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In-Vitro and *In Silico* Antiplatelet Action of the New Piperidin4-One-Thiosemicarbazide Derivative

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ABSTRACT

Platelets are believed to contribute to hemostasis, thrombosis, inflammation, wound healing, and immunology. R2 is a new thiosemicarbazide derivative of piperidone that is effective in the treatment of angina and ischemia.

In healthy volunteers' blood, compound (R2) reduced platelet aggregation induced by collagen (1-2 μg/ml). R2's effect on PRP was determined using an aggregometer.

R2 reduced platelet aggregation substantially. Without R2, collagen-induced platelet aggregation in PRP was showed 80%, while R2 inhibited 100% platelet aggregation at 1.0625 μM. R2 to have collagen-induced antiplatelet IC50 of 0.5555 ± 0.10 μM. The chosen derivative shows binding energy of -5.73 kcal/mol. Molecular docking analysis revealed significant molecular interactions between Piperidone derivatives and platelet aggregation, suggesting that the drug may be developed further as a platelet aggregation inhibitor. Additionally, the new chemical (R2), a novel thiosemicarbazide derivative, may be beneficial in the treatment of platelet-associated thromboembolic diseases.

Key Word: Platelets, Aggregation, Collagen, Thiosemicarbazone derivatives, 4-piperidone, Thrombosis.

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INTRODUCTION

Platelets are essential for hemostasis, thrombosis, wound healing, atherosclerosis, inflammation, and immunology [1–3]. Although platelets' main function after injury or damage is to prevent blood loss, they are often responsible for the formation of dysregulated thrombus, which may result in myocardial infarction, acute coronary syndrome, or ischemia [4]. Numerous agonists (Adenosine diphosphate (ADP), Collagen, Arachidonic acid, Platelet Activating Factor, thrombin, and thromboxane A2) are involved in the activation process of platelets [5]. Aspirin has been used to treat platelet hyperactivity induced by elevated thromboxaneA2 (TxA2) production in several coronary disease conditions to decrease the risk of severe ischemic events [6]. However, following an extended period of follow-up, between 10% and 20% of individuals using aspirin as secondary prophylaxis develop a chronic thrombotic condition. Aspirin's failure is due to aspirin resistance [7–8]. Present antiplatelet agents, such as acetylsalicylic acid, phosphodiesterase inhibitors, P2Y12 antagonists, and main platelet integrin αIIbβ3 antagonists, are concerned with complications and have a limited mode of action [9–10]. Drugs derived from natural compounds, on the other hand, have a low risk of side effects [11]. As a result, it is essential to increase the efficacy of these drugs and to investigate alternative non-aspirin antiplatelet inhibitors that are both safer and more effective. As a result, some compounds derived from natural or synthetic sources that are already used in traditional medicine are being studied more closely to determine their antiplatelet activity [6].

Piperidine alkaloid derivatives isolated from natural Piper nigrum (black pepper) provide a wide variety of compounds with various pharmacological activities [12-15]. Piperidin4-one or 4-piperidone is the most commonly used piperidine derivative as a starting material for the synthesis of several important commercially available bioactive molecules, including propiverine (anticholinergic), piperylone (antipyretic, analgesic), clocapramine (antipsychotic), dorastine (anticancer), fentanyl (anesthetic, analgesic), pimozide (antipsychotic), and others (schizophrenia) [16]. The organic reaction between thiosemicarbazide and the carbonyl group of 4-piperidone yields the strongly functionalized intermediate thiosemicarbazone, which is used to make biologically active heterocyclic compounds like thiazole [17-20]. Thiazoles are anti-inflammatory, antifungal, antiretroviral, antihistaminic, antithyroid, and antimicrobial. Numerous substituted thiazole derivatives have been shown to exhibit substantial analgesic activity [21-22] The bioactivity of thiazole, piperidine, and its novel derivative has led to the discovery of simple methods for the synthesis of antiplatelet activity of 4-piperidone-based carbothioamide derivative. In this study, we evaluated in vitro and *in silico* antiplatelet activities and the medicinal importance of 4-piperidone-based carbothioamide derivative "(Z)-2-(3,3-dimethyl-2,6-diphenylpiperidin-4-ylidene)hydrazinecarbothioamide" in case of cardiovascular complications.

