Kurdish Studies
June 2024
Volume: 12, No: 5, pp. 286-297
ISSN: 2051-4883 (Print) | ISSN 2051-4891 (Online)
www.KurdishStudies.net

DOI: 10.53555/ks.v12i5.3174

In-vitro and In-silico Studies of Thiazole Derivate (R2) Prevents Platelet Stress Fiber Production on Fibrinogen-coated Surfaces.

Usman Ghani Farooqi[1]*, Rehana Perveen[1], Mahwish Akhtar[2], Noor Jahan[3], Sobia Akhter[4], Muhammad Zuhair Yusuf[5]

[1*]Department of Pharmacology, Baqai Institute of Pharmaceutical Sciences, Baqai Medical University, Karachi, Pakistan.
[2]Department of Pharmaceutical Chemistry, Faculty of Pharmacy and Pharmaceutical Sciences, Dow college of Pharmacy, Dow University of Health Sciences, Karachi, Pakistan.
[3] Department of Pharmacology, Faculty of Pharmacy and Pharmaceutical Sciences, Dow college of Pharmacy, Dow University of Health Sciences, Karachi, Pakistan.
[4] Head of Pharmacy, Cancer Foundation Hospital, Karachi, Pakistan.
[5]Department of Biological and Biomedical Sciences, Aga Khan University, Karachi, Pakistan.

*** Corresponding Author:** Usman Ghani Farooqi
Department of Pharmacology, Baqai Institute of Pharmaceutical Sciences, Baqai Medical University, Karachi, Pakistan. dr.usmanfarooqi@gmail.com

Abstract
Platelets undergo aggregation and adhesion at areas of vascular injury in order to preserve hemostasis, although excessive activation can lead to the development of thrombotic diseases. Platelet morphology alterations occur prior to the occurrence of aggregation or adhesion following either physical or pharmacological activation. Platelet shape modifications can be reversed, unlike aggregation and adhesion. This dynamic process encompasses intricate chemical signalling networks and a multitude of diverse cytoskeleton proteins. Thiazole compounds with unique structures have the ability to initiate platelet shape change, hence influencing the formation of blood clots and potentially preventing or treating thrombotic diseases while minimizing the risk of excessive bleeding. This article elucidates the present comprehension of the mechanisms that cause changes in the morphology of platelets, the clinical implications of these changes, and potential targets for therapeutic intervention to regulate the dynamics of the cytoskeleton involved. The study investigated the mechanism of platelet spreading on the fibrinogen matrix with the aim of quantifying platelet activation. In order to facilitate the spreading of platelets, circular cover slips measuring 12mm in diameter were subjected to fibrinogen treatment followed by a washing process. Afterwards, platelets were permitted to attach to the immobilised proteins for a specific duration. Platelet attachment and the existence of actin nodules or stress fibers were seen using a Compound Fluorescent Microscope. The study utilised the AutoDockVina software to conduct in-silico research on the interaction between ligands and proteins (PDB=1EQG and 3ZDY) for antiplatelet activity, as well as antioxidant activity against the protein (PDB=1HD2). Platelets that were subjected to synthetic thiazole derivatives at varying concentrations (0.01μM, 0.05μM, 0.1μM, 0.3μM, and 0.6μM) had different degrees of spreading. The computational investigation demonstrated that the R2 thiazole derivative displayed negative binding energy (BE) values, specifically -7.56 kcal/mol for PDB 1EQG, -8.25 kcal/mol for PDB 3ZDY, and -6.51 kcal/mol for PDB 1HD2. These findings indicate a greater attraction of the ligand to the target protein. The investigation discovered that the R2 novel thiazole derivative had a significant capacity to hinder the clumping together of platelets in reaction to the activation of fibrinogen, particularly at doses of 0.05μM. The molecular docking research results showed that the R2 derivative exhibited greater antiplatelet and antioxidant actions in comparison to the reference molecule. More specifically, it exhibited a stronger inhibitory effect on platelet aggregation for the PDB-1EQG target.

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

Introduction

Platelets create actin-rich structures such as filopodia, lamellipodia, and stress fibers in response to arterial damage and exposure to subendothelial matrix proteins. These structures contribute to the development and stability of blood clots (Hartwig, 1992; Hartwig *et al.,* 1999; McCarty *et al.,* 2006; Calaminus *et al.,* 2007). The actin cytoskeleton can produce several additional actin structures, including as focal adhesions (Lo, 2006) and focal complexes (Zamir and Geiger, 2001). Platelets that adhere to fibrinogen form focal adhesions (Leng *et al.,* 1998). The αIIbβ3 integrin plays a pivotal role in the process of thrombus development by acting as a receptor for specific sticky proteins, including von Willebrand factor (VWF), fibrinogen, and fibronectin (Gardner and Hynes, 1985; Ruggeri, 2002). The presence of αIIbβ3 promotes platelet aggregation (Abulencia *et al.,* 2001) and facilitates the transmission of impulses from the outside to the inside of the platelet, enhancing its activation (Shattil *et al.,* 1998; Naik and Naik,

2003). Prior studies have shown that a range of agonists can induce notable morphological changes in platelets. The activation of the agonist alters the discoid form, mostly supported by the circumferential marginal band of microtubules.Between the ages of (Haydon and Taylor, 1965; Behnke, 1965; Italiano *et al.,* 2008), the cells undergo a change in morphology, becoming spherical and developing filopodia and lamellipodia that resemble sheets (White , 1968; Hartwig, 1992; Bearer *et al.,* 2002). Platelets undergo substantial remodelling of the actin cytoskeleton when they attach to the site of vascular injury. This remodelling allows the platelets to efficiently form blood clots in the high-speed flow of blood vessels. The regulation of actin polymerization is essential for the stepwise generation of filopodia, actin nodules, lamellipodia, and stress fibers during the complex process of actin cytoskeleton reorganization (Aslan and McCarty, 2013).

Patients diagnosed with peripheral arterial disease, coronary heart disease, and stroke have been observed to experience positive effects from a range of antiplatelet medications. These medications include clopidogrel, which is a type of thienopyridine derivative that acts as an antagonist to the P2Y12 receptor. Other medications that have shown benefits are fibrinogen receptor antagonists, aspirin (which inhibits cyclooxygenase), and cilostazol (which is a phosphodiesterase inhibitor) (Fuster and Chesebro, 1981; Herman, 1998; Tendera and Wojakowski, 2003). Although the existing benefits of currently available antiplatelet drugs are well-known, episodes of ischemia nevertheless occur regularly, resulting in high rates of morbidity and mortality (Valgimigli *et al.,* 2012).

This is because all currently available antiplatelet drugs exclusively focus on a single signalling pathway, and most of them, especially clopidogrel and aspirin, hinder platelet activation to a moderate or varying extent. Fibrinogen receptor antagonists effectively inhibit the final shared route of platelet activation. However, their utility in emergency situations is restricted due to the significant danger of bleeding associated with them. As a result, there are many opportunities to improve antiplatelet therapy and develop new drugs that are more effective and safe. This study examined the kinetics of platelet spreading on biologically relevant ligands, such as fibrinogen. The molecular docking experiments against PDB=1EQG and 3ZDY have provided additional insights into the antiplatelet activity of thiazole derivative R2.

