Original Research Article

Novel target of the 4-piperidone thiazole derivative for platelet inhibitions by using inducers collagen and ADP

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ABSTRACT

Platelets are thought to aid in hemostasis, thrombosis, inflammation, wound healing, and immunity. R4 is a novel thiazole derivative that may decrease angina and ischemia. An aggregometer evaluated R4 impact on PRP. Spectrophotometric method measured anti-lipoxygenase activity of R4. In its absence, the platelet aggregation in PRP showed 88 % and 75 % with collagen and ADP, respectively, while R4 inhibited 100 % collagen-induced platelet aggregation at 1.1875 μM and ADP-induced platelet aggregation up to 100 % at 0.9375 μM. Collagen and ADP induced R4 antiplatelet IC50 values were 0.55 ± 0.12 μM, and 0.26 ± 0.20 μM, respectively. R4 inhibited lipoxygenase significantly with an IC50 of 26.65 ± 0.16 μM. The novel compound, R4, may also assist platelet-associated thromboembolic disorders.

***Keywords*:** Aggregation, platelets, collagen, ADP, thiazole derivatives, 4-piperidone, thrombosis

INTRODUCTION

It is well established that platelets play a critical role in hemostasis, thrombosis, wound healing, atherosclerosis, inflammation, and immunity [1–3]. Platelets primary role in response to injury or damage is to inhibit blood loss, but they are often responsible for developing dysregulated thrombus, which may lead to

myocardial infarction, acute coronary syndrome, or ischemia [4]. The activation process of the platelets includes many agonists (Adenosine Diphosphate (ADP), collagen, arachidonic acid, platelet activating factor and thrombin [5]. Aspirin has been used to treat platelet hyperactivity caused by increased Thromboxane A2 (TxA2) synthesis in a variety of coronary disease states to reduce severe ischemic events [6]. However, after a lengthy period of follow-up, between 10 % and 20 % of patients taking aspirin as secondary prophylaxis have a chronic thrombotic case. This failure of aspirin is attributable to resistance to aspirin [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].

Lipoxygenases (LOs) are non-heme iron dioxygenases that catalyze the oxidation of polyunsaturated fatty acid (arachidonic acid or linoleic acid) to form cis and trans-conjugated diene hydroperoxides [12]. The conversion of Arachidonic Acid (AA) to 12(S)-hydroxy-5, 8, 10, 14-eicosatetraenoic acid (12(S)-HETE) was first tested in human and bovine platelets in the mid-1970s [13]. Important species of lipoxygenases and their metabolites can be found in specific cells and tissues. Depending on their kind of cell, three isoforms of 12(S)-lipoxygenases were studied: epiderms, leukocytes and platelets [14]. Bioactive metabolites are formed when arachidonic acid (AA) is oxidized by COX-1 or 12-LOX [13]. In leukocytes, leukotriene B4 (LTB4) and prostaglandins E2 (PGE2) are produced, while in platelets, 12-HETE and thromboxane A2 are produced [15–16]. For platelet secretions of dense granules and for proper platelet aggregation and adhesion, 12-LOX activation is necessary. 12-LOX also plays a major role in calcium mobilization regulation. Baicalein was first identified in the mid-1990s as a classic 12-LOX inhibitor in platelets, inhibiting 12-LOX in

response to AA stimulation, resulting in a substantial reduction in thrombin-induced calcium and aggregation [17].

Piperidine alkaloid derivatives isolated from natural Piper nigrum (black pepper) provide a wide variety of compounds with various pharmacological activities [18-21]. 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) [22]. 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 [23-26]. Thiazoles are anti-inflammatory, antifungal, antiretroviral, antihistaminic, antithyroid, and antimicrobial. Numerous substituted thiazole derivatives have been shown to exhibit substantial analgesic activity [27-28]. 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 thiazole derivative. In this study we evaluated antiplatelet and lipoxygenase inhibition activities and medicinal importance of 4-piperidone-based thiazole derivative (Z)-2-(3,3-dimethyl-2,6-diphenylpiperidin-4-ylidene) hydrazinecarbothioamide in case of cardiovascular complications.