MATERIAL AND METHOD

Material

A novel compound, 4-piperidone -based carbothioamide derivative known as "(Z)-2-(3,3-dimethyl-2,6-diphenylpiperidin-4-ylidene)hydrazinecarbothioamide" (R2) was obtained from the Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Karachi. Chrono-Log Corp. supplied the collagen (Haver-town, PA, USA). The other reagents and solvents used in this study

were of analytical grade, including sodium hydroxide, sodium citrate, dimethyl sulfoxide (DMSO), and phosphate buffer solution.

Instruments

The centrifuge machine was used to prepare both platelet-rich plasma and platelet-poor plasma (Eppendorf Centrifuge 5810R and Mini-Spin Eppendrof AG-22331 Hamburg, Germany, respectively). Platelet aggregation concentration was determined using a dual-channel platelet aggregometer (Model No. 5490 – 2D Chrono – Log Corporation, USA) connected to a personal computer.

Human Subjects

In this study, hundred healthy volunteers (male and female, ages 18–40) were recruited. They had not taken any drug that could affect platelet activity for at least two weeks.

Ethical Approval

The Karachi Institute of Bioengineering and Genetic Engineering's ethics committee approved the experimental protocol.

METHOD

1. In Vitro Anti-platelet Assay

PRP and PPP preparation

A total of 30ml venous blood was drawn from a healthy volunteer using a 21G butterfly needle into a disposable syringe and immediately transferred into polypropylene tubes containing 3.8% sodium citrate (1:9 V/V). According to a previously described method with minor modifications, PRP was obtained by centrifuging the citrated blood tubes at 1400rpm for 15 minutes (Eppendorf centrifuge 5810R). Further centrifugation of PRP at 13000 rpm for 15 minutes resulted in the formation of PPP. [23]

Assay for platelets aggregation

The aggregation responses of platelets were determined using the light transmission system [24] using a Lumi-aggregometer model (5490–2D) (Chrono–Log, Havertown, PA, USA) at 37°C. PRP (350ul) was incubated with constant stirring at 1200rpm in aggregometry sample cuvettes. After 1 minute, 4-piperidone -based carbothioamide derivative R2 (0.3125µM, 0.625µM, 0.9375µM, and 1.0625µM) was applied on Collagen-induced platelet aggregations and incubated for an additional 5 minutes. After that, a threshold concentration of collagen (1-2µg/ml) was applied to cause platelet aggregation. For six minutes, the degree of platelet aggregation was monitored. The turbidity of the platelet sample decreased with increasing platelet aggregation, owing to platelet clearance in PRP and a higher proportion of light propagation. The following formula was used to calculate the percentage of platelet aggregation inhibition:

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$$\text{Percentage inhibition of platelet aggregation } = \frac{\text{A} \times \text{B}}{\text{A}} \times 100$$

A =by using a control sample, the maximum aggregation was reported.

B= Aggregation was observed following the addition of the test compound "(Z)-2-(3,3-dimethyl-2,6-diphenylpiperidin-4-ylidene)hydrazinecarbothioamide" (R2).

2. In silico Anti-platelet Assay

Methodology

Preparation of Inhibitor

The 2D structure of the selected compound was drawn on ChemOffice 16 and save in cdx format. The analog was converted into 3D structures by using ChemDraw 4D ultra (version 16.0) (Chemical Structure Drawing Standard; Cambridge Soft Corporation, USA (2009) [25]. Additionally, the MMFF94X force-field method was used to minimize energies of all analog with 1000 steps iteration. Finally, processed analog was saved into PDB format. The MGL Tools (version 1.5.6) [26] were used to generate PDBQT files.