Material and Method

Regents and Chemicals

The compound R2, specifically [(Z)-4-(4-chlorophenyl)-2-(2-(3-isopropyl-2,6 diphenylpiperidin-4-ylidene) hydrazinyl) thiazole], was synthesised by the Department of Chemistry at the University of Karachi. The human fibrinogen and DPPH were acquired from Sigma-Aldrich (St Louis, MO, USA). All the other reagents used were of reagent grade quality. Deionized water was utilised in all of the studies.

Collection of Blood

Preparation of Washed Platelets

Enlisted individuals who were in good health and had refrained from using aspirin, non-steroidal anti-inflammatory drugs, or any other medications for at least ten days. Prior to the collection of blood, all participants supplied written consent after being fully informed. This informed consent form has been approved by the Institutional Review Board (IRB) of Baqai Medical University. The procedures were conducted in accordance with the established rules and regulations. The procedure for extracting human blood, isolating platelet-rich plasma (PRP), and preparing a suspension of washed platelets is unchanged from previous methods (Fuster and Chesebro, 1981; Aslan and McCarty, 2013). In order to conduct aggregation investigations in distinct trials, a platelet solution was subjected to treatment with R2 at various dilutions (0.01, 0.05, 0.1, 0.3, and 0.6μM) for duration of 2 to 10 minutes prior to its utilisation in the assay. The blood was collected in tubes containing acid citrate dextrose (ACD) in a ratio of 5:1. The blood was subjected to centrifugation at 700rpm for 10 minutes to get platelet-rich plasma (PRP), and then further centrifuged at 900rpm for 10 minutes. After being treated with citric acid (0.3mM) at a pH of 6.8, the PRP was subjected to centrifugation at 1800 rpm for 10 minutes in order to separate and collect the platelets. The platelet pellet was resuspended in 2–3 ml of wash buffer (pH 7.4) and then subjected to centrifugation at a speed of 2200 rpm for duration of 10 minutes. The platelet pellet obtained was reconstituted in modified Tyrode's buffer and subjected to incubation at a temperature of 37°C for duration of 30 minutes. The platelet count was modified to 3×108 platelets per millilitre using Tyrode's buffer (Zhang *et al.,* 2010; Hu *et al.,* 2011).

For Platelet Spreading Assay

Platelet activation is widely recognised as a complex series of steps. After initial platelet attachment, two important platelet agonists (TXA2 and ADP) are released, which induce and promote platelet aggregation. The platelets will undergo pre-incubation with R2 to assess their adhesion to fibrinogen, while minimising the impact of agonist-induced aggregation and activation.

Glass cover slips (12 mm diameter) were covered with a solution of fibrinogen (1mg/10mL) and left overnight at a temperature of 4°C (Duan *et al.,* 2021). The cover slips were washed three times with PBS and any nonspecific

binding was prevented by treating them with a solution of 50mg/10ml denatured fatty acid-free BSA for 1 hour at room temperature (37°C). Platelets undergo pretreatment with antagonist R2 immediately before to their application onto cover slips. The cover slides were coated with platelets that had been pre-treated with the antagonist R2 and then incubated at a temperature of 37°C for duration of 60 minutes. Following duration of 60 minutes, cover slips were rinsed three times with PBS in order to eliminate any platelets that were not attached. Subsequently, the cover slips were treated with 4% PFA and incubated for 10 minutes at a temperature of 37°C within the fume hood. After duration of 10 minutes, the cover slips were subjected to two washes with PBS and subsequently lysed using triton x-100 (0.1%) for a period of 5 minutes. Once more, following a 5-minute interval, cover slips were rinsed twice with PBS. Subsequently, the sample was treated with FITC-phalloidin (diluted 1:100) at room temperature for duration of 30 minutes. Afterward, it was rinsed with PBS and then preserved using DPX, a non-fluorescent medium (Gao *et al.,* 2009). Allow to incubate overnight at a temperature of 4°C in a light-free environment, and then utilise software to visually identify the platelets that contain actin nodules or stress fibers.

Antioxidant Assay

The compound 2, 2'-diphenyl-1-(2, 4, 6, trinitophenyl) hydrazyl was synthesised in ethanol with a concentration of 3mM. Each well of a 96-well plate was labelled with a control chemical, a blank compound, and a test compound R2 at different concentrations. The wells were treated with a 95µl solution of DPPH, followed by the addition of 5µl of the test compounds at concentrations ranging from 10 to 1000µM. The 96-well plate was incubated at a temperature of 37°C for duration of 30 minutes. After the incubation, the microtiter plate was analysed using Spectramax plus 384 Molecular instruments (USA) at a wavelength of 517nm. The radical scavenging activity was evaluated by comparing it to the control, which was gallic acid. The calculation of the radical scavenging activity of DPPH was performed 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

The software ChemOffice 16 was employed to create a 2D representation of the selected compound, which was subsequently stored in CDX format. The software ChemDraw 4D extreme (version 16.0) was employed to convert the analogues into three-dimensional structures. This software is a standard for drawing chemical structures and is developed by Cambridge Soft Corporation, USA (Chemical Structure Drawing Standard, 2009). In addition, the MMFF94X force-field approach was employed to minimise the energy of all analogues through 1000 iterations. Ultimately, the transformed counterpart was stored in PDB format. The MGL Tools (version 1.5.6) (Trott and Olson, 2010), produced PDBQT files.

Preparation of Target Protein

The protein structure for Antiplatelet studies, namely PDB=1EQG and PDB=3ZDY, was obtained from RCSB-PDB at a resolution of 2.60 Å. The source can be found at http://www.rcsb.org/pdb/home/home.do. The bound ligand, water molecules, and other protein chains were removed using BIOVIA Discovery Studio Visualizer. The polar hydrogen and Kollman charges were included into the receptor file using the AutoDockVina programme, resulting in the generation of the PDBQT file.

Molecular Docking Studies

The molecular docking experiments were conducted using AutoDockVina version 4.2. The interaction between the ligand and enzyme was addressed using the Lamarckian genetic algorithm (LGA). A grid box was created at the coordinates 28.433, 28.311, and 198.790, located within the active site residues of a receptor. The AUTOGRID programme generated a grid map with a resolution of 45, where the points are separated by a distance of 0.700 Å. The docking settings were configured to the genetic algorithm's default value. The binding energies accurately determined the optimal conformation of the ligand-enzyme complexes during docking.

Results

Platelet Spreading Assay of R2

Platelet spreading was investigated in the absence of R2, which is the phase of platelet activation that leads to the formation of Actin nodules and stress fibers. The involvement of the Actin cytoskeleton in maintaining the stability

of blood clots has been suggested. In the series of trials, various concentrations of R2 (0.01, 0.05, 0.1, 0.3, and 0.6 μM) were introduced to the dispersed platelets. The absence of R2 (Control) did not significantly change the number of platelets with stress fibers over time (Figure: 1a). Conversely, the existence of R2 resulted in a fast depletion of Actin nodules and stress fibers in extended platelets. The effect reached its maximum at a concentration of 0.05μM (Figure: 1c), resulting in a decrease in the ratio of stress fibers and actin nodules. At concentrations of 0.1, 0.3 and 0.6 μM, the antiplatelet action of R2 is reversed by causing an increase in the number of stress fibers in spread platelets (Figure: 1e,f).

