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IMPACT OF NOVEL THIAZOLE DERIVATE (R1), REVERSES PLATELET STRESS FIBER FORMATION ON FIBRINOGEN-COATED SURFACES: *IN-VITRO* AND *IN-SILICO* STUDIES

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Abstract
Platelets aggregate and adhere to vascular damage sites to maintain hemostasis, but excessive activation can cause thrombotic disorders. Platelet shape changes precede aggregation or adhesion after physical or pharmacological stimulation. Unlike aggregation and adhesion, platelet shape alterations can be reversed. This dynamic phenomenon involves complex chemical signalling networks and many different cytoskeleton proteins. Novel thiazole compounds can prime platelet shape change, modulating thrombosis and preventing or developing thrombotic illnesses with reduced bleeding. In this article, we explained the current understanding of the mechanisms behind platelet shape change, its pathological implications, and potential therapeutic targets for controlling the associated cytoskeleton dynamics. The study investigated platelet spreading on the fibrinogen matrix to measure platelet activation. For platelet spreading, 12mm diameter circular cover slips were incubated with fibrinogen, and washed platelets were allowed to cling to immobilize proteins for a specific duration. Platelet adhesion and actin nodules or stress fibers were observed using a Compound Fluorescent Microscope. The *in-silico* studies used the AutoDockVina version for ligand binding for antiplatelet activity against the protein (PDB=1EQG and 3ZDY) and antioxidant activity against the protein (PDB=1HD2). Platelets treated with synthetic thiazole derivatives at concentrations of (0.01µM, 0.05µM, 0.1µM, 0.3µM, and 0.6µM) had significantly varying degrees of spreading. *In-silico* results indicated that the R1 thiazole derivative had negative binding energy (BE) values, -8.38 kcal/mol for PDB 1EQG and -7.17 kcal/mol for PDB 3ZDY, and -6.84 kcal/mol for PDB=1HD2, implying a greater ligand-binding affinity. The study concluded that the R1 novel thiazole derivative possesses significant antiplatelet action in response to fibrinogen activation of platelet aggregation at a concentration of 0.05 and 0.1µM. Molecular docking showed that the R1 derivative had higher antiplatelet and antioxidant activity than the standard compound, showing more significant antiplatelet action against the PDB-1EQG.

Keywords: Aggregation, Fibrinogen, Platelet Spreading, Ligand binding.

Introduction

In response to arterial injury and subendothelial matrix protein exposure, platelets build actin-rich structures including filopodia, lamellipodia, and stress fibers that abetment thrombus formation and stability.[1-4] Numerous other actin structures, including as focal adhesions[5] and focal complexes[6] can also be formed by the actin cytoskeleton. Focal adhesions have been described in platelets that spread on fibrinogen.[7] The crucial integrin in the formation of thrombus is αIIbβ3, a receptor for certain adhesive proteins such as von willebrand factor (VWF), fibrinogen, and fibronectin.[8,9] Involvement of αIIbβ3 encourages platelet to platelet interaction,[10] and produces outside-inside signals that strengthen activation.[11,12] Previous researches have demonstrated that a variety of agonists can cause significant morphological alterations in platelets. The activation of the agonist changes the discoid shape, which is mostly sustained by the circumferential marginal band of microtubules,[13–15] and the cells take on a spherical shape, extend filopodia, and form sheet-like lamellipodia.[16–18] When platelets adhere to the site of vascular injury, the actin cytoskeleton is significantly remodeled, which enables the platelets to form thrombi efficiently in the high-shear environment of the vasculature. Actin polymerization must be coordinately modulated in order to induce the sequential production of filopodia, actin nodules, lamellipodia, and stress fibers throughout the intricate process of actin cytoskeleton reorganization.[19]

Patients with peripheral arterial disease, coronary heart disease, and stroke have been found to benefit from a variety of antiplatelet medications, including clopidogrel, a thienopyridine derivative of P2Y12 receptor antagonists, fibrinogen receptor antagonists, aspirin, a cyclooxygenase inhibitor, and cilostazol, a phosphodiesterase (PDE) inhibitor.[20–22] Despite the established advantages of currently marketed antiplatelet medications, ischemia episodes continue to occur frequently, and rates of morbidity and mortality remain high.[23]

This is due to the fact that all currently marketed antiplatelet medications only target one signal route, and the majority of them—particularly clopidogrel and aspirin—inhibit platelet activation in a moderate to variable degree. The last common platelet activation pathway is efficiently blocked by fibrinogen receptor antagonists. However, they has only limited used in an emergency because to their severe risk of bleeding. Consequently, there is ample opportunity to enhance antiplatelet therapy and designed novel antiplatelet medications with improved efficacy and safety profiles. In this Study, we evaluated the spreading dynamics of isolated platelets on physiologically relevant ligands, including fibrinogen. Further investigation of the antiplatelet effect of thiazole derivative R1 revealed by the molecular docking studies against PDB=1EQG and 3ZDY.

Material and Method

Regents and Chemicals

Compound R1 [(Z)-2-(2-(3-isopropyl-2,6-diphenylpiperidin-4-ylidene)hydrazinyl)-4-(4-nitrophenyl)thiazole], was synthesized by department of chemistry, university of Karachi. Human fibrinogen and DPPH was purchased from Sigma-Aldrich (St Louis, MO, USA). All other reagents were reagent grade. Deionized water was used throughout the experiments.

