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Platelet protective efficacy of 3,4,5 trisubstituted isoxazole analogue by inhibiting ROS-mediated apoptosis and platelet aggregation

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Abstract Thrombocytopenia is a major hematological concern in oxidative stress-associated pathologies and chronic clinical disorders, where premature platelet destruction severely affects the normal functioning of thrombosis and hemostasis. In addition, frequent exposure of platelets to chemical entities and therapeutic drugs immensely contributes in the development of thrombocytopenia leading to huge platelet loss, which might be fatal sometimes. Till date, there are only few platelet protective molecules known to combat thrombocytopenia. Hence, small molecule therapeutics are extremely in need to relieve the burden on limited treatment strategies of thrombocytopenia. In this study, we have synthesized a series of novel 3,4,5 trisubstituted isoxazole derivatives, among which compound 4a [4-methoxy-*N*'-(5-methyl-3-phenylisoxazole-4-carbonyl) benzenesulfonohydrazide] was found to significantly ameliorate the oxidative stress-induced platelet apoptosis by restoring various apoptotic markers such as ROS content, cytosolic Ca^{2+} levels, eIF2-α phosphorylation, mitochondrial membrane depolarization, cytochrome c release, caspase activation, PS externalization, and cytotoxicity markers. Additionally, compound 4a dose dependently inhibits collagen-induced platelet aggregation. Hence, compound 4a can be considered as a prospective molecule in the treatment regime of platelet activation and apoptosis and other clinical conditions of thrombocytopenia. Further studies might ensure the use of compound 4a as a supplementary therapeutic agent to treat, thrombosis and CVD-associated complications. Over all, the study reveals a platelet protective efficacy of novel isoxazole derivative 4a with a potential to combat oxidative stress-induced platelet apoptosis.

Keywords Isoxazoles derivative · Oxidative stress · Caspases · Platelet apoptosis · Platelet aggregation

Introduction

Platelets are anuclear blood cells derived from bone marrow megakaryocytes, which play an indispensable role in hemostasis, thrombosis, and vascular integrity [1]. After a lifespan of around 7 to 10 days, they are cleared by reticuloendothelial system, primarily in liver and spleen [2]. Similar to other blood cell lineages, a constant number of mature platelets are maintained in the circulation by an antagonistic balance between their production and destruction [3]. Being anuclear, they are more prone to chemical stimuli and free radicals produced during oxidative stress, which trigger platelets to acquire a state of activation. The development of many chronic health diseases such as cardiovascular diseases (CVDs), cancer, and

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arthritis is associated with generation of reactive oxygen species (ROS) that can effectively impair platelet functions and decreased platelet count [4–8]. The reduced platelet count in these conditions is attributed to the ROS-mediated mitochondrial damage and subsequent platelet apoptosis [1]. Various other conditions such as viral infection, connective tissue or lymphoproliferative disorders, blood transfusions, sepsis and acute respiratory distress syndrome also contribute to early platelet death [9–12]. In addition to these major contributing factors, therapeutic drug-induced thrombocytopenia (DIT) is an emerging issue lately. Even though therapeutic drugs are vital in treating many of the incurable diseases, the side effects associated with these drugs are life threatening and might be fatal [13]. Many drugs such as methotrexate, cisplatin, vancomycin, and aspirin are reported to cause platelet apoptosis by ROS and various means, hence causing thrombocytopenia [14–17]. Further, few drugs are also known to stimulate a range of hematologic alterations including reduced blood cell count and subsequent increase in circulating microparticles (MPs) [18], which are known to be involved in the progression of inflammation, coagulation, CVDs, cancer and rheumatoid arthritis [19].