MATERIALS AND METHODS

A novel compound, 4-piperidone -based thiazole derivative known as (Z)-2-(2-(3-methyl-2,6-diphenylpiperidin-4-ylidene) hydrazinyl)-4-(4-nitrophenyl)thiazole (R4) was obtained from the department of pharmaceutical chemistry, faculty of pharmacy, university of Karachi. Chrono-Log Corp. supplied the Adenosine Diphosphate (ADP) and 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.

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. 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.

PRP and PPP Preparation

A total of 30 ml 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 1400 rpm for 15 min (Eppendrof centrifuge 5810R). Further centrifugation of PRP at 13000 rpm for 15 min resulted in the formation of PPP [29].

Assay for platelets aggregation

The aggregation responses of platelets were determined using the light transmission system (Born and Cross, 1963) [30] using a Lumi-aggregometer model (5490–2D) (Chrono–Log, Havertown, PA, USA) at 37 °C. PRP (350 µl) was incubated with constant stirring at 1200 rpm in aggregometry sample cuvettes. After 1 min, 4-piperidone -based thiazole derivative R4 (0.3125 µM, 0.625 µM, 0.9375 µM, 1.0625 µM & 1.1875 µM) for Collagen and R4 (0.3125 µM, 0.625 µM & 0.9375 µM) for ADP was applied and incubated for an additional 5 min. After that, a threshold concentration of collagen (1-2 µg/ml) and ADP (5 µM/ml) was applied to cause platelet aggregation. For six min, 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:

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

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

B= Aggregation was observed following the addition of the 4-piperidone-based thiazole derivative (R4).

Assay for inhibition of lipoxygenase

The inhibition activity of lipoxygenase was determined by changing the Tappel spectrophotometric process [31]. Lipoxygenase enzyme solution in sodium phosphate buffer was prepared to optimize the enzyme concentration in the reaction mixture to 0.05 absorbance / min. The test compound (R4) has been prepared in methanol at different concentrations from 12.5 μM to 500 μM. The reaction mixture contains a sodium phosphate buffer (pH 8.0) of 160 ml (100 μM), a 10 μL solution for the test chemical and a 20 μL solution of the LOX system. The contents were mixed and incubated for 10 min at 258 °C. The reaction was started by adding 10 ml substrate solution (linoleic acid, 0.5 mM, 0.12 % w/v tween 20 in a 1:2 ratio) to each well. After 15 min, the absorbance at 234 nm was calculated. The concentration of the test compound that inhibited lipoxygenase activity by 50 % (IC50) was determined by measuring the degree of inhibition as the concentrations of these compounds were increased in the tests.

The IC50 values were determined by the Enzyme-Kinetics Program EZ-Fit (Perrella Scientific Inc., Amherst, USA) [32].

RESULTS

Platelet Aggregation inhibited by a novel compound R4

In a concentration-dependent manner, the novel compound R4 (0.3125 μM, 0.625 μM, 0.9375 μM, 1.0625 μM & 1.1875 μM) blocked human platelet aggregation mediated by collagen (1 μg/ml) and ADP (5 μM/ml). R4 IC50 values for inhibiting platelet aggregation caused by collagen and ADP were 0.55 ± 0.12 μM and 0.26 ± 0.20 μM, respectively (Tables 1 and 2).

Lipoxygenase Inhibition by a novel compound R4

When R4 was tested *in vitro* for lipoxygenase inhibition function, it displayed strong inhibition in a dose-dependent manner at concentrations ranging from 25 μM to 100 μM. The IC50 value of R4 was determined to be 26.65 ± 0.16 μM, which is almost equal to the IC50 value of the assay norm Baicalein (IC50 = 22.6 ± 0.08 μM) (Table 3, Figures 3 and 4).

Table 1. The IC50 value and inhibitory action of R4 on platelet aggregation caused by collagen

Compound	Concentration (µM)	% Inhibition	IC_{50} (µM)
R4	0.3125	9.090 ± 2.32	0.55 ± 0.12
	0.625	56.818 ± 1.27	
	0.9375	78.409 ± 3.82	
	1.0625	92.045 ± 5.64	
	1.1875	100 ± 0.0	

Values are reported as mean ± standard deviation of Mean, n = 5

Table 2. The IC50 value and inhibitory action of R4 on platelet aggregation caused by ADP

Compound	Concentration (µM)	% Inhibition	IC_{50} (µM)
R4	0.3125	60 ± 1.69	0.26 ± 0.20
	0.6250	92 ± 4.27	
	0.9375	100 ± 3.34	