Collection of Blood

Preparation of Washed Platelets

We recruited exclusively healthy volunteers who had abstained from taking aspirin, non-steroidal anti-inflammatory agents, or any other medications for a minimum of ten days. Before blood collection, all participants provided written informed consent. IRB of Baqai Medical University approved this informed consent form. All procedures were performed following the approved relevant guidelines and regulations. The method for drawing human blood, isolation of platelet-rich plasma (PRP) and preparation of wash platelets suspension are the same as earlier.[19,20] For aggregation studies in separate experiments, platelet suspension was treated with R1 in different dilutions (0.01, 0.05, 0.1, 0.3, and 0.6µM) for 2 – 10 minutes before use in the assay. Blood was drawn into tubes containing acid citrate dextrose (ACD, 5:1).The blood was centrifuged at 700rpm

for 10 minutes to generate platelet-rich plasma (PRP), followed by 900rpm for 10 minutes. The PRP was then centrifuged at 1800 rpm for 10 minutes after being treated with citric acid (0.3mM) [pH 6.8] to pellet the platelets. Platelet pellet was suspended in 2–3 ml wash buffer (pH 7.4) and centrifuged at 2200 rpm for 10 minutes. The resulting platelet pellet was resuspended in modified Tyrode's buffer and incubated at 37°C for 30 minutes. The platelet number was adjusted to 3×10^8 platelets/ml using Tyrode's buffer.[24,25]

For Platelet Spreading Assay

It is commonly acknowledged that platelet activation is a multi-step process and that following initial platelet attachment, the release of two significant platelet agonists (TXA_2 and ADP), that cause and stimulated aggregation. Platelets will be pre-incubation with R1 in order to examine their adherence to fibrinogen, while reducing the effects of agonist-induced aggregation and activation.
Glass cover slips (12 mm in diameter) were coated with fibrinogen (1mg/10mL) overnight at 4°C.[26] Washing the cover slips three times with PBS and nonspecific binding was blocked with 50mg/10ml denatured fatty acid-free BSA for 1 hour at room temperature (37°C). Platelets are pretreated with antagonist R1 just before their addition to cover slips. Cover slides were coated with platelets pretreated with the antagonist R1 and incubated at 37°C for 60 minutes. After 60 minutes, cover slips were washed thrice with PBS to remove non-adhered platelets and fixed with 4% PFA for 10 minutes at 37°C in the fume hood. After 10 minutes, cover slips were washed twice with PBS and then lysed with triton x-100 (0.1%) for 5 minutes. Again, after 5 minutes, cover slips were washed twice with PBS. Then, it was stained with FITC-phalloidin (1:100 dilution) at room temperature for 30 minutes, followed by washing with PBS and mounting with DPX, non-fluorescent media.[27] Leave overnight at 4°C in the dark and visualized using software to identify the platelet containing actin nodules or stress fibers.

Antioxidant Assay

The 2, 2'-diphenyl-1-(2, 4, 6, trinitophenyl) hydrazyl was made in ethanol at a concentration of 3mM. A control compound, a blank compound, and a test compound R1 at varied concentrations were written on each well of a 96-well plate. DPPH solution (95µl) was added to the wells and then supplemented with the test compounds (5µl) at concentrations ranging from 10 to 1000µM. Now, at 37°C, a 96-well plate was incubated for 30 minutes, and the microtiter plate was examined at a wavelength of 517nm after 30 minutes using Spectramax plus 384 Molecular instruments (USA). Radical scavenging activity was assessed compared to control, which was gallic acid. The radical scavenging activity of DPPH was calculated using the following equation.

$$DPPH\ radical\ scavenging\ effect\ (\%) = Ac - \frac{As}{Ac} * 100$$

Where,
Ac is the absorbance of control.
As is the absorbance of the test compound.

In-Silico Antiplatelet Assay

Methodology

Preparation of Inhibitor

ChemOffice 16 was utilized to generate a two-dimensional model of the chosen compound, which was then saved in CDX format. To transform the analogue into three-dimensional structures, ChemDraw 4D ultra was utilized (version 16.0) (Chemical Structure Drawing Standard; Cambridge Soft Corporation, USA (2009).[28] Additionally, the MMFF94X force-field method was used to minimize the energies of all analogues with 1000 steps iteration. Finally, the processed analogue was saved into PDB format. The MGL Tools (version 1.5.6).[29] generated PDBQT files.

Preparation of Target Protein

The protein structure for Antiplatelet studies (PDB=1EQG and PDB=3ZDY) 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 produced a grid map of 45, with points spaced at 0.700 Å. The docking parameters were set to the default setting of the genetic algorithm. Binding energies predicted the best pose of docked ligand-enzyme complexes.