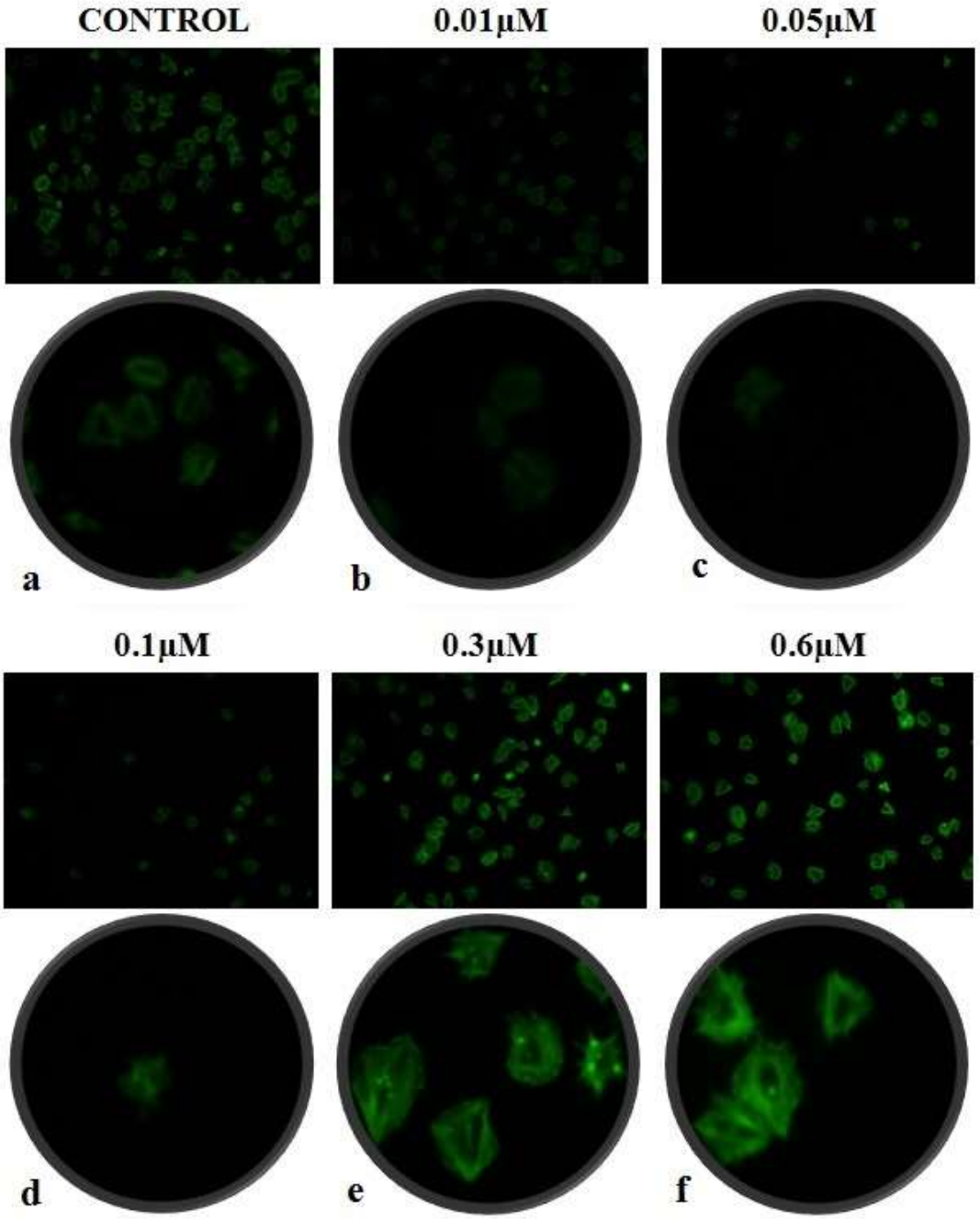


Figure: 1. Synthetic compound R2 inhibits platelet spreading on immobilized fibrinogen.

Human platelets were seeded in the presence of various R2 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.

R2 Reverses Stress Fiber Formation in Spread Platelets on Fibrinogen

Fibrinogen attaches to the active integrin GPIIb/IIIa through inside-out signal pathways, resulting in the aggregation of platelets. GPIIb/IIIa receptor engages with fibrinogen, triggering outside-in signalling that induces

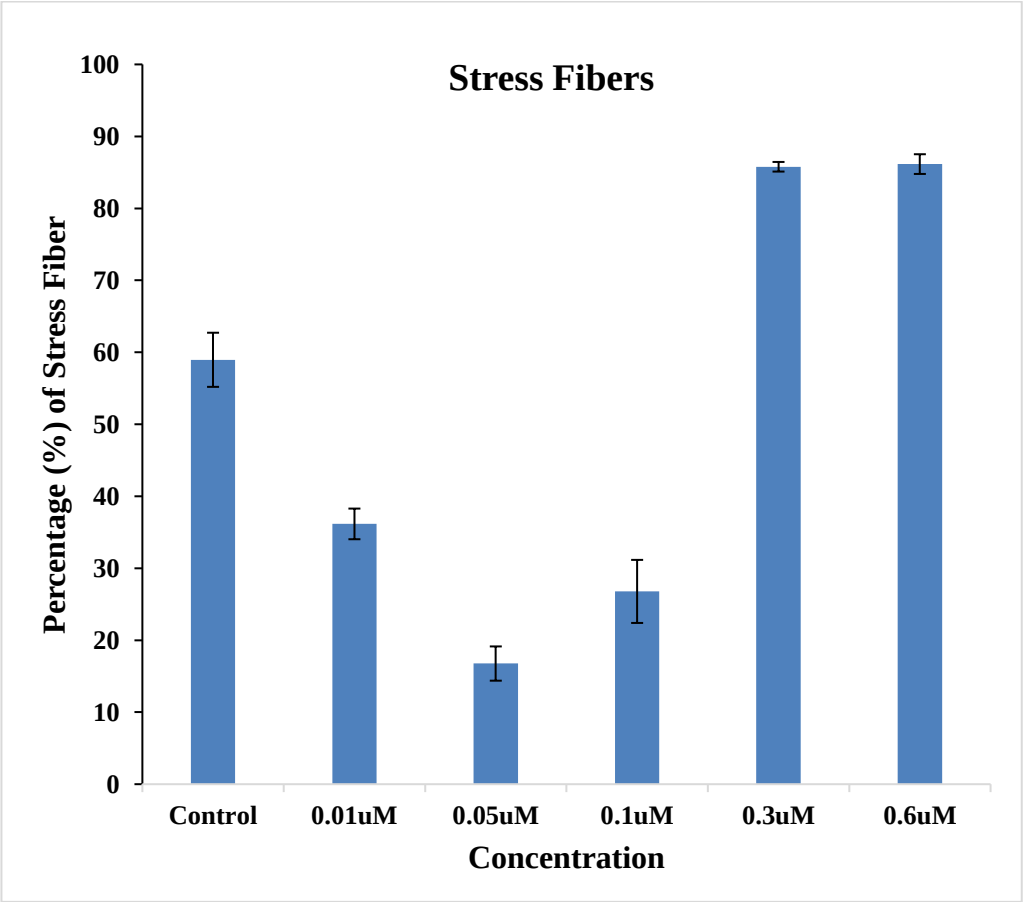
clot retraction and platelet spreading. These activities play a crucial role in thrombosis. Table 1 demonstrates that R2 effectively hindered platelet spreading on immobilised fibrinogen within the concentration range of 0.01–0.6 μM. Specifically, at concentrations of 0.01, 0.05, and 0.1 μM, R2 exhibited inhibitory effects. R2 had a notable inhibitory effect on platelet spreading through the reversal of fibrinogen activation. This effect was observed at concentrations of 0.05 and 0.1μM, resulting in an average percentage of stress fibers of 16.76±2.38 and 26.78±4.38, respectively (Graph: 1). Concentrations of 0.3 and 0.6 μM cause a reversal of the inhibitory effect of R2 Compound and lead to an increase in stress fibers in spreading platelets, with values of 85.78±0.67 and 86.15±1.37 (Graph: 1 and 2).

