Role of 4-substituted 2,6-bispyrazinyl-(4-dimethoxyphenyl) Pyridines on Plasma Re-calciﬁcation Time and Platelet

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ABSTRACT

Present study deals with exploring the antithrombotic properties of synthesized 2,2'-[4-(n,n'-dimethoxyphenyl) pyridine-2,6-diyl] dipyrazines (1-4) compounds. These compounds prolonged the clotting time of citrated human plasma in both PRP and PPP but surprisingly compound 2 exhibit pro-coagulant activity by decreasing the clotting time in both PRP and PPP. In PRP compounds 1, 3 and 4 enhanced clotting time from control to 60-100 sec whereas in PPP enhanced the clotting time from control up to 80-140 sec. In addition, only compound 3 inhibited the ADP induced platelet aggregation whereas remaining 1, 2 and 4 did not show any role on platelets aggregation. The inhibition percentage of 3 was found to be 14%. Moreover, these compounds were found non-toxic to RBCs.

Keywords: Anticoagulant, Antiplatelet and Non-toxic.

1. Introduction

Hemostasis is highly regulated pathway which was meant to prevent blood loss during tissue injury [1]. Due to some genetic imbalance and some environmental factors several disorders such as hemophilia (uncontrolled blood loss) and thrombotic disorders (unusual blood clot) [2]. Several anticoagulants from snake venom (Ancrod), fungi Aspergillusoryzae (Brina), coumarins derivative from sweet clover, hirudin derivative from saliva of leach are currently being used to treat thrombotic disorders [3-5].

Flax seed, Bitter gourd, Pea pod, Jackfruit seed extracts and also lignin capped silver nano-particles found to be exhibit the anticoagulant effect [6-9]. Although many synthetic anti-coagulants and pro-coagulants were available in the market but they cause side effects such as nausea, vomiting and diarrhea.

2. Experimental Section

2.1 Materials and Methods

Reagent grade chemicals obtained from different commercial sources were used as received and solvents were purified and dried according to standard methods. Adenosine diophosphate (ADP) and Fresh human blood were collected from healthy donors. Lumi aggregation system (Model-700) was used to determine platelet aggregation.

2.2 Synthesis of compounds (1 to 4)

A 100mL round-bottom flask charged with 2-acetylpyrazine (1.47g, 2mmol) was added MeOH (30mL), KOH pellets (0.675g, 2mmol) and 2mL of water. The whole mixture was stirred for 10min and then was added corresponding n,n’-dimethoxy benzaldehydes (1.0g, 1mmol). Upon stirring at room temperature for 4hr, a white precipitate obtained was filtered, washed with MeOH and diethyl ether.

The products on recrystallization in a mixture of CHCl\textsubscript{3}:MeOH (1:1) gave white crystals of 1 to 4 [10].
2.3 Preparation of Platelet Rich Plasma (PRP) and Platelet Poor Plasma (PPP)

The method of Ardlie and Han [11] was employed for the preparation PRP and PPP. The platelet concentration of PRP was adjusted to $3.1 \times 10^8$ platelets/mL with PPP.

The PRP maintained at 37°C was used within 2hr for the aggregation process. All the above preparations were carried out using plastic wares or siliconized glass wares.

2.4 Plasma re-calcification time

The plasma re-calcification time was determined according to the method of Quick et al., [12]. Briefly, the ligand 1 to 4 (0-40μg) was pre-incubated with 0.2 mL of citrated human plasma in the presence of 10mM Tris-HCl (20μL) buffer of pH 7.4 for 1min at 37°C. 20μL of 0.25M CaCl$_2$ was added to this pre-incubated mixture and clotting time was recorded with respect to control without sample.

2.5 Platelet aggregation

Platelet aggregation was performed according to the method of Born [13]. Briefly, aliquots of PRP were pre-incubated with various concentrations of 1 to 4 (0–30μg) in 0.25mL reaction volume.

The aggregation was initiated independently by the addition of agonist ADP followed for 6min.

2.6 Direct haemolytic activity

Direct haemolytic activity of compounds 1 to 4 was determined by using washed human erythrocytes as described previously [14]. Briefly, packed human erythrocytes and Phosphate Buffered Saline (PBS) (1:9 v/v) were mixed; 1mL of this suspension was incubated independently with the various concentrations of compounds (0-200μg) for 1h at 37°C. The reaction was stopped by adding 9mL of ice cold PBS and centrifuged at 1000g for 10min at 37°C. RBC cells lysis percentages were calculated based on the amount of haemoglobin released in the supernatant was measured at 540 nm.

Activity was expressed as percent of haemolysis against 100% lysis of cells due to addition of water that served as positive control and PBS served as negative control.

3. Results and discussion

3.1 Plasma re-calcification time

To understand the role of newly synthesised 2,6-bispyrazinylpyridine derivatives 1 to 4 on blood coagulation cascade, plasma recalification time was analysed using fresh human plasma drawn from healthy volunteers in both PRP and PPP. Plasma re-calcification time was recorded for pre-incubated compounds with citrated human plasma either as PRP or PPP in the presence of Tris–HCl buffer at pH, 7.4 for 1min at 37°C.

When the CaCl$_2$ was added to this pre-incubated mixture clotting of blood was initiated and time of clotting with varying concentration was recorded. Interestingly, compounds 1 to 4 enhanced clotting time from control 154 to 300, 330, 178, and 250sec respectively, similarly in PPP the clotting time was enhanced from control 194 to 340, 370, 210 and 280sec respectively as shown in Fig.1. Therefore these can act as anticoagulation agents.
3.2 Antiplatelet Activity

Platelets are components in the blood stream, they play a major role in blood clotting process by forming the platelet plug at the site of injury [15-18]. In some cases unusual clots were formed in arteries and veins which is known as thrombosis that ultimately leads to death due to environmental factors and genetic aberrations. In these cases in-order to inhibit the activation of platelets anti-platelet agents plays a pivotal role [19-21]. Eptifibatide, derivative from rattle snake venom that inhibits glycoprotein IIb/IIIa receptor on platelets is currently using in the treatment of coagulation disorders [22]. Thus, the current established unknown ligands may leads to be potent antiplatelet agent in future to treat thrombotic disorders. The role of compounds 1 to 4 on platelet aggregation was assayed. Fascinatingly, only compound 3 found to inhibit the ADP induced platelet aggregation whereas remaining compounds did not show any role on platelets aggregation. The inhibition percentage was found to be 18%.
L3). The values represent ± SD of three independent experiments. (b) Dose dependent platelet aggregation inhibition % (c) Dose dependent platelet aggregation %.

3.3 Non-toxic property of ligands

In order to identify the role of compounds (1 to 4) on the membrane of RBC direct haemolytic assay was performed. The experimental data revealed that these compounds did not hydrolyse RBC up to the concentration of 100 μg. These observations suggested that they are nontoxic to RBCs. Thus, they could be better therapeutic agents to treat thrombotic disorders.

![Graphs a to d showing haemolytic activity](image)

**Fig.3.** Haemolytic activity plotted for percentage of RBC lysis against variation of concentration of compounds 1 to 4

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**Consent to participate**

Not Applicable

**Consent for publication**

We declare that we consented for the publication of this research work.

**Availability of data and material**

Authors are willing to share data and material according to the relevant needs.

**Author’s contribution**

All authors participated in overseeing laboratory work, data analysis, and manuscript writing and review.
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