Results

Platelet Spreading Assay of R1

Analysis of platelet spreading in the absence of R1, the platelets activation phase to form Actin nodules and stress fibers. The Actin cytoskeleton has been implicated in thrombus stability. In the series of experiments different concentration of R1 (0.01, 0.05, 0.1, 0.3, and 0.6 µM) was added to the spread platelets. In the absence of R1 (Control) the number of platelets with stress fibers did not alter appreciably over the time (Figure: 1a). In contrast the presence of R1 caused a rapid loss of Actin nodules and stress fibers in spread platelets. This effect was maximal at Concentration of 0.1µM (Figure: 1d), decreasing the ratio of Stress Fibres, and Actin Nodules. Although increased concentration 0.3 and 0.6 µM, reverse the R1 effect of antiplatelet by increases the number of stress fibres in spread platelet (Figure: 1e,f).

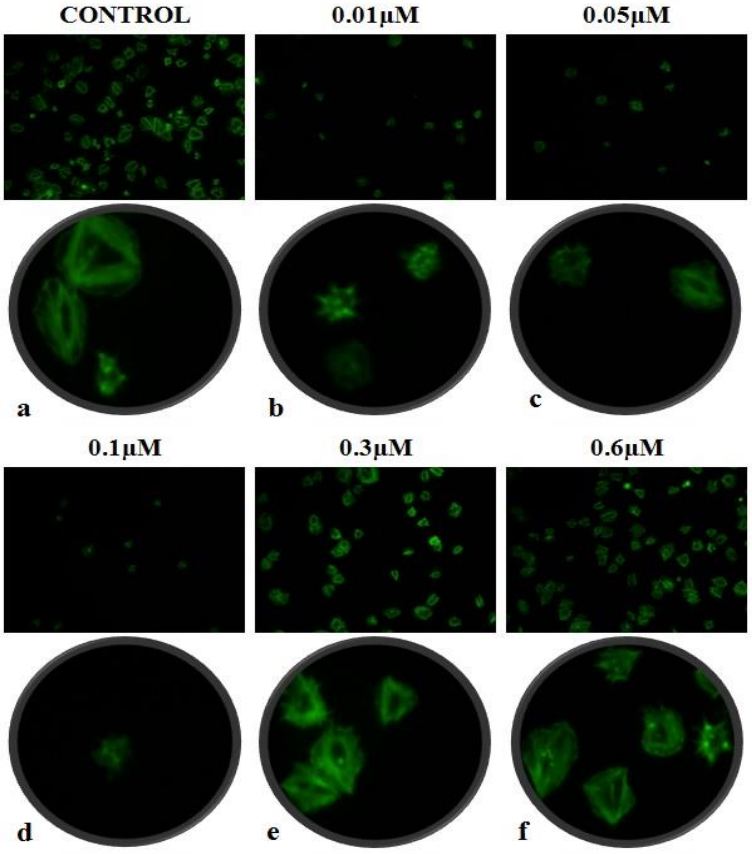


Figure: 1. Synthetic compound R1 inhibits platelet spreading on immobilized fibrinogen.

Human platelets were seeded in the presence of various R1 concentrations (0.01, 0.05, 0.1, 0.3, and 0.6 µM) on fibrinogen-coated cover slips. Platelets were stained with FITC-Phalloidin and examined by using an objective of 40x.

R1 Reverses Stress Fiber Formation in Spread Platelets on Fibrinogen

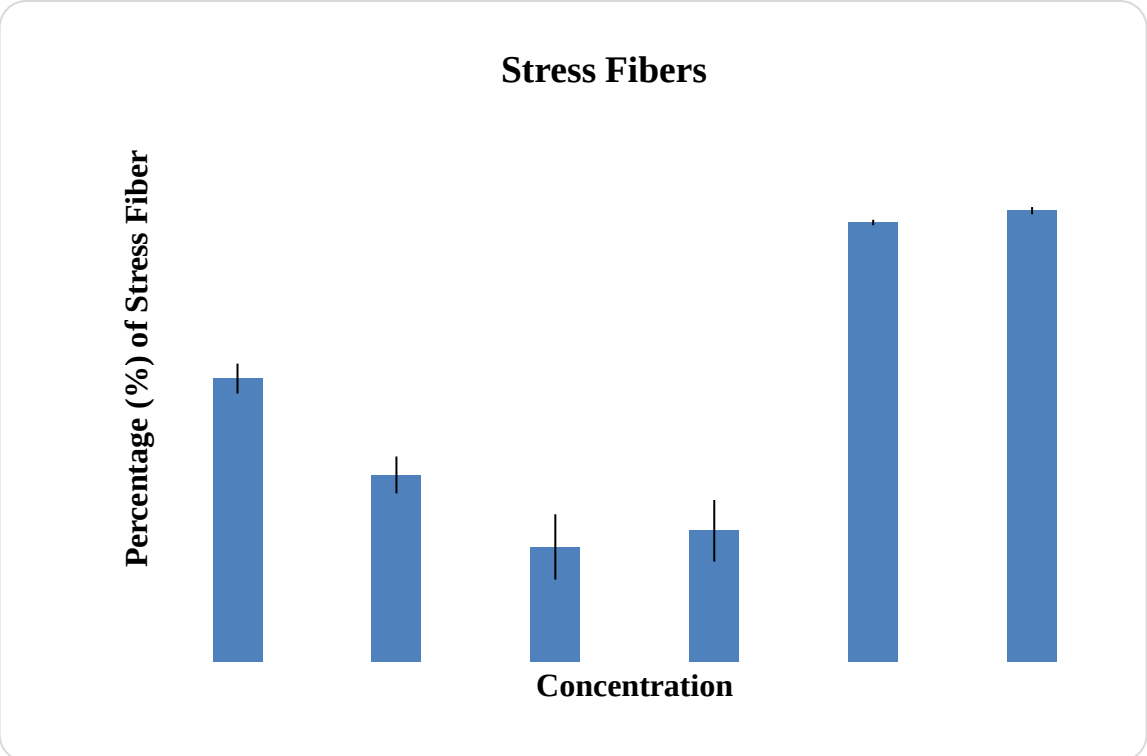
Fibrinogen binds to active integrin GPIIb/IIIa, which is mediated by inside-out signal pathways, causing platelet aggregation. GPIIb/IIIa binds to fibrinogen and then initiates outside-in signaling that leads to clot retraction and platelet spreading, both of which are critical processes in thrombosis. As shown in Table: 1 in the range of 0.01–0.6 µM, R1 inhibited platelet spreading on immobilized fibrinogen for the Concentration of 0.01, 0.05 and 0.1 µM. Significant antiplatelet action of R1 in response to reverse fibrinogen activation of platelet spreading showed at a concentration of 0.05 and 0.1µM with the average percentage of stress fibers was 18.91±5.37 and 21.57±5.06 (Graph: 1). Further increase concentrations 0.3 and 0.6 µM, reverse the inhibition effect of R1 Compound, increases the stress fibers of spreading platelets 72.29±0.44 and 74.24±0.59 (Graph: 1 and 2).

