (0.64U/ml), calcium ionophore A23187 (2.5μg/ml), or PMA (1.5μM) induced platelet aggregation (Fig 13Bi). These results suggest that the compound might be acting directly on arachidonic acid metabolism resulting in reduced generation of TXA$_2$ thereby attenuating the collagen-induced aggregation. It has been reported that generation of TXA$_2$ from collagen activated platelet leads to the further activation, aggregation and granule secretion of platelets.
Results: COX & platelet inhibitors; S001-556

Fig 13Bi: Percent platelet aggregation induced by ADP, collagen, thrombin, and calcium ionophore A23187, arachidonic acid, and PMA in the absence and presence of S001-556 (30μM/kg, po) (for **: p<0.01).

Fig 13Bii: Percent inhibition of platelet aggregation induced by ADP, thrombin, and calcium ionophore A23187, arachidonic acid, and PMA in the presence of S001-556 or aspirin (30μM/kg, po).
Moreover, aspirin and S001-556 are reduced platelet aggregation significantly in case of ADP and arachidonic acid induced platelet aggregation (Fig 13Bii).


**Fig 14Bii:** In vitro effect of S001-556 on coagulation parameters-thrombin time (TT), prothrombin time (PT) and activated partial thromboplastin time (APTT) at 100 and 200μg/ml concentrations (for ****: \( p < 0.001 \)).

**Fig 14Biii:** Ex vivo effect of Ticlopidine & Aspirin on coagulation parameters-thrombin time (TT), prothrombin time (PT) and activated partial thromboplastin time (APTT).
Results: COX & platelet inhibitors; S001-556

S001-556 had no significant effect on thrombin time (TT) and activated partial thromboplastin time (APTT) however it increases prothrombin time (PT) significantly at 200µg/ml concentration (Fig 14Bi). Moreover, *ex vivo* effect of Ticlopidine and Aspirin were evaluated on clotting parameters and they did not affect the clotting parameters (Fig 14Bii).

Results obtained thus clearly suggest that S000-556 is exhibiting promising antithrombotic activity through its action on platelets and it may affect PT at higher concentrations.
(iii). Discussion

GPIIb/IIIa (αIIbβ3) binds to several adhesive ligands like fibrinogen, vitronectin, and vWF. αIIbβ3 is a major platelet membrane glycoprotein that contains Arg-Gly-Asp (RGD) sequences is the recognition sequences for binding to αIIbβ3 (Mohri and Ohkubo, 1993). It is a complex consisting of two large, non-identical glycoproteins αIIb and β3 which bind non-covalently in a Ca$^{2+}$ dependent way. This shows the importance of calcium in platelet activation (Vallar et al., 1999). Since various ligands can interact with αIIbβ3, no complete protection in animals was observed in case of Fg and vWF knockout animals (Ni et al., 2000). Therefore, it is desired to block GPIIb/IIIa receptor to prevent platelet activation and aggregation. Moreover, drugs like aspirin, clopidogrel and ticlopidine have limited efficacy as other pathways for platelet activation still seems to be operational. Aspirin did not increase significant benefit even though it was administered singly or in combination with ADP receptor antagonists. Blood flow viscosity, shear rate and other cellular components also play important role in intravascular thrombosis (Xiao et al., 2006).

Importance of GPIIb/IIIa was identified in Glanzmann’s thrombasthenia patients which is a rare autosomal recessive bleeding disorder characterized by a quantitative deficiency or a functional abnormality of the major platelet membrane integrin receptor: the GPIIb/IIIa complex. Thrombasthenic platelets are severely deficient in GPIIb/IIIa content or function, and fail to aggregate or form the hemostatic plug at the site of vessel injury. On the other hand, heterozygous subjects (having about half the number of normal GPIIb/IIIa complexes) do not show bleeding problems (Perutelli and Mori, 1992). Moreover, knock out studies by deletion of β3 confirmed Glanzmann’s thrombasthenia type phenotypes in mice and the animals were found to be protected against ferric chloride induced thrombosis. This observation further validated the importance of GPIIb/IIIa receptor in haemostasis and thrombosis (Hodivala-Dilke et al., 1999). Potential role of αIIbβ3 in platelet activation and aggregation, led to the development of inhibitors of αIIbβ3 integrin receptor.
However, most of these agents exhibited severe bleeding complication, thrombotic thrombocytopenic purpura and increased mortality restricted its wider use (Peter et al., 1998; Ferguson et al., 1998). Inhibition of GPIIb/IIIa (αIIbβ3) is an important strategy for the development of anti-platelet therapies. Therefore, GPIIb/IIIa antagonists with higher safety margin and efficacy are being evaluated. The test compound 99/353 and its analogs which were firstly screened for their anti-thrombotic activity against collagen-epinephrine induced pulmonary embolism model in mice, among them 99/353 conferred significant protection against pulmonary thromboembolism without adversely affecting the bleeding time, which is a desired characteristic of an ideal anti-thrombotic compound (Table 1B). 99/353 conferred better protection than aspirin treated mice (Table 1B). 99/353 was therefore undertaken to determine its efficacy in other animal models of thrombosis.

99/353 conferred significant protection against hardened RBCs induced death in mice at both 30μM/kg and 100μM/kg dose (Fig 1B). Moreover, in vitro studies suggested that 99/353 also possess marginal vasodilatory effect as evident by the vasorelaxant effect on phenyl epinephrine pre-contracted aortic rings (Fig 5B).

