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Materials

Adenosine 5'-diphosphate (ADP), thrombin (Lyophilized powder 500 NIH Units/ml), collagen type I, collagen type III, 12-phorbol 13-myristate acetate (PMA), arachidonic acid, calcium ionophore A23187, ethylene glycol-bis- (β-amioethyl ether)-N, N', N'-tetra acetic acid (EGTA), calcium chloride, Dimethyl sulfoxide (DMSO), NaCl, tri-sodium citrate, citric acid, Epinephrine and Apyrase (Grade VI Lyophilized powder, ATPase ≥ 200 Units/mg protein) were procured from Sigma, USA. Collagen suspension was procured from Chrono-Log Corp. (Havertown, USA), STA thrombin reagent, Neoplastin CI plus, Fibri-Prest, CK Prest were purchased from Stago France to assess thrombin time, prothrombin time, fibrinogen time and activated partial thromboplastin time.

Thrombin substrate III fluourogenic, PPACK (thrombin inhibitor), recombinant tissue factor (rTF) were obtained from Calbiochem. All other chemicals used in the above mentioned studies were either procured from SRL (India). Oregon green labeled collagen was obtained from Molecular Probes, India. The synthetic compounds synthesized at CDRI were evaluated such as 99/353 (Mol wt. 587.0), S000-20 (Mol wt. 429), 99/259 (Mol wt. 405), S002-329 (Mol wt. 439) and S002-333 (Mol wt. 385).

Methods

1. Animal Models of Thrombosis

Experimental animals: Swiss male mice (20-25 g), SD male rats (150-300 g) bred and maintained at central animal house facility of Central Drug Research Institute, Lucknow, were used in the present study. The animals were maintained on standard chow diet and water ad-libitum and on 12hr/12hr light-dark cycle at temperature 25±2°C, humidity 45-55%. They were housed individually in polypropylene or
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metallic cages and were handled gently. All the experiments were done according to the approved guidelines of institute animal ethics committee (IAEC).

In some experiments blood was obtained from human volunteers free from cardiovascular complications and who had not taken any drugs for 15 days prior to the time of blood collection.

1.1 Mouse thrombosis model

Pulmonary thromboembolism was induced by a method as described by DiMinno and Silver (1983). The compounds to be tested, standard drugs or the vehicle were administered by oral route 60 min prior to the thrombotic challenge. Ten mice were used for evaluating the effect of test compound, while a group of 5 mice was used to evaluate the effect of aspirin or vehicle. A mixture of collagen (150μg/ml) and adrenaline (50μg/ml) was injected into the tail vein to induce hind limb paralysis or death. Results have been reported as %, which represent protection against collagen and epinephrine induced thrombosis. The test compounds having more than 40% protection were considered significant.

1.2 Bleeding time

Bleeding time in mice was evaluated by the method of Dejana et al, (1979). The tail 2mm from tip of mice was incised and the blood oozed was soaked on a filter paper, which was monitored at an interval of 10-15 sec till the bleeding stops. The time elapsed from the tip incision to the stoppage of bleeding was determined as the bleeding time. The CDRI compounds (30μM/kg), aspirin (30mg/kg) or vehicle was given orally 60 min prior to the tail incision in a group of 5 mice each.

1.3 Arachidonic acid induced-death

Mice (20-25g) were grouped into control, vehicle, standard drug and test substances treated groups. Mice were grouped into vehicle, test compound and aspirin treated or other standard drug treated groups, each group having 5 animals each. The animals were administered test substances by the oral route. Arachidonic acid (80mg/kg) was
injected into the tail vein of each animal after 1 hour of test agent administration. The animals survived and died in each group were recorded and percent protection was calculated and reported (Nabata et al., 1987; Sakai et al., 1985).

1.4 Hardened RBCs induced death

The study was conducted according to the protocol described earlier (Momi et al., 2000). Blood was collected from SD rats by cardiac puncture under ether anesthesia into (3.8%) tri-sodium citrate, which was then centrifuged at 150g for 10 minutes. The buffy coat was removed and discarded; remaining red blood cells were treated with chlorpromazine (0.2mg/ml). The suspension was carefully mixed and incubated for 15 minutes at room temperature. Red blood cells were centrifuged at 2000g for 10 minutes. The cell pellet was resuspended in 3% glutaraldehyde in Sorenson buffer, pH: 7.4 and incubated for 30 min at 4° C under continuous mixing. The cells were then washed three times in saline (NaCl 0.154M). It was finally suspended in saline at a hematocrit of 12.5%.