Therefore, in the present work, we are targeting the often neglected ROS-induced platelet apoptosis, which is a common feature in many of the pathophysiological conditions leading to thrombocytopenia. The development of thrombocytopenia in numerous human pathologies raises a need for the development of new molecules, which have the potential to alleviate stress-induced early platelet death. However, till date, very few molecules with platelet protective efficacy are reported, which includes natural compounds like crocin, cinnamtannin B1, and a synthetic derivatives of ibuprofen and N- acetyl cysteine with platelet protective efficacy [20]. Hence, search for novel synthetic platelet protective molecules with potential to combat against ROS-induced pathological conditions is essential in vascular pharmacology. In this context, we have made an attempt to synthesize novel isoxazole derivatives, as isoxazole moiety has been recognized to possess a wide range of biological properties such as, anticoagulant, antiviral, anti-apoptotic, and anti-inflammatory properties [21–24]. Apart from these, many of the isoxazole-based drugs such as sulfamethoxazole, cycloserine, and acivicin are being commercially used for various treatments [25]. Upon consideration of the above facts, a series of novel isoxazole molecules (4a-h) were synthesized, and for the first time, we have made an effort to explore the platelet protective property of isoxazole derivatives with calcium ionophore (A23187) as agonist to induce platelet apotosis as earlier studies have reported that A23187 enhances ROS mediated apoptosis in several cell lines including platelets [26].

Materials and methods

Chemicals

Benzamidine hydrochloride, acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (AC-DEVD-AMC), *N*-acetyl-Leu-Glu-His-Asp trifluoromethyl coumarin (AC-LEHD-FMC), CHAPS, dithiothreitol (DTT), dimethyl sulfoxide (DMSO), *N*-(2-Hydroxyethyl) piperazine-*N*-ethanesulfonic acid (HEPES), sodium orthovanadate, fluorescein isothiocyanate (FITC)-labeled annexin, 10-nonyl acridine orange (NAO), fura-2/AM, fluorogenic, non-fluorogenic dyes, calcium ionophore (A23187), and MTT were from Sigma Chemicals, USA. Monoclonal anti-cytochrome c antibody was from Epitomics, USA. Anti-caspase-3 antibody was from Santa Cruz Biotechnology, USA. Antibodies against phospho-eIF2-α, eIF2-α, and β-tubulin were from Cell Signalling Technology, USA. Collagen type-I was from Chrono-log Corporation, USA. LysoSensor green DND-189 was from Molecular probes, USA. Lactate dehydrogenase (LDH) estimation kit was purchased from Agappe Diagnostics Limited, India. 1,1-diphenyl-2-picrylhdrazyl (DPPH) was from HiMedia Laboratories, India. All the synthesized compounds (4a-h) were solubilized in 50 % (v/v) DMSO as stock solutions and further diluted in respective assay buffers.

The synthesis and characterization of compounds (4a-h) are provided as supplementary information.

Antioxidant activity

The effect of isoxazole derivatives (4a-h) on DPPH radical scavenging activity was determined according to the method of Yamaguchi et al. [27], with slight modification, quercetin was used as reference standard. Briefly, 0.1 mM solution of DPPH was incubated with 0–50 μM of isoxazole derivatives (4a-h) for 20 min at ambient temperature in dark, and the resulting absorbance was measured using UV/Vis spectrophotometer at 517 nm against blank (BioMate 3S, Thermo Scientific).

Determination of ferric reducing ability

The reductive ability of the isoxazole derivatives (4a-h) was determined according to the method of Hsieh and Yan [28]. Briefly, test compounds (0–50 μM) were incubated with 2.5 mL of sodium phosphate buffer (0.2 M, pH 6.6) containing 1 % potassium ferricyanide, and the mixture was incubated at 50 °C for 20 min. At the end of incubation, 2.5 mL of 5 % TCA was added and centrifuged at 450×*g* for 10 min. The upper layer (2.5 mL) was separated and mixed with 0.5 mL of 0.1 % ferric chloride solution. Finally absorbance was measured at 700 nm against blank using UV/Vis spectrophotometer (BioMate 3S, Thermo Scientific).