Preparation of Target Protein

The protein structure for Antiplatelet studies (PDB=1EQG) was downloaded from RCSB-PDB with the resolution of 2.60 Å (http://www.rcsb.org/pdb/home/home.do). The bound ligand, water molecules, and extra protein chains were deleted using BIOVIA Discovery Studio Visualizer. The polar hydrogen and Kollman Charges were introduced in the receptor file using AutoDockVina Program and generated the PDBQT file.

Molecular Docking Studies

The molecular docking studies were performed using the AutoDockVina version (4.2). The ligand-enzyme interaction was deal with the Lamarckian genetic algorithm (LGA). The grid box was generated in the middle of active site residues of a receptor with 28.433, 28.311, and 198.790. The AUTOGRID program was used to produced a grid map was 45, 45, 45 with points spaced at 0.700 Å. The docking parameters were set to the default setting of the genetic algorithm. The best pose of docked ligand-enzyme complexes was predicted by binding energies.

Analysis of Results

The dlg file of the docked complex was used to predict the best pose. Furthermore, the BIOVIA Discovery Studio Visualizer (DSV), PyMol molecular visualization tool [27], and PLIP [28]were used to visualize the interaction of ligand and protein complexes.

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Results

In vitro platelet aggregation inhibited by a novel compound R2.

Figures 1 and 2 illustrate the inhibitory activity of the 4-piperidone-based carbothioamide derivative (R2) on platelet aggregation caused by collagen. The findings indicated that R2 had a strong inhibitory effect on platelet aggregation induced by Collagen.

In a concentration-dependent manner, the novel compound R2 (0.3125µM, 0.625µM, 0.9375µM, and 1.0625µM) blocked human platelet aggregation mediated by collagen (1ug/ml). The R2 IC50 value for inhibiting platelet aggregation caused by collagen was 0.55µM ±0.10 (Table 1, Figure 1).

Table 1.The IC50 value and inhibitory action of R2 on platelet aggregation caused by collagen

Compound	Concentration (µM)	% of Inhibition	IC_{50} (µM)
R2	0.3125	37.5 ± 2.32	0.5555 ± 0.10
	0.625	56.25 ± 1.27	
	0.9375	87.5 ± 3.82	
	1.0625	100 ± 0.0	

*** Values are reported as mean ± Standard Error of Mean (SEM), n = 5**

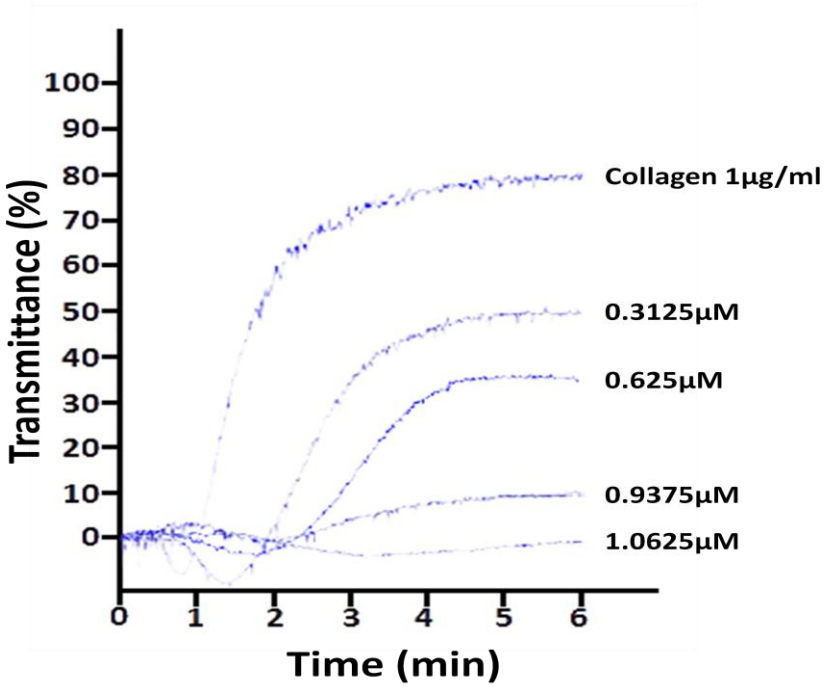

Figure 1. Dose-response curve of R2 novel compound on platelet aggregation induced by collagen

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***In silico* platelet aggregation is inhibited by a novel compound R2**.