The hardened RBCs (200μl) were injected intravenously through tail vein in mice to induce death. Mice were grouped into vehicle, test compound, nifedipine, clopidogrel, ticlopidine and aspirin treated groups, each group having 5 animals each. The protection offered by the test compounds were evaluated against death subsequent to thrombotic challenge. Results are expressed as percent protection in the compound or standard drug treated group.

1.5 Arterio-venous shunt model

Rats were grouped into control, aspirin and test compound group, each group having six animals. Rats were anesthetized with urethane (1.25gm/kg). A cervical incision was made and carotid artery and its contra lateral jugular vein was exposed to prepare a shunt by using polyethylene tubes. Two 7-cm siliconized polyethylene tubes (0.5/1.0mm inner/outer diameter) were linked to a central 6 cm silicone tube (1.0/1.5mm, inner/outer diameter) containing a 5 cm silk thread (pre-weighed) and
were filled with heparin solution (50U/kg). The shunt assembly was cannulated between the jugular vein and contra-lateral carotid artery and blood was allowed to circulate through the shunt. Blood flow through the shunt was maintained for 10 min, subsequently the central part of the shunt was removed and silk thread having thrombus deposit was taken out and weighed. The thrombus adhered/deposited on thread was calculated by subtracting the wet weight of the silk thread. The standard drugs and test compounds were given 1hr prior to the establishment of arterio-venous shunt (Hayashi et al., 2001).

1.6 FeCl₃ induced thrombosis

The rats were grouped into control, heparin, aspirin, ticlopidine and test compounds treated groups, each group having six animals. Ferric chloride induced thrombosis experiments were performed according to the methods described earlier (Kurz et al., 1990). Rats were anaesthetized with urethane (1.25gm/kg). The left carotid artery was carefully dissected and a pulsed Doppler Probe (DBF-120A-CPx, CBI-8000, Crystal Biotech, and USA) was placed to record the blood flow velocity and the patency of the blood vessels. The carotid artery thrombosis was induced by FeCl₃ (20%, wt/vol) saturated piece of Whatman blotting paper (2x1mm²). Thrombosis was monitored as the cessation in carotid artery blood flow. The time at which the blood-flow velocity was decreased to zero was recorded as total time of occlusion (TTO) of the vessel. When the blood flow velocity did not occlude within 120 min, time to thrombotic occlusion was assigned a value of 120 min. Each of the drugs or vehicle was administered either through intra venous (right jugular vein) or oral route prior to the application of filter paper to the carotid artery.

2. In vitro and ex vivo test systems:

2.1 Platelet aggregation in platelet rich plasma:

Platelet aggregation in rat PRP was monitored according to the protocol described earlier (Dikshit et al., 1993). To assess effect of highly lipophilic agents, compound
was administered via intra peritoneal or oral route 60 min prior to blood collection. Rats were anaesthetized with ether and blood (9 ml) was drawn from the heart into a plastic syringe containing 1 ml of 1.9% tri- Sodium citrate. The blood was centrifuged at 300g for 20 min and the platelet rich plasma (PRP) was collected. The remaining blood was further centrifuged at 2500g for 15 min at 20°C to obtain platelet poor plasma (PPP). The platelet count in the PRP was adjusted to 2x10^8 cells/ml by using PPP. Aggregation was induced by adenosine-5'-diphosphate (ADP), thrombin, collagen, or calcium ionophore A23187 and PMA was monitored on a dual channel aggregometer. Minimum of 4 numbers of observations was recorded for each experiment. Percent inhibition of the test groups were calculated as follows:

\[
\% \text{ Inhibition} = \left[ \frac{\text{Aggregation}_{\text{vehicle}} - \text{Aggregation}_{\text{test}}}{\text{Aggregation}_{\text{vehicle}}} \right] \times 100
\]

Where, Aggregation_{vehicle} = Aggregation obtained in vehicle/control group and Aggregation_{test} = Aggregation obtained in test group.

2.2 Platelet aggregation in human PRP in vitro

The platelet aggregation in human PRP was perfumed with slight modification of the earlier described protocol (Dikshit et al., 1993). Blood was collected in syringe in tri-sodium citrate (3.8%) in the ratio of 1:10 (v/v) from healthy human volunteers who had not taken any aspirin or COX inhibitors at least two weeks before. The blood was centrifuged at 300g for 20 min at 20°C to obtain platelet rich plasma (PRP). The remaining blood was further centrifuged at 2500g for 15 min at 20°C to get platelet poor plasma (PPP). The platelet count in the PRP was adjusted to 2x10^8 cells/ml by using PPP. Aggregation was induced by ADP, collagen, thrombin, and thrombin receptor activating peptide (TRAP) on a dual channel aggregometer. The vehicle or test substance was incubated for 5 min prior to addition of inducer and the percent
aggregation was calculated as described above (section 2.1). Minimum of 4 numbers of observations was recorded for each experiment.