R2 Reverses Actin Nodules Formation in Spread Platelets on Fibrinogen

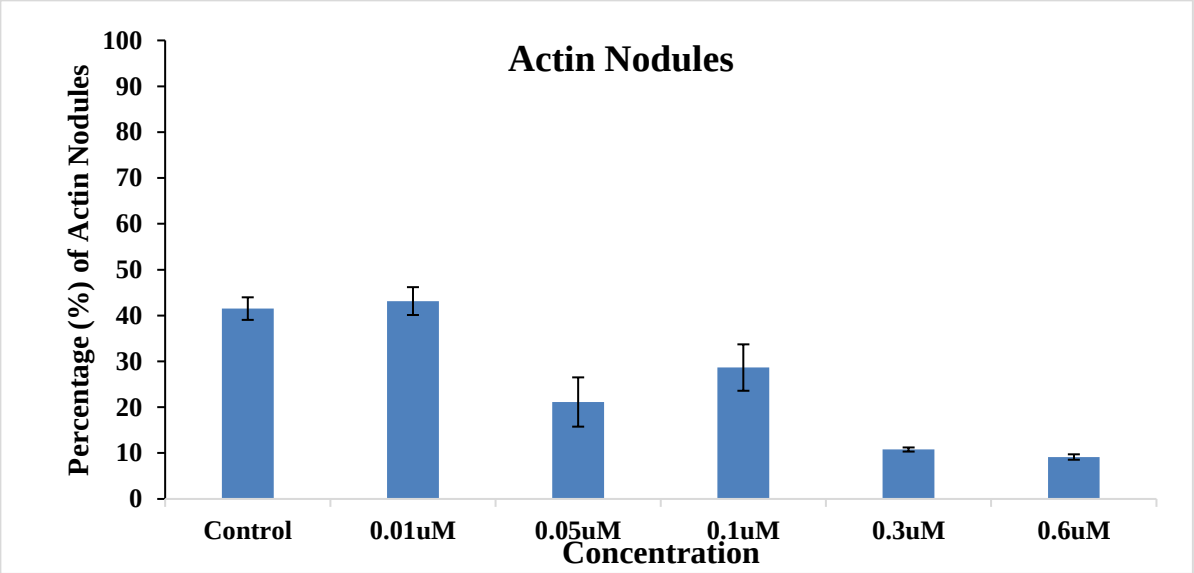
Table 1 demonstrates that R2 exhibits significant antiplatelet activity in the range of 0.01-0.6 μM. Specifically, at concentrations of 0.05 and 0.1 μM, R2 effectively inhibits platelet spreading induced by reverse fibrinogen activation. The average percentage of Actin nodules seen at these concentrations is 21.14±5.62 and 28.65±1.67, as shown in Graph 2. Increasing the doses of 0.3 and 0.6 μM further enhances the inhibitory action of R2 Compound on the actin nodule of spreading platelets, resulting in average values of 10.78±0.91 and 9.14±2.32 (Graph: 2), while also increasing the presence of stress fibers.

Table: 1. Percentage of R2 Reverse Stress Fiber and Actin Nodule on Fibrinogen Mediated Platelet Spreading

Platelet Spreading Conditions	Control	R2 Concentration				
		0.01 μM	0.05 μM	0.1 μM	0.3 μM	0.6 μM
Stress Fibers	58.96±3.76	36.16±2.13	16.76±2.38	26.78±4.38	85.78±0.67	86.15±1.37
Actin Nodules	41.51±2.17	43.15±3.67	21.14±5.62	28.65±1.67	10.78±0.91	9.14±2.32



Graph: 1. Percentage of Platelet Spreading formed Stress Fibers



Graph: 2. Percentage of Platelet Spreading formed Actin Nodules

DPPH Inhibiting Antioxidant Activity of R2 Compound

The antioxidant efficacy of compound R2 was evaluated through in vitro investigations employing DPPH as a generator of free radicals. Free radicals are inherently stable molecules and can be counteracted by the presence of antioxidants. Compound R2 demonstrated concentration-dependent antioxidant activity when evaluated at 100, 250, and 500 µM.

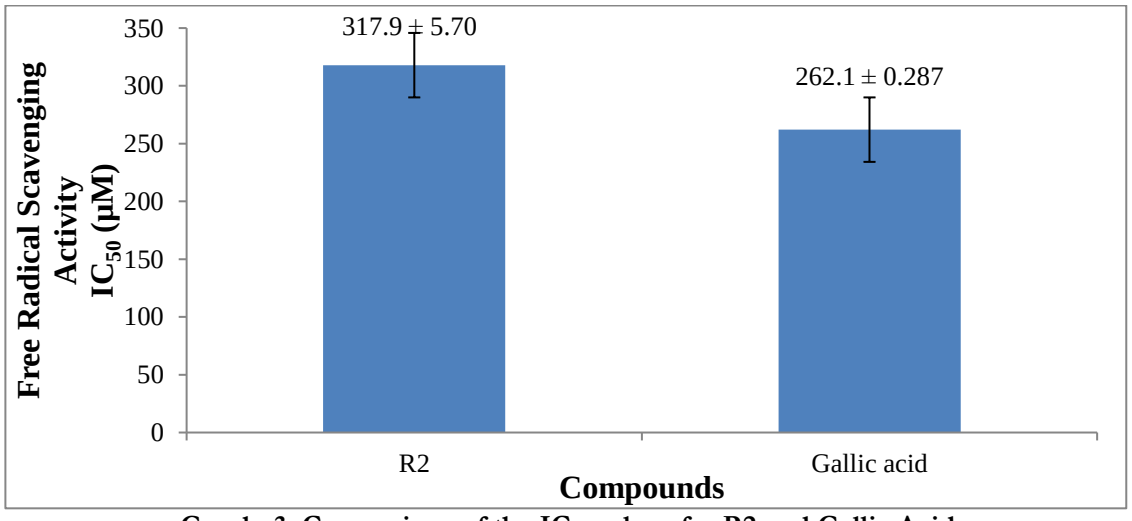
The chemical R2 exhibited antioxidant characteristics that varied depending on the dose, as indicated in Table 2. Compound R2 at a concentration of 100 µM inhibits DPPH activity by 15.6%. The inhibitory efficiency of the compound is within the micro-molar range. It shows a linear pattern of inhibition, with 39.20% inhibition of DPPH activity at a concentration of 250 µM and 78.40% inhibition at a concentration of 500 µM (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 R2 IC50 value of 317.9±5.70µM. (Graph: 3; Table: 2).