R1 Reverses Actin Nodules Formation in Spread Platelets on Fibrinogen

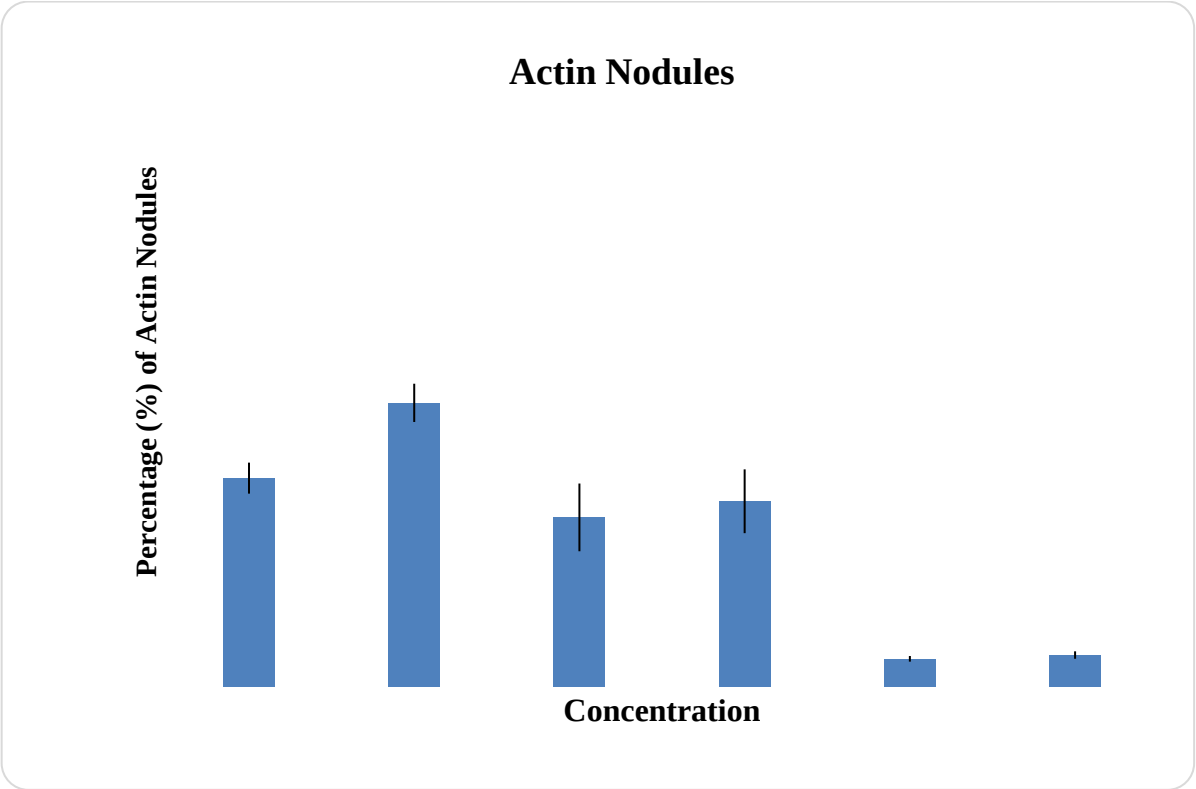
As shown in Table: 1 in the range of 0.01–0.6 µM, Significant antiplatelet action of R1 in response to reverse fibrinogen activation of platelet spreading showed at a concentration of 0.05 and 0.1µM with the average percentage of Actin nodules was 26.88±6.56 and 29.44±2.42 (Graph: 2). Further increase concentrations 0.3 and 0.6 µM, increases the inhibition effect of R1 Compound, against actin nodule of spreading platelets 4.44±1.18 and 5.04±1.71 (Graph: 2), meanwhile increases the stress fiber.