2.3 Thrombin generation assay *in vitro.*

Thrombin generation assay was done with slight modifications of the protocol described earlier (Hemker et al., 2000). Rats were anesthetized and blood was collected by cardiac puncture in a syringe containing 3.8% tri-sodium citrate in a ratio of 1:9. The blood was centrifuged at 300g for collecting PRP (Platelet rich plasma) and at 2500g for 15 minutes at 20°C for obtaining PPP (platelet poor plasma). To PPP or PRP (120μl), 45 μl thrombin substrate (1.1mM) was added, followed by 50 μl of Tris buffer pH: 7.5 and incubated for 5 minutes in 96-well black plate. To each well calcium chloride (10μl from 415mM), 0.5pM Recombinant tissue factor (rTF) or platelet activators (ADP, collagen, thrombin, ristocetin, arachidonic acid or calcium ionophore) were added. The 96-well plate was placed in the well plate reader and shaken for 30 seconds. Thrombin generation was assayed using a fluorimeter, GeminiLX (Excitation: 320nm and emission: 405nm) or High throughput screening systems, cycle interval=15 seconds, duration 2.30hr at 37°C. Thrombogram was plotted and these results were analyzed and interpreted. Minimum of 4 numbers of observations was recorded for each experiment.

2.4 Thrombin generation assay *ex vivo.*

Thrombin generation assay was performed as per Hemker et al., 2000. Rats were grouped in to two groups (i) vehicle/control group and (ii) test molecule-treated groups. The vehicle or drug was treated at 30μM/kg dose 1 hour prior to blood collection. Then the rats were anesthetized and blood was collected by cardiac puncture in a syringe containing 3.8% tri-sodium citrate in a ratio of 1:9. The blood was centrifuged at 300g for collecting PRP (Platelet rich plasma) and at 2500g for 15 minutes at 20°C for obtaining PPP (platelet poor plasma). To PPP or PRP (120μl), 45
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μl thrombin substrate, (1.1mM) was added, followed by 50 μl of Tris buffer pH: 7.5 and incubated for 5 minutes in 96-well black plate. To each well calcium chloride (10μl from 415mM), 0.5pM Recombinant tissue factor (rTF) or platelet activators (ADP, collagen, thrombin, ristocetin, arachidonic acid or calcium ionophore) were added. The 96-well plate was placed in the well plate reader and shaken for 30 seconds. Thrombin generation was assayed using a fluorimeter, Gemini LX (Excitation: 320nm and emission: 405nm) or High throughput screening systems, cycle interval=15 seconds, duration 2.30hr at 37°C. Thrombogram was plotted and these results were analyzed and interpreted. Minimum of 4 numbers of observations was recorded for each experiment.

2.5 Coagulation Parameters

Blood was collected by cardiac puncture from ether-anaesthetized rats or hamsters into a syringe containing (3.2%) tri-sodium citrate (9:1, v/v). It was centrifuged at 2500g for 15 minute at 20°C to obtain PPP. Coagulation parameters, that is, thrombin time (TT), prothrombin time (PT), activated partial thromboplastin time (aPTT) and fibrinogen level (FT) were assessed in PPP according to the manufacturer’s instructions and measured by using a Coagulometer (Batra et al., 2004; Essler et al., 2000). Minimum of 4 numbers of observations was recorded for each experiment.

2.6 Preparation of human washed platelets and antibody labeling for flow cytometry studies

The flow cytometry studies were conducted according to the protocol described earlier (Wadhawan et al., 2004). Fresh human blood (9ml) was collected into a tube containing 1ml of 3.8% tri-sodium citrate and centrifuged at 300g for 20 minutes at 20°C to obtain PRP. Aspirin (1mM) 1μl/ml and Na-EDTA (1mM) was added to PRP and incubated for 15 minutes. Apyrase was added to it. It was centrifuged again at 800g. Pellet obtained was taken and suspended in wash buffer A(20mM HEPES, 138mM NaCl, 2.9mM KCl, 1mM MgCl2, 0.36mM NaH2PO4, 1mM EGTA, pH:6.2)
and again centrifuged at 800g for 20 minutes at 20°C. Supernatant was discarded & pellet was collected. The pellet was suspended in buffer B (same as buffer ‘A’ but without EGTA & apyrase, pH: 7.4) for study. To the washed platelet PAC-1 FITC was added and incubated at room temperature. The platelet suspension was activated with a particular agonist for specific time. The sample was fixed with para formaldehyde. The samples were kept overnight at 2-8°C for flow-cytometry studies.