Table: 2. Scavenging of DPPH radical by Gallic Acid and R2

	Concentration (µM)	Percentage Inhibition (%)	IC_{50} (µM)
R2	100	15.6 ± 2.16	317.9 ± 5.70
	250	39.20 ± 2.41	
	500	78.40 ± 3.73	
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 R2 and Gallic Acid

In-Silico Platelet Aggregation Inhibited by a Novel Compound R2

R2 forms nine hydrophobic interactions with specific residues in PDB 1EQG, namely ARG-83A (3.48), PRO-84A (3.31), PRO 86A (3.96), PHE-88A (3.43), ILE-89A (3.98), ILE 89A (3.51), HIS-90A (3.43), LEU-92A (3.69), LEU-93A (3.82). These interactions have a binding energy of -7.56 kcal/mol (Figures: 2). The new thiazole derivative R2 forms hydrophobic interactions with seven specific sites in PDB3ZDY, namely ALA-218B (3.81), PRO-228A (3.45), PHE-231A (3.57), PHE-231A (3.37), LYS-253B (3.97), TRP-262A (3.91), ASP-336D (3.32) and three hydrogen bonding with specific sites, namely TRP-129D (2.98), SER-130D (2.70), LYS-137D (3.30). These interactions result in a binding energy of -8.25 kcal/mol, as indicated in Tables 3 and illustrated in (Figure 3).

Table 3: Docking studies with PDB 1EQG and PDB 3ZDY screened R2, 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)
R2 for PDB 1EQG	--	--	**9**	**ARG-83A (3.48), PRO-84A (3.31), PRO 86A (3.96), PHE-88A (3.43), ILE-89A (3.98), ILE 89A (3.51), HIS-90A (3.43), LEU-92A (3.69), LEU-93A (3.82)**	**ARG-83A (5.97), ARG-120A (4.09)**	-7.56
R2 for PDB 3ZDY	3	TRP-129D (2.98), SER-130D (2.70), LYS-137D (3.30)	7	ALA-218B (3.81), PRO-228A (3.45), PHE-231A (3.57), PHE-231A (3.37), LYS-253B (3.97), TRP-262A (3.91), ASP-336D (3.32)	LYS-137D (4.14)	-8.25

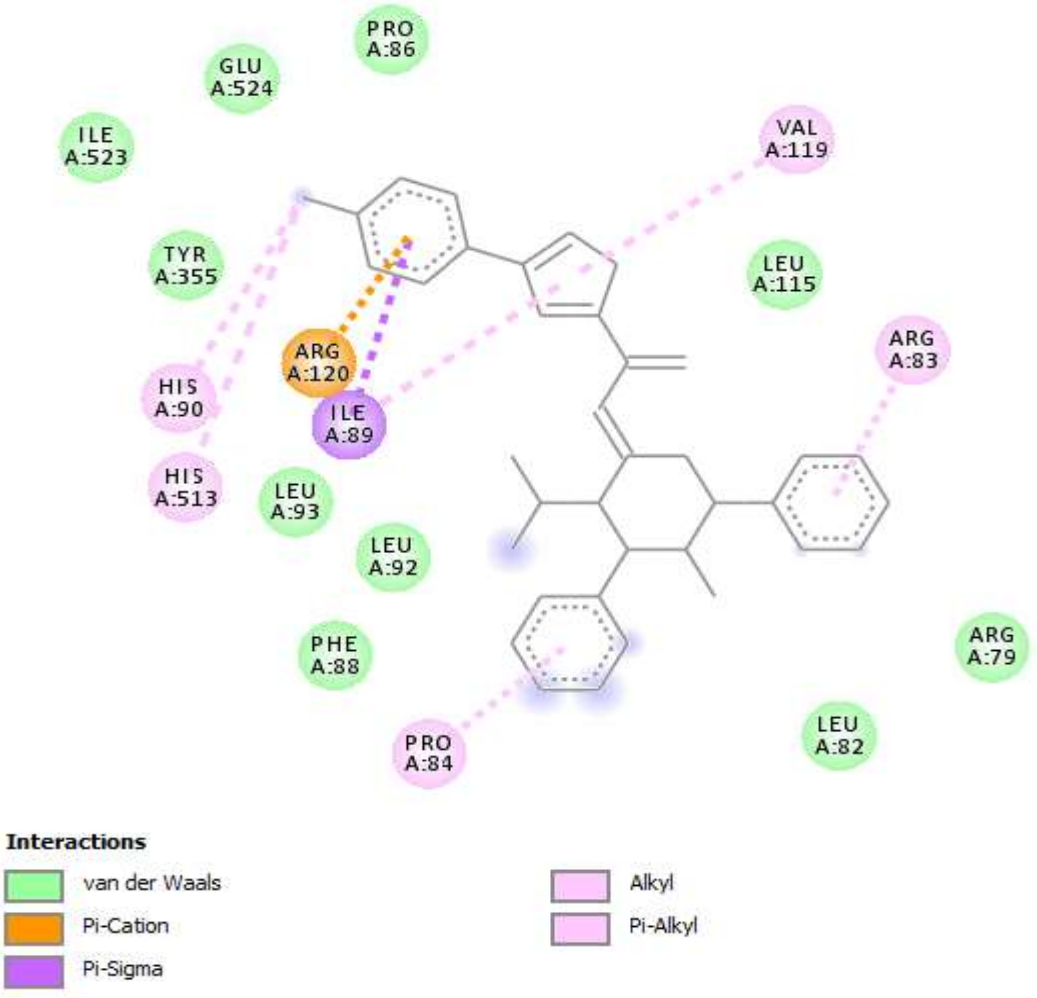


Figure: 2. 2D projection of the interactions of compound R2 with the active site for antiplatelet activity using PDB-1EQG