Preparation of platelet-rich plasma and washed platelets

Venous blood was drawn from healthy human volunteers with written consent as per the norms of Institutional Human ethical committee. The study was approved and was in accordance with the guidelines of the Institutional Human Ethical Committee (IHEC-UOM No. 95/Ph.D./2013-14) University of Mysore, Mysore. The blood drawn was instantly mixed with acid citrate dextrose (ACD) anti-coagulant (85 mM sodium citrate, 78 mM citric acid and 111 mM D-glucose) in the ratio 6:1 (blood: ACD *v/v*) following centrifugation at $90\times g$ for 15 min to obtain platelet-rich plasma (PRP). Further in order to get washed platelets, PRP was centrifuged at $1700\times g$ and washed twice with CGS buffer (123 mM NaCl, 33 mM D-glucose, 13 mM trisodium citrate, and pH 6.5) and re-suspended in HEPES-buffered saline [HBS, 2.5 mM HEPES, 150 mM NaCl, 2.5 mM KCl, 12 mM $NaHCO_3$, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5.5 mM D-glucose, and 0.3 % bovine serum albumin (BSA) pH 7.4].The platelet count was determined using a Neubauer chamber and adjusted to 5×10^8 cells/mL in the final suspension using Tyrode's albumin buffer [145 mM NaCl, 5 mM KCl, 10 mM HEPES, 0.5 mM Na_2HPO_4, 1 mM $MgCl_2$, 6 mM glucose, and 0.3 % BSA] pH 7.4 [29].

Determination of endogenously generated ROS

The method of Lopez et al. [30] was followed to determine the endogenous ROS production in platelets using CMH2DCFDA, a ROS-sensitive fluorescent probe. PRP and washed platelets were treated with A23187 (5 μM) as agonist. For inhibition studies, pre-loaded platelets with agonist were incubated with compound 4a in increasing concentration (0–50 μM), and the final volume was made up to 200 μL with HBS and incubated at 37 °C for 1 h. The control (untreated) and treated platelets were then incubated with 10 μM CMH2DCFDA (excitation 488 nm and emission at 530 nm) for 30 min at 37 °C and analyzed by Varioskan multimode plate reader (Thermo Scientifics, USA) or flow cytometer (FACSVerse™, BD Biosciences, USA).

Estimation of intracellular calcium

Intracellular Ca^{2+} level in PRP as well as washed platelets was determined as described previously [31]. PRP and washed platelet suspensions were independently treated with A23187 (5 μM), and for inhibition studies, pre-loaded platelets with agonist were incubated with compound 4a in increasing concentration (0–50 μM). The final volume was made up to 200 μL with HBS containing 1 mM $CaCl_2$ and incubated for 1 h at 37 °C. Test samples were then incubated with 2 μM fura-2/AM for 45 min at 37 °C. The resulting fura-2/AM fluorescence was measured by exciting the samples at 340 and 380 nm, and the resulting fluorescence was measured at 500 nm. Data were presented as absorption ratios (340/380 nm).

Determination of changes in mitochondrial membrane potential ($\Delta\Psi m$)

JC-1 dye was used to detect changes in the $\Delta\Psi m$ according to the method of Salvioli et al. [32]. Both PRP and washed platelets were treated with A23187 (5 μM) as agonist, and for inhibition studies, pre-loaded platelets with agonist were incubated with compound 4a in increasing concentration (0–50 μM) and kept for 1 h at 37 °C. After incubation, samples were then loaded with JC-1 (10 μg/mL) at 37 °C for 10 min. The cells were then excited at 488 nm and emission was detected at 585 nm for JC-1 aggregates and 516 nm for JC-1 monomers using multimode plate reader. Data were presented as emission ratios (585/516).

Assessment of cardiolipin peroxidation

Cardiolipin peroxidation was determined by a fluorescent probe 10-NAO [33]. Both PRP and washed platelets were processed as discussed above with A23187 (5 μM) as agonist or pre-loaded platelets with agonist were incubated with 4a in increasing concentration (0–50 μM) for inhibition studies. After incubation, samples were treated with NAO (5 μM) for 30 min at 37 °C, and the resulting fluorescence was measured by exciting the samples at 499 nm, and emission was recorded at 530 nm.

Assessment of mitochondrial permeability transition pore (MPTP) formation

MPTP formation in platelets was assessed using calcein AM. Washed platelets (5×10^6 cells/mL) were incubated with agonist A23187 (5 μM) for 10 min, and for inhibition studies, pre-loaded platelets with agonist were treated with compound 4a in increasing concentration (0–50 μM) and incubated at 37 °C for 1 h. After incubation, calcein AM (1 μM) was added and incubated for 30 min at 37 °C. To quench cytosolic calcein fluorescence, $CoCl_2$ (1 mM) was added and the resulting mitochondrial fluorescence was measured by exciting the samples at 488 nm, and emission was detected at 585 nm [34].