Typically, the binding energy (BE) of a receptor-ligand complex is calculated by modeling the molecular dynamics of the complex and calculating the free energy of tiny ligands binding to biological macromolecules. BE values were calculated in this research to evaluate the binding affinity of proteins (PDB 1EQG) for the target compound quantitatively. As shown in Fig. 2, the selected compound had a negative BE value of -5.73 kcal/mol. Given that a lower BE value usually implies a greater ligand-binding affinity, the docking results imply that all of the test compounds may be powerful ligands with substantially distinct binding mechanisms.

R2 binds to six hydrophobic bonds at the following locations in PDB 1EQG: ARG83, LEU112, LEU115, VAL116, VAL119, and ARG120, with a binding energy of -5.73 kcal/mol. R2 inhibited PDB 1EQG at a concentration of 63.11 mM.

This demonstrates that the newly synthesized carbothioamide compounds possess active binding sites and the ability to inhibit platelet aggregation (Table 2, Figure 2).

The Carbothioamide derivate chosen had higher binding energy. This shows that R2 has potential antiplatelet binding sites and suggesting a greater potential for antiplatelet action at PDB 1EQG

Table 2: Chemical details and binding energy of Piperidin4-one-thiosemicarbazide derivative.

Derivative	H-Bond	H-Bond (an amino acid with bond length)	No. of Hydrophobic Bond	Hydrophobic Bonding (Amino acid and bond length)	Binding Energy (kcal/mol)
R2	**No**	**No**	**06**	**ARG83 (3.49), LEU112 (3.82), LEU115 (3.92), VAL116 (3.88), VAL 119 (3.63), ARG120 (3.86)**	**-5.73**

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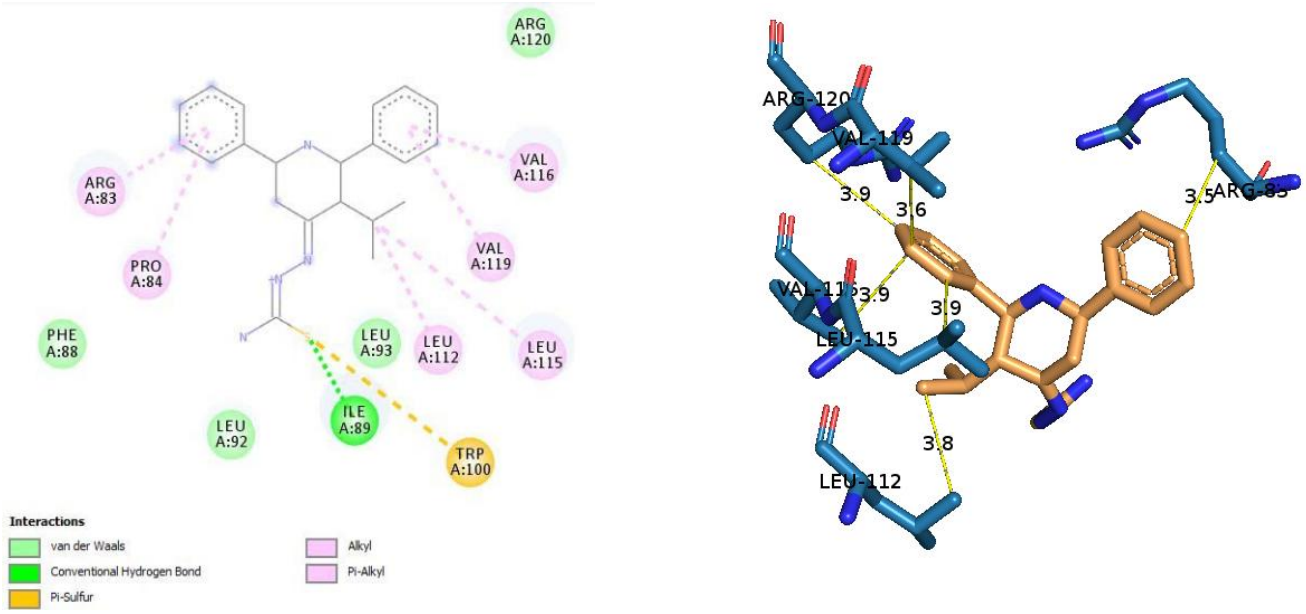