In AV-shunt model, 99/353 significantly reduced thrombus weight at 30μM/kg (Fig 2B), which was found to be better than aspirin. Evaluation of 99/353 was therefore undertaken to investigate its mechanisms of action.

Since, antithrombotic compounds bring about their actions either by acting on platelets or interfering with clotting factors (Packham, 1994; Mackman, 2004). Synthetic analogs of 99/353 were evaluated against ADP induced platelet aggregation in vitro. S002-671, S000-390, S002-674, S002-721 and S002-723 had more inhibitory effect in vitro than 99/353 (Table 2B). However, similar effect was not translated against collagen & adrenaline induced thrombosis (Table 1B). 99/353 was therefore taken as a lead molecule to investigate its mechanism of action. The pathways being affected by 99/353 was investigated by assessing platelet aggregation and coagulation parameters. 99/353 significantly inhibited platelet aggregation induced by most of the
Discussion: COX & platelet inhibitors

inducers. Moreover, the inhibition was more consistent against inducers (ADP, collagen and thrombin) which directly act through platelet membrane glycoprotein or platelet surface receptors. Test molecule 99/353 inhibited aggregation induced by all the inducers such as collagen, ADP, thrombin, PMA, AA and calcium ionophore A23187 (Fig 3B). The IC₅₀ of 99/353 against collagen, ADP and thrombin are comparable (Fig 3B). 99/353 did not alter thrombin time (TT), prothrombin time (PT) and activated partial thromboplastin time (APTT) even at higher concentrations (200µg/ml) (Fig 4Bi & 4Bii). It is therefore imperative that it might be acting at surface receptors. It is reported that antagonizing or inhibiting GPIIb/IIIa is a promising strategy for new drug development (Casserly and Topol, 2002). GPIIb/IIIa receptor antagonists are considered better antithrombotic drugs and have been considered more effective in conditions like aspirin resistance; however, bleeding complications and thrombotic thrombocytopenic purpura (TTP) have been found to associate with their use (Harrington et al., 2000).

Flow cytometry experiments further suggested that 99/353 was acting through GPIIb/IIIa receptor as 99/353 dose dependently inhibited PAC-1 binding in thrombin induced activation of platelets (Fig 6B).

Activation of GPIIb/IIIa from a low affinity state to high affinity state during inside-out signaling is the final event in the platelet activation. The conformation change in GPIIb/IIIa leads to enhanced binding of fibrinogen to GPIIb/IIIa receptor. Fibrinogen bridges the binding to adjacent platelets (Basani et al., 2001). As it is the final step in the platelet aggregation induced by agonists like ADP, collagen, thrombin and TxA2. Second important criterion of opting for this target is that it is specific to only platelets, thus minimizing side effects by reducing the non-specific actions on other cells (Frishman et al., 1995).

There are different types of GPIIb/IIIa receptor blockers which have been developed so far

(i) Monoclononal antibody {Abciximab (Reopro™)}
Abciximab is relatively non-specific and binds to other receptor like, vitronectin receptor. It is also associated with thrombocytopenia and excessive bleeding. Eptifibatide is more specific than abciximab; however, it needs continuous infusion to maintain anti-platelet effect (Frishman et al., 1995, Peter et al., 1998).

Lamifiban and Tirofiban are GPIIb/IIIa inhibitors which are administered by intravenous route whereas Xemilofiban, orofiban and sibrafiban are orally active GPIIb/IIIa receptor blockers. Various clinical trials have shown that oral GPIIb/IIIa inhibitors are associated with high mortality (Ferguson et al., 1998).

Cyclooxygenase is one of the important enzymes responsible for the formation of potent activator TxA₂ in platelets. Platelet activation by collagen, thrombin, ADP and epinephrine leads to the formation of TxA₂ and subsequently activation of platelets. Platelet activation leads to activation of membrane phospholipase A₂, release of membrane phospholipids and arachidonic acid, which is metabolized by cyclooxygenase and thromboxane synthetase to generate TxA₂. TxA₂ acts through prostanoid receptors which are the potent activator of platelets (FitzGerald et al., 1987; Armstrong, 1996). COX inhibitors, thromboxane synthetase inhibitors and TxA₂ receptor antagonist development is one of the favorable approaches in new antithrombotic drug development (De Clerck et al., 1989a; De Clerck et al., 1989b). Aspirin inhibits COX, however gastric irritability and bleeding complications associated with it restricts its use in various clinical conditions.

The test agent S001-556 conferred protection against collagen-epinephrine induced thrombosis (Fig 7B). Moreover, S001-556 did not affect bleeding time (Fig 8B). Moreover, it conferred significant protection against arachidonic induced death at higher concentration and it was more effective as compared to aspirin (Fig 9B). S001-556 also conferred protection against hardened RBCs induced thrombotic challenge in mice (Fig 10B), Suggesting that it might possess vasodilatory effect or
protective effective in this model might be due to its platelet inhibitory effect. Moreover, efficacy profile studies in ferric chloride, AV-shunt and stasis induced thrombosis models suggested that S001-556 is effective against intravascular thrombosis (Fig 11B & 12B).

S001-556 was specifically acting through platelet inhibition and it did not affect TT and APTT. However, PT was altered at higher concentration. Since, this compound possess significant antioxidant effect apart from direct inhibition of COX-1, it seems to be better than aspirin. Present study exhibited that S001-556 is better antithrombotic agent than aspirin with lesser side effects.