Determination of caspase activity

Caspase activity was determined with slight modification according to the method of Amor et al. [35]. Both PRP and washed platelets were incubated with A23187 (5 μM) and

for inhibition studies, pre-loaded platelets with agonist were incubated with compound 4a in increasing concentration (0–50 μM) and kept at 37 °C for 1 h. Platelet lysate was prepared by adding an equal volume of 2× Triton buffer (2 % Triton X-100, 2 mM EGTA, 100 mM Tris-HCl—pH 7.2, 10 μg/mL leupeptin, 2 mM PMSF, 10 mM benzamidine, and 2 mM Na_3VO_4) and allowed to undergo lysis for about 30 min at 4 °C. The lysate was subjected to centrifugation at 16,000×*g* for 5 min. The pellet thus obtained is the Triton-insoluble cytoskeleton-rich fraction. Further caspase activity was measured by incubating cell lysate in a 96-well plate with substrate solution [20 mM HEPES, pH 7.4, 2 mM EDTA, 0.1 % CHAPS, 5 mM DTT, and 8.25 μM caspase substrate (AC-DEVD-AMC for caspase-3 and AC-LEHD-AFC for caspase-9)] for 2 h at 37 °C. Substrate cleavage was recorded using a multimode plate reader by excitation the samples at 360 nm and emission at 460 nm.

Determination of phosphatidylserine (PS) externalization

Annexin V binding of PS can be used as a reliable marker for platelets undergoing apoptosis. Washed platelets were incubated with A23187 (5 μM), and for inhibition studies, pre-loaded platelets with agonist were incubated with compound 4a in increasing concentration (0–50 μM) at 37 °C for 1 h. After incubation samples were stained using Annexin-FITC washed and analyzed by flow cytometer [17].

Immunoblot

Washed platelets (5 × 10^8/mL) were stimulated by adding A23187 (5 μM) as agonist and for inhibition studies pre-treated platelets with agonist were treated with compound 4a (0–50 μM). After 1 h, cells were lysed with 10 μL of HEPES buffer (10 % SDS, 20 mM *N*-ethylmaleimide, 20 mM sodium *o*-vanadate, 50 mM EDTA, and 10 mM PMSF) and centrifuged. Following centrifugation, the supernatants were separated on 10 % SDS-PAGE, and proteins were transferred on to a PVDF membrane. After blocking with 5 % non-fat milk powder in TBST, the blots were probed with cytochrome c, caspase-3, phospho-eIF2-α, and eIF2-α overnight at 4 °C. After incubation, blots were then treated with HRP-conjugated anti-IgG antibody and finally developed by chemiluminescence imaging system (Alliance 2.7, Uvitec, UK). β-tubulin was used as loading control [36]. For re-probing, blots were incubated in stripping buffer (200 mM glycine, pH 2.2, 1 % Tween-20, and 0.1 % SDS) for 2 min and washed with TBST. Blots were again treated with stripping buffer for 5 min and washed thrice with TBST, 2 min each wash. The stripped membrane was blocked in TBST containing 5 % non-fat milk powder over night at 4 °C and probed with desired antibodies. Each membrane was stripped three times maximum.

Determination of cytotoxicity by MTT and LDH release assays

Both MTT and LDH leakage assays have been used extensively as a marker for cell death. For MTT assay, washed platelets in polystyrene 96-well microtiter plates were treated with agonist A23187 (5 μM), and for inhibition studies, pre-loaded platelets with agonist were incubated with compound 4a in increasing concentration (0–50 μM), and the reaction volume was made up to 200 μL with Tyrode's buffer. After 1 h of incubation, 250 μM of MTT was added and incubated for additional 3 h at 37 °C. Finally, MTT was removed and remaining formazan crystals were dissolved in DMSO, and the absorbance was measured at 570 nm, using multimode plate reader. For LDH release assay, washed platelets were treated with agonist A23187 (5 μM), and for inhibition studies, pre-loaded platelets with agonist were incubated with compound 4a in increasing dose (0–50 μM) for 1 h at 37 °C. After incubation, platelets were pelleted by centrifugation at 1700×*g* for 10 min, and supernatants were used to detect LDH release by kit method, according to the manufacturer's protocol. The assay was performed in a time course of decrease in NADH absorbance at 340 nm for 3 min using spectrophotometer (BioMate 3S, Thermo Scientific) [36].