Values are reported as mean ± standard deviation of Mean, n = 5

Table 3. The IC50 value and inhibitory action of R4 on lipoxygenase enzyme

LIPOXYGENASE INHIBITION ACTIVITY			
	Concentration (µM)	**Percentage Inhibition**	**IC_{50}**
R4	25	46.9 ± 0.54	26.65 ± 0.16
	50	68.7 ± 0.89	
	100	97.4 ± 0.92	
Baicalein			22.6±0.08

Values are reported as mean ± standard deviation of Mean, n = 10

Figures 1 and 2 illustrate the inhibitory activity of the 4-piperidone-based thiazole derivative (R4) on platelet aggregation caused by collagen and ADP. The findings indicated that R4 had a strong inhibitory effect on platelet aggregation induced by two inducers, collagen and ADP. The IC50 value of R4 was determined to be 26.65 ± 0.16 µM, which is almost equal to the IC50 value of the assay norm Baicalein (IC50 = 22.6 ± 0.08 µM) (Figures 3 and 4).

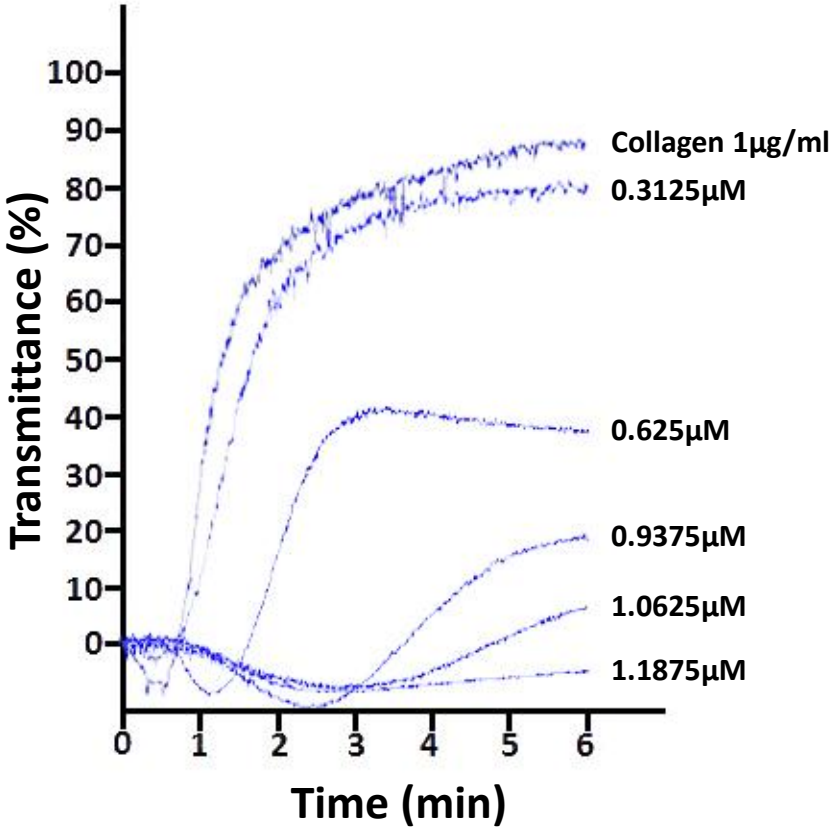

Figure 1. Dose-response curve of R4 novel compound on platelet aggregation induced by collagen.