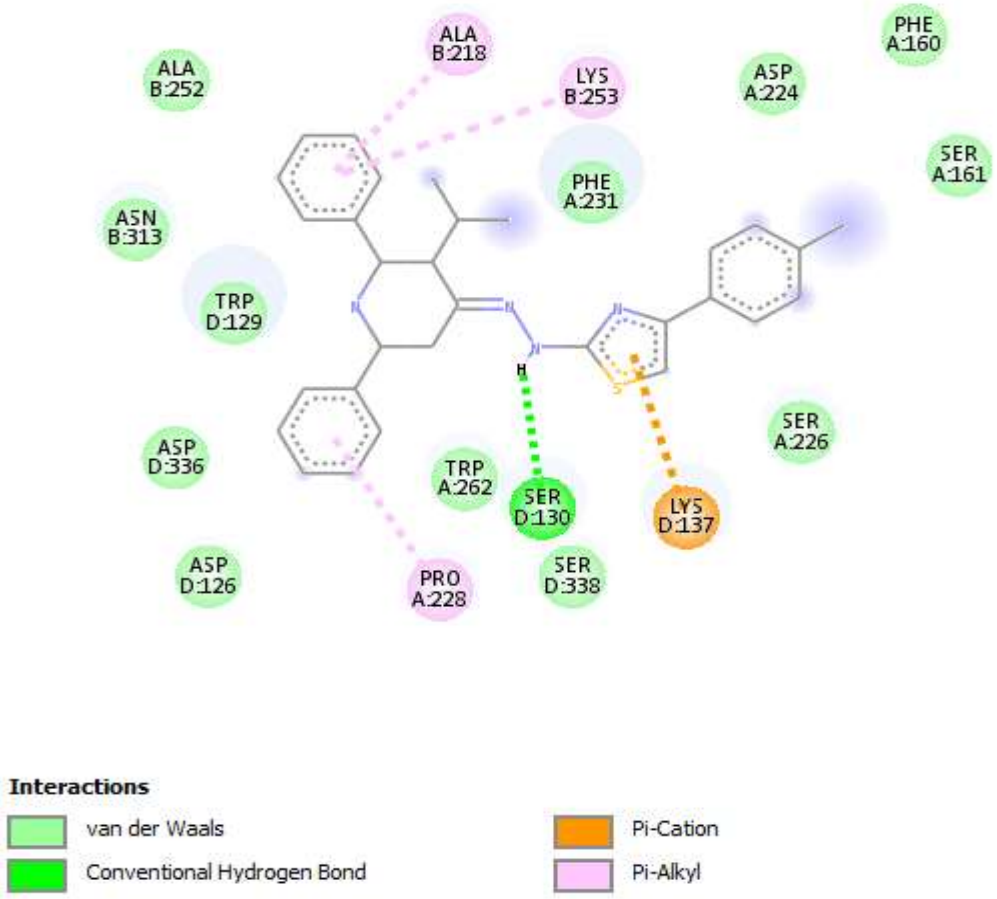


Figure: 3. 2D projection of the interactions of compound R2 with the active site for antiplatelet activity using PDB-3ZDY

In-Silico Antioxidant Activity by a Novel Compound R2

The docking computation of butylated hydroxyanisole showed that the projected pose formed two hydrophobic bonding contacts with amino acid residues PRO-40 and PRO-45, as well as three H-bonding interactions with amino acid residues THR-44, CYS-47, and ARG-127 (Table: 4). The thiazole derivative R2 exhibits binding affinity to certain sites, including six hydrophobic sites PRO-45A, LEU-112A, ASP-113A, LEU-116A, THR-147A, LEU-149A and one hydrogen bond site THR-147A as depicted in Figures 4. R2 exhibits a binding energy that is twice as strong as that of Standard butylated hydroxyanisole, with a value of -6.51 kcal/mol compared to -3.59 kcal/mol (Table 4).

Table 4: Docking studies with PDB 1HD2 for the screening of free radicals scavenging complex of BHA and R2

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
R2	1	THR-147A (2.42)	6	PRO-45A (3.68), LEU-112A (3.64), ASP-113A (3.43), LEU-116A (3.36), THR-147A (3.27), LEU-149A (3.39)	-	-6.51

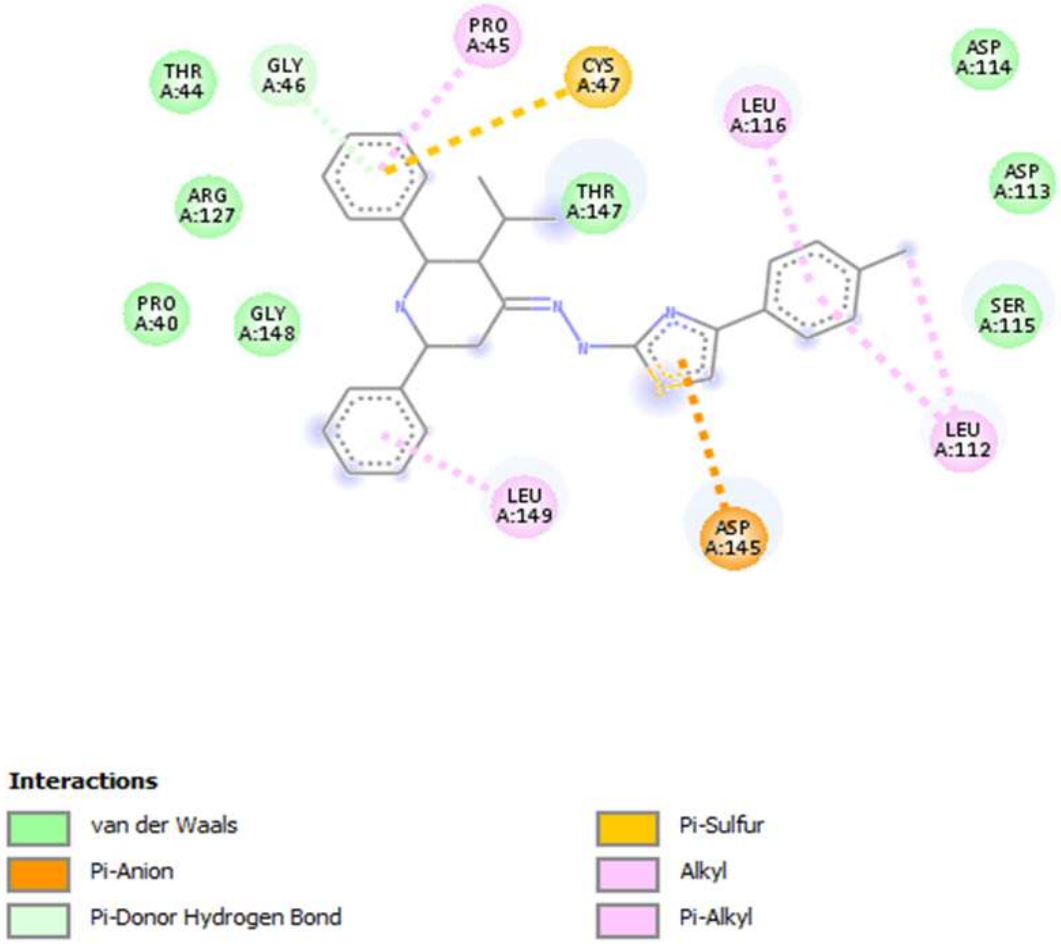


Figure: 4. 2D projection of the interactions of compound R2 with the active site for antioxidant activity using PDB-1HD2

Discussion

Persistent platelet attachment is crucial for the development of future thrombus formation. The fast restructuring of the actin cytoskeleton in platelets is essential for their capacity to withstand shear stress. Thrombus instability and embolisation have been linked to the incapacity of platelets to produce lamellipodia or stress fibers during activation and spreading (Schoenwaelder *et al.,* 2002; McCarty *et al.,* 2005).

The platelet outside-in signalling pathway mostly contributes to platelet thrombosis rather than hemostasis. To enhance the development of efficient and secure antiplatelet medications, it is advisable to focus on the platelet outside-in signal pathway (Su *et al.,* 2008). Integrin signalling relies on changes in shape and the grouping together of integrins, which enhance their ability to bind to the ligand with greater strength and stability. Ligand binding initiates a complex network of signalling, and structural cytoskeletal proteins lead to outside-in signalling, characterised by an increase in tyrosine phosphorylation. The relationship between αIIbβ3 and fibrinogen has been thoroughly characterised in platelets (Ruggeri, 2002). Phencyclidine specifically hindered the process of platelet aggregation and secretion, as well as the effects of other commonly occurring platelet activators such as a-thrombin, ADP, and collagen. Adrenaline is distinct as a platelet agonist since it does not induce shape alteration and solely leads to localised increases in cytoplasmic Ca+2 levels (Jamieson, 1988). There is a proposal suggesting that these specific variations in the concentration of calcium ions may lead to the exposure of fibrinogen receptors through a second messenger pathway that has not yet been identified (Shattil *et al.,* 1989).