Table: 1. Percentage of R1 Reverse Stress Fibre and Actin Nodule on Fibrinogen Mediated Platelet Spreading

Platelet Spreading Conditions	Control	R1 Concentration				
		0.01 µM	0.05 µM	0.1 µM	0.3 µM	0.6 µM
Stress Fibres	46.62±2.46	30.76±3.03	18.91±5.37	21.57±5.06	72.29±0.44	74.24±0.59
Actin Nodules	33.11±3.08	45.05±2.60	26.88±6.56	29.44±2.42	4.44±1.18	5.04±1.71



Graph: 1. Percentage of Platelet Spreading formed Stress Fibers



Graph: 2. Percentage of Platelet Spreading formed Actin Nodules

DPPH Inhibiting Antioxidant Activity of R1 Compound

The antioxidant activity of the chemical R1 was assessed by in vitro studies using DPPH as a source of free radicals. Free radicals are stable compounds and can be reduced by the presence of antioxidants. When tested at 100, 250, and 500 µM, compound R1 exhibited concentration-dependent antioxidant activity.

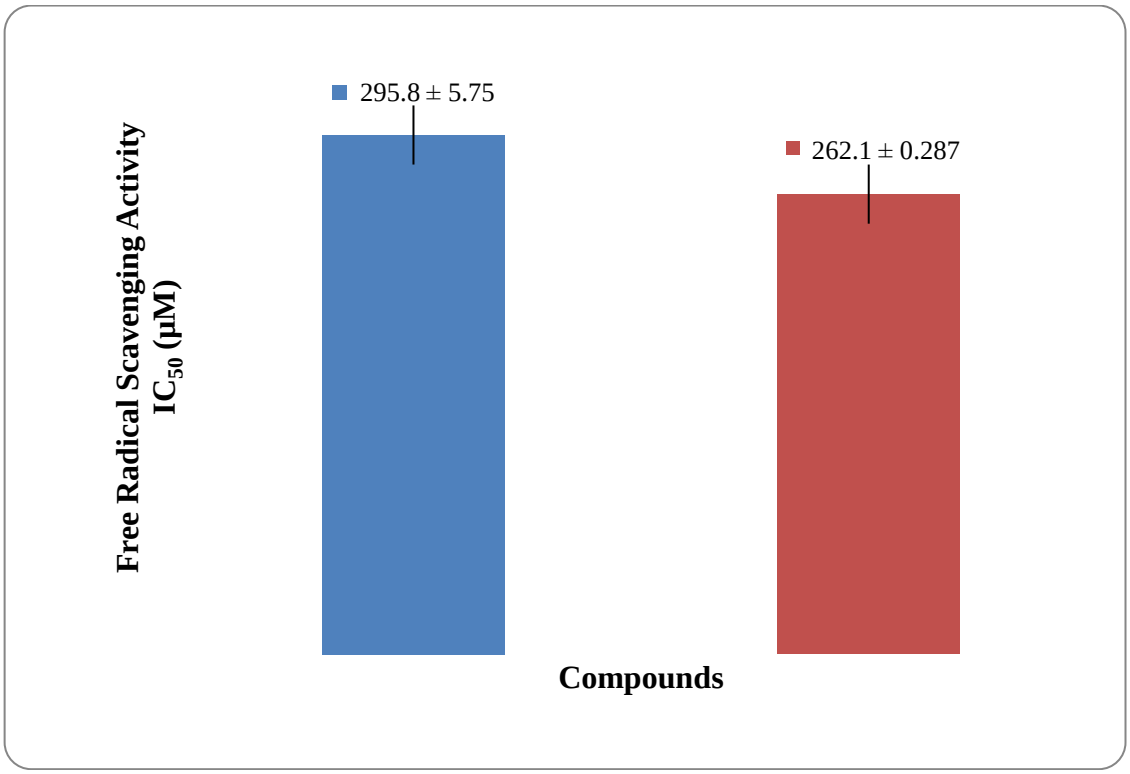
A compound R1 dose-dependent antioxidant properties at various doses showed in (Table: 2). DPPH activity is 16.9% inhibited by compound R1 at a concentration of 100 µM. Its inhibitory efficiency is in the micro-molar range, and it exhibits a linear pattern of inhibition, 42.25% of DPPH activity at 250 µM and 84.5% at 500µM (Table: 2).

As IC_{50} values were derived by comparison with the reference antioxidant, (Gallic Acid), the antioxidant DPPH activity was also assessed. The gallic acid concentration used in the experiment, 262.1±0.287µM, is equivalent to the compound R1 IC50 value of 295.8±5.75µM. (Graph: 3; Table: 2).