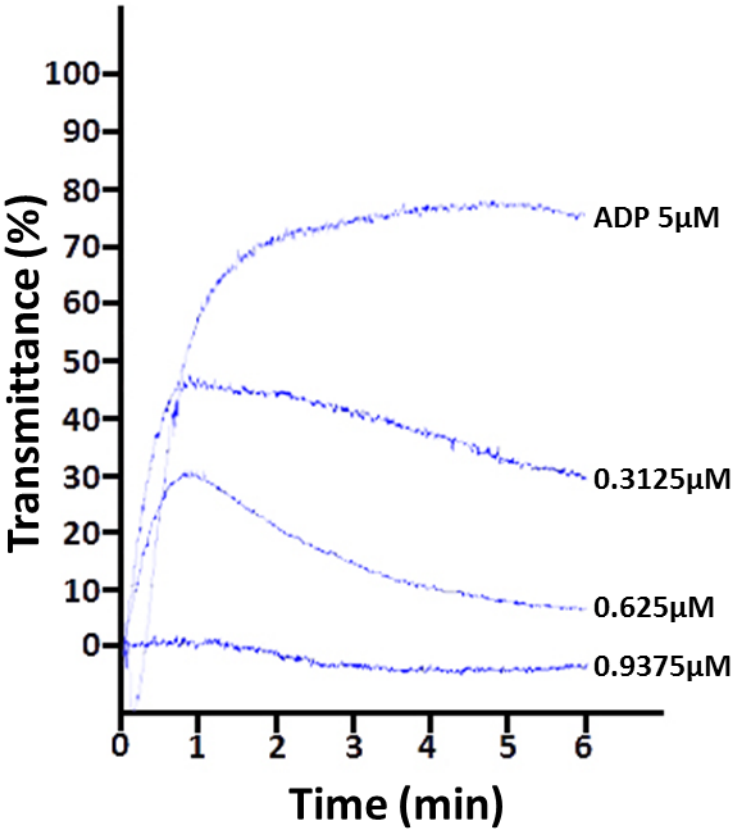

Figure 2. The dose-response curve of the R4 novel compound on platelet aggregation induced by ADP.

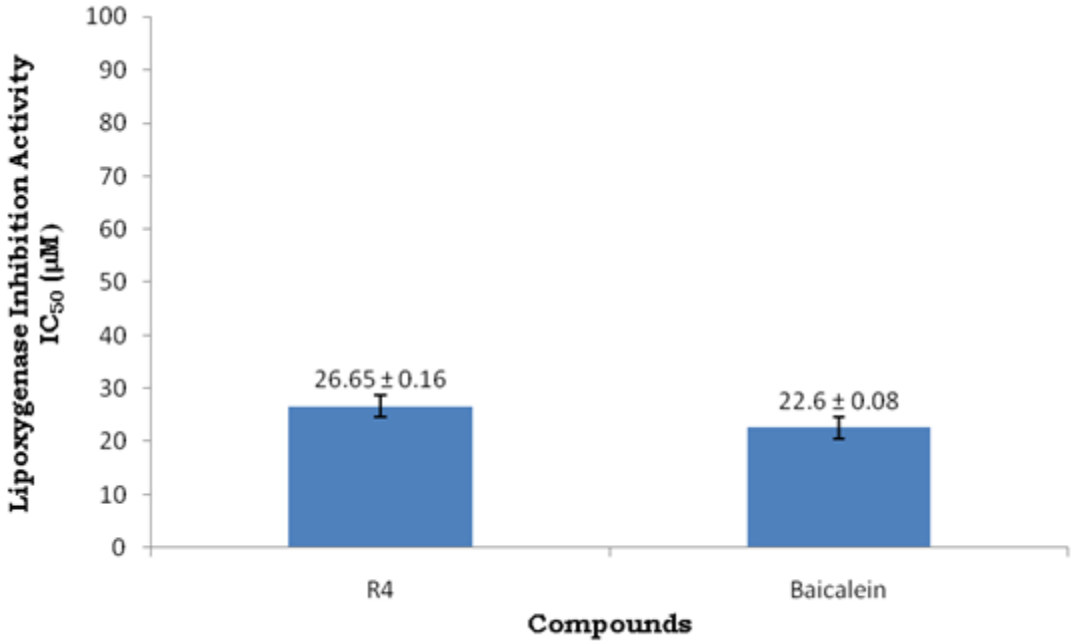

Figure 3. Comparison of the IC50 values for R4 and a reference compound (Baicalein).