2.7 Preparation of human washed platelets and collagen-binding assay: a flow cytometry study

Fresh human blood (9ml) was collected into a tube containing 1ml of 3.8% tri-sodium citrate and centrifuged at 300g for 20 minutes at 20°C to obtain PRP. Aspirin (1mM) 1µl/ml and Na-EDTA (1mM) was added to PRP and incubated for 15 minutes. Apyrase was added to it. It was centrifuged again at 800g. Pellet obtained was taken and suspended in wash buffer A (20mM HEPES, 138mM NaCl, 2.9mM KCl, 1mM MgCl2, 0.36mM NaH2PO4, 1mM EGTA, pH:6.2) and again centrifuged. Supernatant was discarded and pellet was collected. The pellet was suspended in buffer B (same as buffer ‘A’ but without EGTA & apyrase, pH: 7.4) for study.

The cell count was adjusted by spectrophotometer. The absorbance of 0.650 at 630nm refers to 2X10^8 numbers of platelets per ml in the sample or cell suspension. 2X10^7 cells were taken for assessing collagen-hinging to the platelets using various concentration of collagen Oregon green 488 assay such as 0.1µg/ml, 0.3µg/ml, 1µg/ml, 3µg/ml, 10µg/ml, 30µg/ml and 50µg/ml.

In another experiments the platelets (1X10^7) were pre-incubated with test molecules (S000-20 or S002-329) for 5 minutes, subsequently fluorescent collagen (10µg/ml) was added and then, the samples were incubated for 30 minutes at 37°C. Then, collagen binding was subsequently assessed by flow cytometry.
2.8 Preparation of aortic rings and vascular contraction and relaxation studies

The aortic ring contraction and relaxation studies were carried out with slight modification of the earlier protocol described from our lab (Raghavan and Dikshit, 2001). Rats were anaesthetized by inhalation of ether. After opening the chest thoracic aorta was excised and immediately placed in ice-cold Krebs bicarbonate medium of the following composition (mM): NaCl 118; KCl 5; CaCl₂ 2.5; MgSO₄ 1.2; KH₂PO₄ 1.2; NaHCO₃ 25; Glucose 11; disodium EDTA 0.030; pH 7.4. It was dissected free of connective tissue and fat and was cut into rings (5 mm in length). The rings were mounted vertically between two stirrups in organ chambers filled with 10 ml Krebs solution maintained at 37±0.5°C, constantly bubbled with 95%O₂-5%CO₂. One stirrup was connected to an anchor associated with force transducer (FSG-01 transducer, Experimentria Limited, Budapest, Hungary) and the other to the tissue holder for recording of isometric tension. The rings were equilibrated for 90 min, during which the bathing fluid was changed every 15 min and the tissue was kept under a constant tension of 2 g throughout the experiment. After equilibration, the rings were evaluated for the presence of a functional endothelium by exposing the rings to a KCl (80 mM) physiological salt solution before starting the experiments. The aortic rings were contracted with sub-maximal concentration of phenylephrine (1X10⁻⁷ M). The pre-contracted rings were then exposed to increasing concentration of 99/353(10nM to 300μM) or acetylcholine (10nM to 300μM) to assess their vasorelaxation effects. The percent relaxation was calculated and plotted.

3. Statistical Analysis

The results have been reported as Mean±SEM. The comparisons have been made among the control and test substance treated groups by using unpaired, Student t-test. The p value <0.05 was considered significant.
4. Lists of Instruments Used:

Blood flow meter (CBI-8000, Crystal biotech, USA)
Coagulometer (Start4 Semi automated, Young Instruments, Stago, France)
Dual channel aggregometer (560 Ca, 230 VAC, Chrono-log Corp, USA)
FACS Calibur (BD Biosciences, USA)
Goose neck lamp (Havard Apparatus, GmbH, Germany)
Heat controlled operation table (Havard Apparatus, GmbH, Germany)
High speed centrifuges, Sigma 3K12, Sigma 3K30 (Sigma Laborzentrifugen GmbH, Germany)
Fluorimeter, GeminiLX (Molecular Devices, USA)
Isolated tissue bath system (Experimentria, Budapest, Hungary)
pH meter (Toshniwal Instruments Pvt. Ltd., India)
Small animal ventilator (Havard Apparatus, GmbH, Germany)
Spectrophotometer (Shimadzu, Japan)