Table: 2. Scavenging of DPPH radical by Gallic Acid and R1

R1	**Concentration (µM)**	**Percentage Inhibition (%)**	**IC_{50} (µM)**
	100	16.9 ± 3.17	295.8 ± 5.75
	250	42.25 ± 2.63	
	500	84.5 ± 4.98	
Gallic Acid			262.1 ± 0.287

* The data are expressed as mean ± Standard Error of Mean (SEM), n = 3



Graph: 3. Comparison of the IC_{50} values for R1 and Gallic Acid

***In-Silico* Platelet Aggregation Inhibited by a Novel Compound R1**

With a binding energy of -8.38 kcal/mol, R1 attaches to six hydrophobic bonds at the following positions in PDB 1EQG: GLN-44A, ILE-46A, THR-60A, PRO-125A, and TYP-130A (Figures: 2). With the following sites: ASN-133D, TYR-190A, ALA-218B, PHE-231A, and PHE-231A, the novel thiazole derivative R1 makes five hydrophobic bonds with PDB3ZDY (Figures: 3), with a binding energy of -7.17 kcal/mol (Tables: 3).

Table 3: Docking studies with PDB 1EQG and PDB 3ZDY screened R1, for antiplatelet activity

Derivatives	No. of Hydrogen Bonding.	Hydrogen Bonding (an amino acid with bond length)	No. of Hydrophobic Bond	Hydrophobic Bonding (Amino acid and bond length)	PI-Cation Interaction	Binding Energy (kcal/mol)
R1 for PDB 1EQG	--	--	6	GLN-44A (3.21), ILE-46A (3.45), THR-60A (3.83), PRO-125A (2.93), PRO-125A (3.43), TYP-130A (3.87)	--	-8.38
R1 for PDB 3ZDY	--	--	5	ASN-133D (3.78), TYR-190A (3.77), ALA-218B (3.66), PHE-231A (3.61), PHE-231A (3.80)	--	-7.17

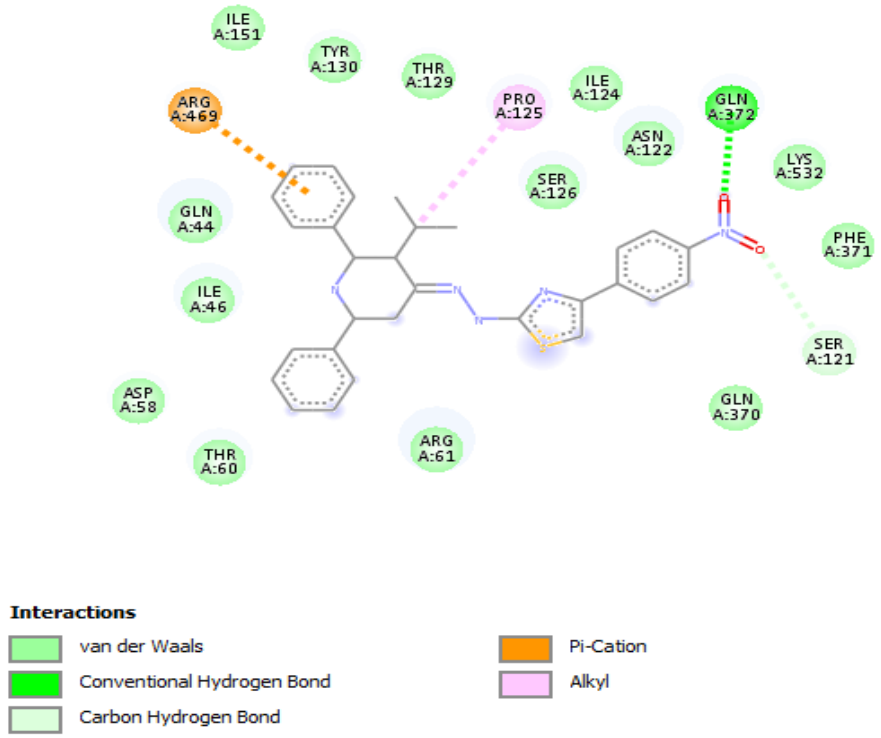


Figure: 2. 2D projection of the interactions of compound R1 with the active site for antiplatelet activity using PDB-1EQG