Measurement of γ -glutamyltransferase (GGT) activity

Washed platelets (5 × 10^6 platelets/mL) were treated with agonist A23187 (5 μM), and for inhibition studies, pre-treated platelets with agonist were incubated with 4a (0–50 μM) for 1 h at 37 °C. After incubation, platelets were pelleted, suspended in distilled water, and lysed by sonication. The resulting lysate was used to determine GGT activity according to the previously reported method [36],which comprises of assay mixture (1 mL) containing 4 mM γ-glutamyl *p*-nitroanilide and 40 mM glycylglycine in 185 mM Tris–HCl buffer, pH 8.2. The results were calculated using molar extinction coefficient of *p*-nitroanilide (9900 M^{-1} cm^{-1}) at 405 nm and expressed as mM *p*-nitroanilide formed/min/mg protein.

Determination of GSH/GSSG levels

GSH and GSSG levels were determined by previously reported [37]. In brief, pre-treated washed platelets

(5×10^6 platelets/mL) with agonist A23187 (5 μM) were incubated with compound 4a (0–50 μM) for 1 h at 37 °C. After incubation, platelets were incubated with *o*-phthalaldehyde (1 mg/mL) at room temperature for 15 min to determine GSH levels, and to determine GSSG, platelets were treated with *N*-ethylmaleimide (10 μM) prior to *o*-phthalaldehyde addition, and the resulting fluorescence was measured at 430 nm by exciting the samples at 365 nm. The concentration of GSH and GSSG were determined from the standard curve.

Determination of glucose-6-phosphate dehydrogenase (G6PDH) activity

G6PDH activity was estimated by monitoring the increase in absorbance at 340 nm for 3 min due to $NADP^+$-dependent glucose 6-phosphate transformation [38]. Briefly, washed platelets (5×10^6 platelets/mL) were treated with agonist A23187 (5 μM), and for inhibition studies, agonist pre-treated platelets were incubated with compound 4a (0–50 μM) for 1 h at 37 °C. After incubation, platelets were pelleted, suspended in distilled water, and lysed by sonication. The lysates were used to determine G6PDH activity in the reaction mixture (1 mL) containing Tris-HCl buffer (0.05 mM, pH 7.5), 3.8 mM NADP, 3.3 mM glucose-6-phosphate, and 6.3 mM $MgCl_2$. The activity was expressed as nmol NADPH formed/min/mg protein.

Platelet aggregation

Turbidimetric method was employed to determine platelet aggregation using a dual channel Chrono-log model 700-2 aggregometer (Havertown, USA) as described previously [39]. Briefly, 250 μL of PRP was taken in siliconized glass cuvette and pre-incubated with different concentrations of compound 4a (0–50 μM) for 3 min at 37 °C and aggregation was stimulated by the addition of collagen (2 μg/mL). The resulting aggregation of platelets was followed for 6 min with constant stirring at 1200 rpm.

Protein estimation

The method of Lowry et al. [40] was adopted for protein estimation using BSA as standard.

Statistical analysis

Results were expressed as mean ± SEM of five independent experiments. Statistical significance among groups were determined by one-way analysis of variance (ANOVA) followed by Tukey's test for comparison of means [$n = 5$, $p\,*/^{\#} < 0.05, p\,**/^{\#\#} < 0.01, p\,***/^{\#\#\#} < 0.001$; * significant compared to untreated platelets. # significant compared to A23187-treated platelets].