The significant suppression of R2 on platelet spreading may contribute to its minimal propensity for bleeding while maintaining exceptional antithrombotic effectiveness. The spreading of platelets in the control group demonstrated that both stress fibers and actin nodules increased similarly at a specified time interval. However, the spreading of platelets treated with different concentrations of R2 (0.01, 0.05, 0.1, 0.3, and 0.6 μM) showed a significantly varying

degree of spreading. Treatment with R2 at concentrations of 0.05μM effectively inhibits the production of stress fibers and actin nodules by blocking the activation of fibrinogen in platelets, hence preventing their aggregation. The previous study also found that the R1 new thiazole derivative had a notable antiplatelet effect when platelet aggregation is activated by fibrinogen, at concentrations of 0.05 and 0.1μM (Farooqi *et al.,* 2023).

The formation of nodules is not contingent upon the generation of filopodia; instead, they are present from the initial stages of lamellipodia development and exhibit continuous movement until fully formed lamellipodia are produced. Nevertheless, they vanish upon the formation of stress fibers. The development of actin nodules necessitates actin polymerization and continuous Src kinase activity. When stress fibers are formed, focal adhesions are often found near the ends of the stress fibers. Therefore, it is improbable that actin nodules represent the focal adhesions (Calaminus *et al.,* 2008). At higher levels of 0.1, 0.3 and 0.6 μM, R2 reverses the impact by enhancing the creation of stress fibers. However, at lower concentrations of 0.05μM, R2 may suppress the activity of Src Kinase, as indicated by the low concentration of actin nodule.

Reactive oxygen species (ROS) can harm the cellular components of the vascular wall and trigger several redox-sensitive transcriptional pathways, resulting in a proatherogenic transcriptome profile. Endothelial dysfunction in the development of atherosclerosis is initiated by an increase in the production of reactive oxygen species (ROS) in the blood vessel wall and a decrease in the amount of nitric oxide (NO) available (Lee et *al.,* 2012). An antioxidant medication can reduce platelet activity by scavenging lipid peroxides and free radicals (ROS) that harm the vascular endothelium and inhibit prostacyclin synthetase. Additionally, the drug can interact with specific target proteins to achieve this effect. Antioxidants possess anticoagulant and antiplatelet aggregation effects (Singh *et al.,* 2008). The current investigation observed that the free-radical scavenging capacity of R2 was marginally inferior to that evaluated by Gallic acid. The chemical R2 had antioxidant activities that varied in intensity depending on the amount administered (100, 250, and 500 μM). The IC50 value of R2, which is 317.9±5.70μM, is comparable to the IC50 value of the reference antioxidant, Gallic Acid, which is 262.1±0.287μM.

COX-1, an isozyme of the enzyme cyclooxygenase that is consistently produced in platelets, stimulates the production of eicosanoids such as TXA2 and PGD2, which play a role in the activation and clumping together of platelets. TXA2 synthase is a crucial enzyme involved in the metabolism of amino acids. It converts PGH2 to TXA2 when COX-1 produces PGH2 from AA in platelets (Son *et al.,* 2014). Nonsteroidal anti-inflammatory medications (NSAIDs) suppress the activity of prostaglandin H2 synthase, hence inhibiting the formation of prostanoids. We selected the complex of prostaglandin H2 synthase (COX-1), the biologically active form of ibuprofen (PDB: 1EQG) (Selinsky *et al.,* 2001), and glycoprotein llb/llla protein (PDB-3ZDY). The purpose of the docking experiment was to determine the binding score, which helps forecast the activity of synthesised derivatives. COX-1 and glycoprotein llb/llla have essential functions in regulating platelet aggregation, with ADP, arachidonic acid, and collagen serving as agonists for each, respectively (Al-Saad *et al.,* 2019). The R2 residue exhibited negative binding energy (BE) values of -8.25 and -7.56 kcal/mol for PDB 3ZDY and PDB 1EQG, respectively, indicating a higher affinity for ligand binding. The results of molecular docking analysis indicate that R2 has a little stronger binding affinity for PDB-3ZDY compared to PDB-1EQG. This suggests that R2 has a greater potential for inhibiting glycoprotein llb/llla protein receptors binding to its ligands and therefore may be more effective in its antiplatelet activity.

Conclusion

The thiazole derivative exhibited antiplatelet activity through the fibrinogen inhibitor pathway, as evidenced by the inhibition of platelet activation and a reduction in the formation of actin nodules and stress fibers, utilising the platelet spreading technique. The medication examined in molecular docking tests shown antiplatelet efficacy by inhibiting the glycoprotein llb/llla receptor route and the COX-1 pathway. However, the glycoprotein pathway can be considered a secondary mechanism which enhance the antiplatelet activity of the compound R2.

References

1. Abulencia, J. P., Tien, N., McCarty, O. J., Plymire, D., Mousa, S. A., Konstantopoulos, K. (2001). Comparative antiplatelet efficacy of a novel, nonpeptide GPIIb/IIIa antagonist (XV454) and abciximab (c7E3) in flow models of thrombosis. *Arteriosclerosis, Thrombosis, and Vascular Biology, 21*, 149–56.
2. Al-Saad, H. N., RAZZAK MAHMOOD, A. A., & Al-Bayati, R. I. (2019). Design, Synthesis, Docking Study and Antiplatelet Evaluation of New Thiosemicarbazide Derivatives, Derived From Captopril. *Oriental journal of chemistry*, *35*(2), 829-838.
3. Aslan, J. E., & McCarty, O. J. (2013). Rho GTPases in platelet function. *Journal of Thrombosis and Haemostasis*, *11*(1), 35-46.
4. Bearer, E. L., Prakash, J. M., & Li, Z. (2002). Actin dynamics in platelets. *International review of cytology*, *217*, 137-182.