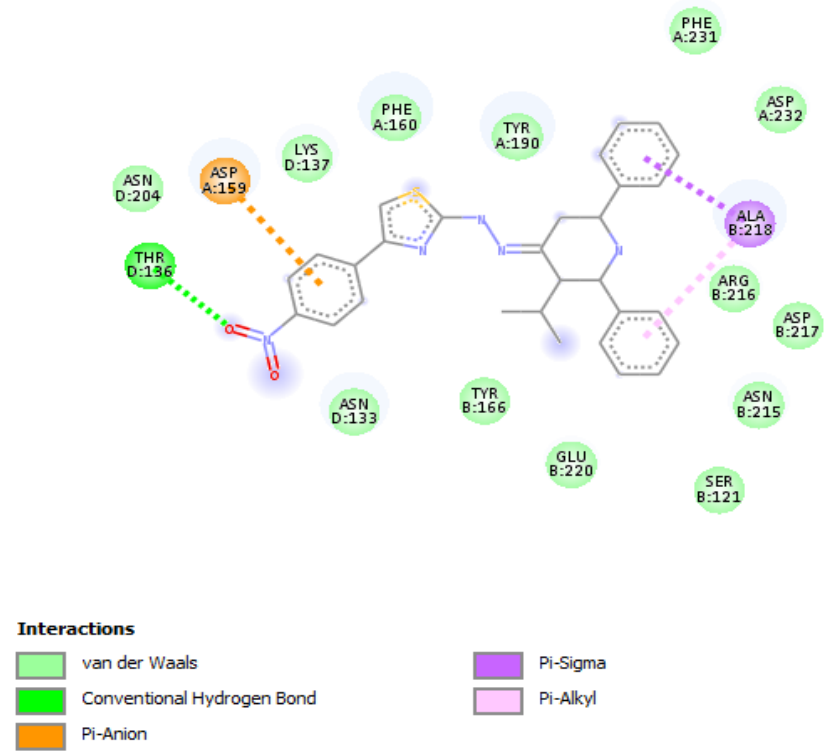


Figure: 3. 2D projection of the interactions of compound R1 with the active site for antiplatelet activity using PDB-3ZDY

In-Silico Antioxidant Activity by a Novel Compound R1

The standard butylated hydroxyanisole docking computation revealed that the projected pose displayed two hydrophobic bonding contacts with amino acid residues PRO-40 and PRO-45 and three H-bonding interactions with amino acid residues THR-44, CYS-47, and ARG-127 (Table: 4). The novel thiazole derivative R1 binds to the following sites: four hydrophobic PRO-45A, LEU-112A, LEU-116A, and LEU-149A; and one hydrogen bond, THR-147 (Figures: 4). R1 has double binding energy -6.84 kcal/mol when compared to Standard butylated hydroxyanisole which has -3.59 kcal/mol (Table 4).

Table 4: Docking studies with PDB 1HD2 for the screening of free radicals scavenging complex of BHA and R1

Derivatives	No. of Hydrogen Bonding	Hydrogen Bonding (an amino acid with bond length)	No. of Hydrophobic Bond	Hydrophobic Bonding (Amino acid and bond length)	PI-Cation Interaction	Binding Energy (kcal/mol)
BHA	3	THR-44 (1.95), CYS-47 (2.11), ARG-127 (2.99)	2	PRO-40 (3.62), PRO-45 (3.48)	-	-3.59
R1	1	THR-147A (2.42)	4	PRO-45A (3.65), LEU-112A (3.33), LEU-116A (3.48), LEU-149A (3.25)	-	-6.84

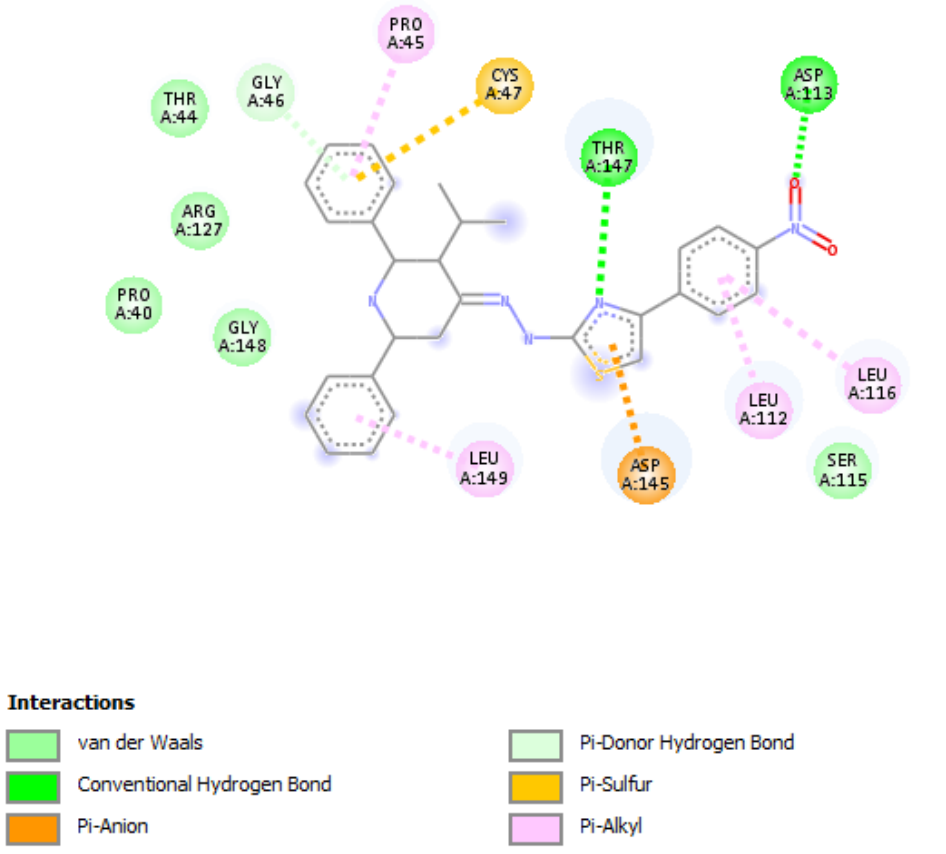


Figure: 4. 2D projection of the interactions of compound R1 with the active site for antioxidant activity using PDB-1HD2