Results

Synthesis of new isoxazole derivatives

Structurally diversified 3,5-disubstituted isoxazole-4-hydrazide derivatives were synthesized through the adaptable stepwise reactions given in Fig. 1. The reaction between ethyl 5-methyl-3-phenylisoxazole-4-carboxylate **1** with hydrazine hydrate in ethanol under reflux condition gave the desired scaffold **2** in good yield. The hydrazide **2** was derivatized with different sulfonyl and benzoyl chlorides. Interestingly both electron donating and electron withdrawing substituents on the phenyl sulfonyl and benzoyl chlorides underwent reaction smoothly to give desired products in good to excellent yield. The structure of all the synthesized compounds (Table 1) was confirmed by NMR spectroscopic and HRMS data (supplementary information).

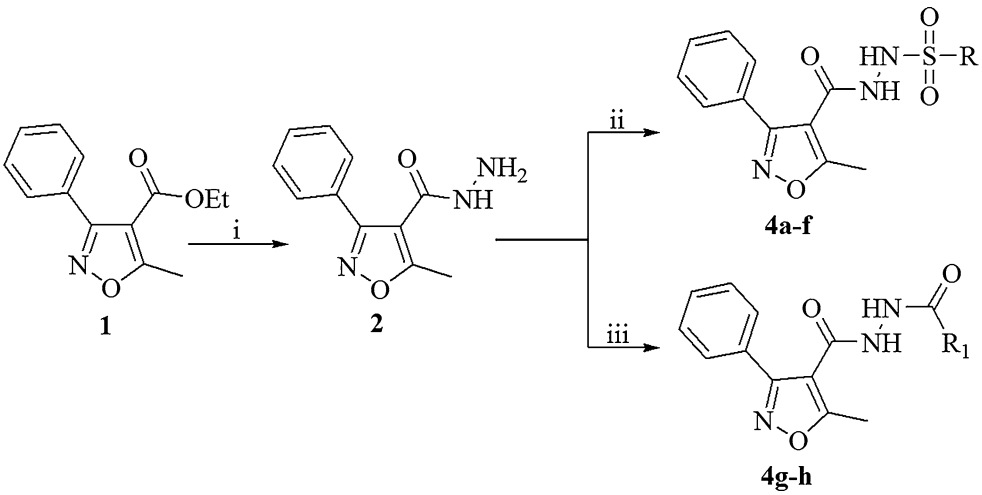
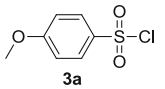
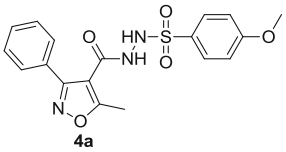
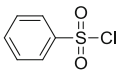
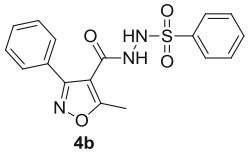
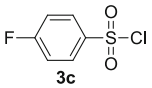
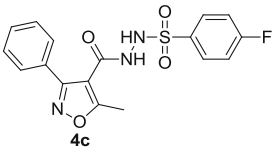
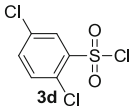
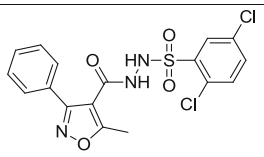
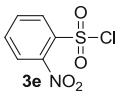
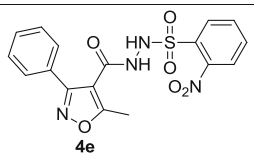
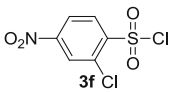
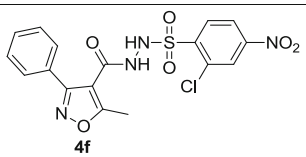
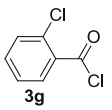
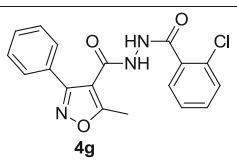
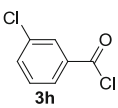
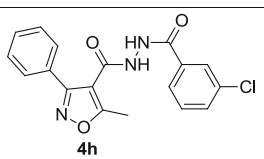


Fig. 1 Reaction sequence for the synthesis of new isoxazole derivatives: *i* $NH_2NH_2.H_2O$, Ethanol, 70 °C, *ii* phenylsulfonyl chlorides (**3a-f**), pyridine, 0 °C-R.T and *iii* benzoyl chlorides (**3g-h**), pyridine, 0 °C-R.T