A: 2D Image **B:** 3D Image

Figure 2: Binding energy and Ligand interactions of Piperidin4-one-thiosemicarbazide derivative A: 2D Image, B: 3D Image of Piperidin4-one-thiosemicarbazide

DISCUSSION

Heart disease and stroke are the main causes of mortality worldwide. They are caused by a blood clot or thrombus formed as a result of platelet and other protein activation. Numerous medications are available for this purpose. However, the researcher continued to work on new compounds to improve the patient's effectiveness and safety profile while minimizing adverse effects.

Diseases caused by blood clots or thrombi, such as heart failure and stroke, are the leading cause of mortality globally. This disease may be treated with a variety of medicines. However, the researcher proceeded to experiment with different medicines to enhance the efficacy and safety profile of the patient by using side effects [29].

Novel synthetic products or natural products derived from plants with antiplatelet activity can be a source of a major compound with significant effectiveness and little adverse effects. At a dosage of 75–150 mg, aspirin is the most often used antiplatelet agent for the prevention of vascular accidents. [7]. Also, at minimal doses, aspirin may induce adverse effects such as gastric erosion and GI bleeding [30]. As a result, novel therapeutic agents are needed for precisely targeting the desired degree of platelet activation while minimizing adverse effects. With high reliability and reproducibility, light transmission aggregometry is used to determine the effect of Collagen on platelet aggregation as agonists. [31]. The antiplatelet effect of R2 induced by collagen was demonstrated in the current study at a concentration of 1.0625µM. (Figure 1). Thus, our novel compound R2 can exert antiplatelet activity by inhibiting the cyclooxygenase and lipoxygenase pathways, as well as platelet aggregation.

New pathways to pharmacological care for blood clot-related coronary disorders such as angina, stroke, and MI greatly decrease the morbidity profile of CVD patients, either through medication alone or in conjunction, such as aspirin and clopidogrel. These treatments are not always effective in reducing mortality in these patients, which may be due to their prolonged duration of actions, which results in bleeding complications.[32–33]. The current treatment of platelet activation in CVD therapy involves primary consideration for adverse side effects such as bleeding, especially before and during surgical procedures. [34]Therefore, alternate methods are warranted which would inhibit platelet activation, thus minimizing the bleeding side effect.

While treatments targeting COX-1 or surface receptors such as PAR1, P2Y12, and integrin receptor IIb3 have been highly effective in decreasing MI-related morbidity, they have failed to meaningfully reduce death in these patients. This might be because antiplatelet medications do not inhibit platelet activation, have a protracted onset and duration of action, and can cause considerable morbidity due to bleeding problems. [32–33].

New treatment methods are thus needed to reduce platelet activity when the vessels are occluded and stroke without producing bleeding problems. It can be addressed by blocking the secondary path of platelet activation, which further inhibits coagulation formation without changing the bleeding profile, as seen with COX-1 secondary pathway suppression.

CONCLUSION

Although the exact mechanism of action is unknown, our findings suggest that the novel 4-piperidone-based carbothioamide derivative "(Z)-2-(3,3-dimethyl-2,6-diphenylpiperidin-4-ylidene)hydrazinecarbothioamide" (R2) can inhibit platelet aggregation by inhibiting secondary aggregation pathways such as Lipoxygenase and cyclooxygenase. Based on these findings, further study with other platelet aggregation-inducing drugs, as well as genetic experimentation, is required to investigate broader approaches for treating these disabling medical conditions. Following additional studies, we conclude that a 4-piperidone-based carbothioamide derivative (R2) could be used at defined concentrations as a platelet inhibitor in cardiovascular disorders.

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