5. Behnke, O. (1965). Further studies on microtubules: A marginal bundle in human and rat thrombocytes. *Journal of ultrastructure research*, *13*(5-6), 469-477.
6. Calaminus, S. D., Auger, J. M., McCarty, O. J. T., Wakelam, M. J. O., Machesky, L. M., & Watson, S. P. (2007). MyosinIIa contractility is required for maintenance of platelet structure during spreading on collagen and contributes to thrombus stability. *Journal of Thrombosis and Haemostasis*, *5*(10), 2136-2145.
7. Calaminus, S. D., Thomas, S., McCarty, O. J. T., Machesky, L. M., & Watson, S. P. (2008). Identification of a novel, actin-rich structure, the actin nodule, in the early stages of platelet spreading. *Journal of Thrombosis and Haemostasis*, *6*(11), 1944-1952.
8. Chemical Structure Drawing Standard (2009); Cambridge Soft Corporation, USA.
9. Duan, X., Perveen, R., Dandamudi, A., Adili, R., Johnson, J., Funk, K., & Akbar, H. (2021). Pharmacologic targeting of Cdc42 GTPase by a small molecule Cdc42 activity-specific inhibitor prevents platelet activation and thrombosis. *Scientific Reports*, *11*(1), 13170.
10. Farooqi, U. G., Perveen, R., Akram, Z., Hussain, M., Siddiqui, R., & Akhtar, M. (2023). IMPACT OF NOVEL THIAZOLE DERIVATE (R1), REVERSES PLATELET STRESS FIBER FORMATION ON FIBRINOGEN-COATED SURFACES: IN-VITRO AND IN-SILICO STUDIES. *Journal of Population Therapeutics and Clinical Pharmacology*, *30*(18), 2779-2791.
11. Fuster, V., & Chesebro, J. H. (1981). Antithrombotic therapy: role of platelet-inhibitor drugs. I. Current concepts of thrombogenesis: role of platelets.(first of three parts). In *Mayo Clinic Proceedings*, *6*(2), 102-112.
12. Gao, G., Chen, L., Dong, B., Gu, H., Dong, H., Pan, Y., ... & Chen, X. (2009). RhoA effector mDia1 is required for PI 3-kinase-dependent actin remodeling and spreading by thrombin in platelets. *Biochemical and biophysical research communications*, *385*(3), 439-444.
13. Gardner, J. M., & Hynes, R. O. (1985). Interaction of fibronectin with its receptor on platelets. *Cell*, *42*(2), 439-448.
14. Hartwig, J. H. (1992). Mechanisms of actin rearrangements mediating platelet activation. *The Journal of cell biology*, *118*(6), 1421-1442.
15. Hartwig, J. H., Barkalow, K., Azim, A., & Italiano, J. (1999). The elegant platelet: signals controlling actin assembly. *Thrombosis and haemostasis*, *82*(08), 392-398.
16. Haydon, G. B., & Taylor, D. A. (1965). Microtubules in hamster platelets. *The Journal of cell biology*, *26*(2), 673.
17. Herman, A. G. (1998). Rationale for the combination of anti-aggregating drugs. *Thrombosis Research*, *92*(1), S17-S21.
18. Hu, L., Fan, Z., Du, H., Ni, R., Zhang, S., Yin, K., ... & Ding, Z. (2011). BF061, a novel antiplatelet and antithrombotic agent targeting P2Y12 receptor and phosphodiesterase. *Thrombosis and haemostasis*, *106*(12), 1203-1214.
19. Italiano Jr, J. E., Richardson, J. L., Patel-Hett, S., Battinelli, E., Zaslavsky, A., Short, S., & Klement, G. L. (2008). Angiogenesis is regulated by a novel mechanism: pro-and antiangiogenic proteins are organized into separate platelet α granules and differentially released. *Blood, The Journal of the American Society of Hematology*, *111*(3), 1227-1233.
20. Jamieson, G. A. (1988). *Platelet Membrane Receptors: Molecular Biology, Immunology, Biochemistry, and Pathology: Proceedings of the XIXth Annual Scientific Symposium of the American Red Cross Held in Washington, DC, October 20-22, 1987* (Vol. 283). Alan R. Liss.
21. Lee, R., Margaritis, M., M Channon, K., & Antoniades, C. (2012). Evaluating oxidative stress in human cardiovascular disease: methodological aspects and considerations. *Current medicinal chemistry*, *19*(16), 2504-2520.
22. Leng, L., Kashiwagi, H., Ren, X. D., & Shattil, S. J. (1998). RhoA and the function of platelet integrin αIIbβ3. *Blood, The Journal of the American Society of Hematology*, *91*(11), 4206-4215.
23. Lo, S. H. (2006). Focal adhesions: what's new inside. *Developmental biology*, *294*(2), 280-291.
24. McCarty, O. J., Larson, M. K., Auger, J. M., Kalia, N., Atkinson, B. T., Pearce, A. C., & Watson, S. P. (2005). Rac1 is essential for platelet lamellipodia formation and aggregate stability under flow. *Journal of biological chemistry*, *280*(47), 39474-39484.
25. Mccarty, O. J., Calaminus, S. D. J., Berndt, M. C., Machesky, L. M., & Watson, S. P. (2006). von Willebrand factor mediates platelet spreading through glycoprotein Ib and αIIbβ3 in the presence of botrocetin and ristocetin, respectively. *Journal of thrombosis and haemostasis*, *4*(6), 1367-1378.
26. Naik, U. P., & Naik, M. U. (2003). Association of CIB with GPIIb/IIIa during outside-in signaling is required for platelet spreading on fibrinogen. *Blood*, *102*(4), 1355-1362.
27. Ruggeri, Z. M. (2002). Platelets in atherothrombosis. *Nature medicine*, *8*(11), 1227-1234.
28. Schoenwaelder, S. M., Hughan, S. C., Boniface, K., Fernando, S., Holdsworth, M., Thompson, P. E., & Jackson, S. P. (2002). RhoA Sustains Integrin αIIbβ3Adhesion Contacts under High Shear. *Journal of Biological Chemistry*, *277*(17), 14738-14746.

29. Selinsky, B. S., Gupta, K., Sharkey, C. T., & Loll, P. J. (2001). Structural analysis of NSAID binding by prostaglandin H2 synthase: time-dependent and time-independent inhibitors elicit identical enzyme conformations. *Biochemistry*, *40*(17), 5172-5180.
30. Shattil, S. J., Budzynski, A., & Scrutton, M. C. (1989). Epinephrine induces platelet fibrinogen receptor expression, fibrinogen binding, and aggregation in whole blood in the absence of other excitatory agonists.
31. Shattil, S. J., Kashiwagi, H., & Pampori, N. (1998). Integrin signaling: the platelet paradigm. *Blood, The Journal of the American Society of Hematology*, *91*(8), 2645-2657.
32. Singh, I., Mok, M., Christensen, A. M., Turner, A. H., & Hawley, J. A. (2008). The effects of polyphenols in olive leaves on platelet function. *Nutrition, metabolism and cardiovascular diseases*, *18*(2), 127-132.
33. Son, D. J., Akiba, S., Hong, J. T., Yun, Y. P., Hwang, S. Y., Park, Y. H., & Lee, S. E. (2014). Piperine inhibits the activities of platelet cytosolic phospholipase A2 and thromboxane A2 synthase without affecting cyclooxygenase-1 activity: Different mechanisms of action are involved in the inhibition of platelet aggregation and macrophage inflammatory response. *Nutrients*, *6*(8), 3336-3352.
34. Su, X., Mi, J., Yan, J., Flevaris, P., Lu, Y., Liu, H., ... & Xi, X. (2008). RGT, a synthetic peptide corresponding to the integrin β3 cytoplasmic C-terminal sequence, selectively inhibits outside-in signaling in human platelets by disrupting the interaction of integrin αIIbβ3 with Src kinase. *Blood, The Journal of the American Society of Hematology*, *112*(3), 592-602.
35. Tendera, M., & Wojakowski, W. (2003). Role of antiplatelet drugs in the prevention of cardiovascular events. *Thrombosis research*, *110*(5-6), 355-359.
36. Trott, O., & Olson, A. J. (2010). AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *Journal of computational chemistry*, *31*(2), 455-461.
37. Valgimigli, M., Campo, G., Monti, M., Vranckx, P., Percoco, G., Tumscitz, C., ... & Ferrari, R. (2012). Short-versus long-term duration of dual-antiplatelet therapy after coronary stenting: a randomized multicenter trial. *Circulation*, *125*(16), 2015-2026.
38. White, J. G. (1968). Fine structural alterations induced in platelets by adenosine diphosphate. *Blood*, *31*(5), 604-622.
39. Zamir, E., & Geiger, B. (2001). Molecular complexity and dynamics of cell-matrix adhesions. *Journal of cell science*, *114*(20), 3583-3590.
40. Zhang, S., Hu, L., Du, H., Guo, Y., Zhang, Y., Niu, H., ... & Ding, Z. (2010). BF0801, a novel adenine derivative, inhibits platelet activation via phosphodiesterase inhibition and P2Y12 antagonism. *Thrombosis and haemostasis*, *104*(10), 845-857.