Discussion

For subsequent thrombus development, hemostasis is dependent on persistent platelet adhesion. Platelets' ability to rapidly remodel their actin cytoskeleton is crucial for their ability to endure shear stress. The inability of platelets to generate lamellipodia or stress fibers after activation and spreading has been associated to thrombus instability and embolisation.[30,31]

The platelet outside-in signal manner is mostly involved in platelet thrombosis rather than hemostasis. A better approach to developing effective and safe antiplatelet drugs is to target the platelet outside-in signal pathway.[32] Integrin signaling depends on conformational modifications and integrin clustering that improve affinity/avidity for the ligand. In turn, ligand interaction starts a complicated network of signaling, and structural cytoskeletal proteins result in outside-in signaling, which is characterized by a rise in tyrosine phosphorylation. This pathway has been fully characterized in platelets for αIIbβ3- interaction by fibrinogen.[33] Phencyclidine selectively inhibited aggregation secretion and other common platelet agonists such as a-thrombin, ADP, and collagen. Adrenaline is a unique platelet agonist in that it does not promote shape change and only causes localized elevations in cytoplasmic Ca^{+2} concentration.[34] It has been proposed that these localized changes in [Ca^{+2}] may cause fibrinogen receptors to be exposed via an as-yet unidentified second messenger pathway.[35]

The profound inhibition of R1 on platelet spreading may contribute to its negligible bleeding tendency while retaining excellent antithrombotic efficacy. Spreading of platelet in control illustrated that at the specific time interval both the stress fibers and actin nodules were equally increase, while spreading in different concentration of R1-treated platelets (0.01, 0.05, 0.1, 0.3, and 0.6 µM) exhibited a markedly different degree of spreading. R1 at 0.05 and 0.1 µM significantly reverse the formation of stress fibre along with actin nodules by inhibited the fibrinogen activation of platelet for aggregation.

The nodules are not dependent on the production of filopodia; rather, they are present from the very beginning of lamellipodia development and move continuously until complete lamellipodia are generated. However, they disappear when stress fibers form. Actin polymerization and constant Src kinase activity are required for the formation of actin nodules. When astress fibers form it present the focal adhesions occur at the tips of stress fibers, so it is unlikely to represent the focal adhesions by actin nodules.[36] R1 at higher doses 0.3 and 0.6 µM reverse the effect by increasing the formation of stress fibres, meanwhile the low concentration of actin nodule at these two doses of R1 (0.05 and 0.1 µM) may be represent inhibiting the Src Kinase activity.

Reactive oxygen species (ROS) have the ability to cause damage to the vascular wall's cellular structures as well as activate a number of redox-sensitive transcriptional pathways that lead to a transcriptome profile that is proatherogenic. Endothelial dysfunction in atherogenesis is triggered by a rise in reactive oxygen species (ROS) generation in the vascular wall and a reduction in the bioavailability of nitric oxide (NO).[37] By scavenging lipid peroxides and free radicals (ROS) which damage vascular endothelium and inhibit prostacyclin synthetase, as well as through particular interactions with target proteins, an antioxidant drug may lower platelet activity. Antioxidants may therefore have anticoagulant and antiplatelet aggregation properties.[38] It was observed in the present study that the free-radical scavenging ability of R1 was slightly lower than the one assessed by Gallic acid. A compound R1 exhibited dose-dependent antioxidant properties at various doses (100, 250 and 500 µM). IC_{50} value of R1 295.8±5.75µM is equivalent to the reference antioxidant, (Gallic Acid), IC_{50} value which is 262.1±0.287µM.

COX-1, a COX isozyme that is constitutively expressed in platelets, causes the synthesis of eicosanoids such as TXA2 and PGD2, which are involved in platelet activation and aggregation. TXA2 synthase is an essential AA-metabolizing enzyme that transforms PGH2 to TXA2 when COX-1 generates PGH2 from AA in platelets.[39] Nonsteroidal anti-inflammatory agents (NSAIDs) inhibits prostaglandin H2 synthase, which inhibits prostanoid production. We picked the complex of prostaglandin H2 synthase (COX-1), the active enantiomer of ibuprofen (PDB: 1EQG),[40] and glycoprotein llb/llla protein (PDB-3ZDY). In general, the docking investigation was carried out to

calculate the binding score, which aids in the prediction of the activity of synthesized derivatives. COX-1 and glycoprotein llb/llla play critical roles in controlling platelet aggregation, with ADP, arachidonic acid, and collagen acting as agonists, respectively.[41] R1 had negative binding energy (BE) values, -7.17 and -8.38 kcal/mol for PDB 3ZDY and PDB 1EQG, implies a greater ligand-binding affinity. Molecular docking concluded R1 exhibited a slightly higher affinity towards PDB-1EQG than PDB-3ZDY, suggesting a more significant potential for antiplatelet action by inhibiting COX-1.

Conclusion

By using the platelet spreading technique, the thiazole derivative demonstrated antiplatelet efficacy via a fibrinogen inhibitor pathway, demonstrating the inhibition of platelet activation and a decrease in the number of actin nodules and stress fiber production. In molecular docking experiments, the tested drug demonstrated antiplatelet efficacy via the glycoprotein llb/llla receptor inhibitor pathway as well as the COX-1 inhibitor pathway; however, the glycoprotein pathway may be regarded as a second pathway.

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