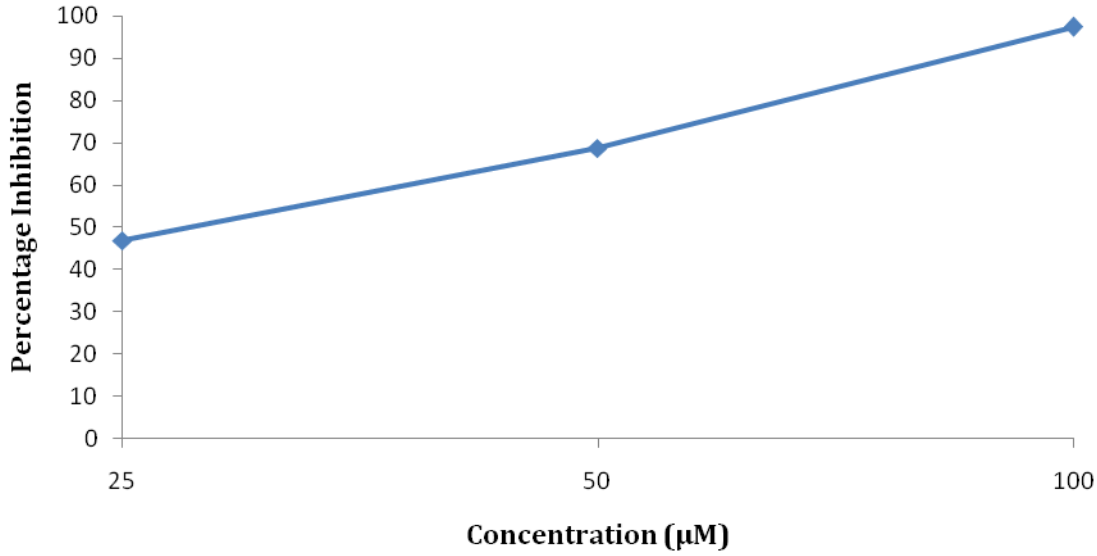

Figure 4. R4 has an inhibitory role on the lipoxygenase enzyme.

DISCUSSION

Diseases caused by a blood clot or thrombus due to activation of platelet and other proteins, such as heart diseases and stroke, are the leading cause of death in the world. Several drugs are available for this treatment. But the researcher continuously worked on different compounds for improving the efficacy and safety profile of the patient using side effects.

Blood clot or thrombus-related illnesses, such as heart failure and stroke, are the primary cause of death worldwide. Numerous medications are available to treat this condition. However, the researcher continued to experiment on various drugs to improve the patient's effectiveness and safety profile through the use of side effects [33].

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 [34]. 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 and ADP on platelet aggregation as agonists [35]. The antiplatelet effect of R4 induced by collagen was demonstrated in the current study at a concentration of 1.187 μM. (Figure 1). In the case of ADP also, it showed moderate activity at 0.937 μM (Figure 2). Additionally, it demonstrated mild activity at 0.312 μM for ADP (Figure 2). Thus, our novel compound R4 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 [36–37]. 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 [38]. Therefore, alternate methods are warranted which would inhibit platelet activation, thus minimizing the bleeding side effect.12-lipoxygenase (12-LOX) in humans may be a novel antiplatelet target in patients with cardiovascular disorders. To develop potential 12-LOX inhibitors, though, we must first gain a better understanding of their pathophysiological and biochemical consequences. Although our current understanding of this enzyme and its oxidized products in platelets and other tissues is limited, tentative data suggest that inhibiting 12-LOX on the platelet may be beneficial in treating human diseases.

With the inhibition of COX-1 and P2Y12, Lipoxygenase can become a viable antiplatelet target in the future. This approach can reduce platelet aggregation without increasing the risk of bleeding. As compared to the naturally occurring compound baicalein, which was described as a selective inhibitor of 12-LOX in human platelets, R4 inhibited the lipoxygenase enzyme strongly, with an IC50 of 26.65 ± 0.16 μM in our experiments [39] without impairing the function of cyclooxygenase [40]. The role of 12-HETE, an AA-oxidized metabolite found in platelets, is unknown. Additionally, 12-HPETE stimulated 12-LOX in lysed platelets, thus inhibited COX-1 [41]. According to a study, 12(S)-HETE inhibits the activity of neutrophil PLA2 and platelet [42]. Other eicosanoid compounds, such as 12-HPEPE and 12-HEPE, which are generated from the 12-LOX enzyme oxidation of EPA, suppress platelet aggregation [39]. 12-HPEPE and 12-HEPE have been shown to attenuate the AA and collagen-mediated release of serotonin (5-HT) in a dose-dependent way, in addition to their effects on platelet aggregation [43].

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 [36–37].

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 thiazole derivative (Z)-2-(3,3-dimethyl-2,6-diphenylpiperidin-4-ylidene)hydrazinecarbothioamide (R4) 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 thiazole derivative (R4) could be used at defined concentrations as a platelet inhibitor in cardiovascular disorders.

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