Table 1 Step-wise synthesis of 3,5-disubstituted isoxazole-4-hydrazides 4a-h

Entry	Sulfonyl/acyl chlorides	Product	Time (h)	Yield (%)[a]
1	 **3a**	 **4a**	5.0	91
2	 **3b**	 **4b**	5.0	89
3	 **3c**	 **4c**	5.0	86
4	 **3d**	 **4d**	5.5	85
5	 **3e**	 **4e**	5.5	83
6	 **3f**	 **4f**	6.0	80
7	 **3g**	 **4g**	4.0	93
8	 **3h**	 **4h**	4.0	94

[a] Isolated yield based on the previous step

Free radical scavenging potentials of isoxazole and its derivatives

Deleterious-free radical generation is the key event in progression of many pathological conditions. During oxidative stress, these free radicals contribute the major role in inducing cell death. Therefore, isoxazole derivatives (Table 1) were evaluated for their free radial scavenging potentiality prior to their determination of platelet protective property. To begin with, reducing potential of isoxazole derivatives (4a-4h) were assessed by ferric reducing assay. Among the derivatives, compound 4a showed increased radical reducing ability (Fig. 2a). Further DPPH scavenging property of isoxazole derivatives (4a-4h) were evaluated, where compound 4a showed potent radical scavenging activity when compared with other isoxazole derivatives (Fig. 2b). Based on these results, compound 4a was considered as potent candidate among other isoxazole derivatives and therefore appeared to be promising for the evaluation of its anti-platelet properties.

Compound 4a alleviates A23187-induced ROS and intracellular calcium levels

Cellular oxidative stress plays a vital role in intrinsic mode of platelet apoptosis, therefore compound 4a was initially analyzed for its ROS scavenging potential in platelets. Platelets treated with standard agonist A23187 evoked significant ROS levels in PRP and washed platelets, whereas compound 4a was able to reduce A23187-induced ROS generation in a dose-dependent manner, which was further confirmed by FACS analysis (Fig. 3a, b). Increased levels of intracellular calcium is yet another important oxidative stress marker, which drives platelets toward intrinsic apoptotic pathway. Treatment of platelets with A23187 caused an increased level of Ca^{2+} in platelets. However, compound 4a dose-dependently ameliorated increased Ca^{2+} level, and significant inhibition was observed at 50 μM concentration (Fig. 3c). Further, 4a dose dependently inhibited the phosphorylation of eIF2α, which signifies the inhibition of endoplasmic reticulum stress in platelets (Fig. 3d). Altogether, compound 4a was found to be potent in reducing ROS content, intracellular calcium levels, and phosphorylation of eIF2α in platelets.

Effect of compound 4a on mitochondrial membrane potential depolarization and peroxidation of cardiolipin

Depolarization of mitochondrial membrane potential ($\Delta\Psi m$) and cardiolipin peroxidation are the key manifestation in the intrinsic apoptotic event. $\Delta\Psi m$ is a crucial parameter for evaluation of mitochondrial function, which was measured using JC-1 dye. Compound 4a was able to efficiently inhibit A23187-induced $\Delta\Psi m$ dissipation and restore the $\Delta\Psi m$ completely in both PRP and washed platelets (Fig. 4a).

Further, compound 4a was assessed for its effects on cardiolipin peroxidation using NAO. Peroxidized cardiolipin loses its affinity for NAO resulting in reduced fluorescence. Treatment of platelets with A23187 induced significant reduction in fluorescence suggesting that there was increased peroxidation of cardiolipin. Upon treatment with compound 4a, there was dose-dependently restoration of cardiolipin peroxidation in A23187-treated platelets (Fig. 4b).

Inhibition of MPTP formation and subsequent cytochrome c release by compound 4a

Formation of MPTP results in release of cytochrome c from mitochondria to cytosol causing activation of caspases, which are the critical regulatory events in the intrinsic apoptotic pathway. Therefore, MPTP formation was determined by calcein–$CoCl_2$ quenching assay using

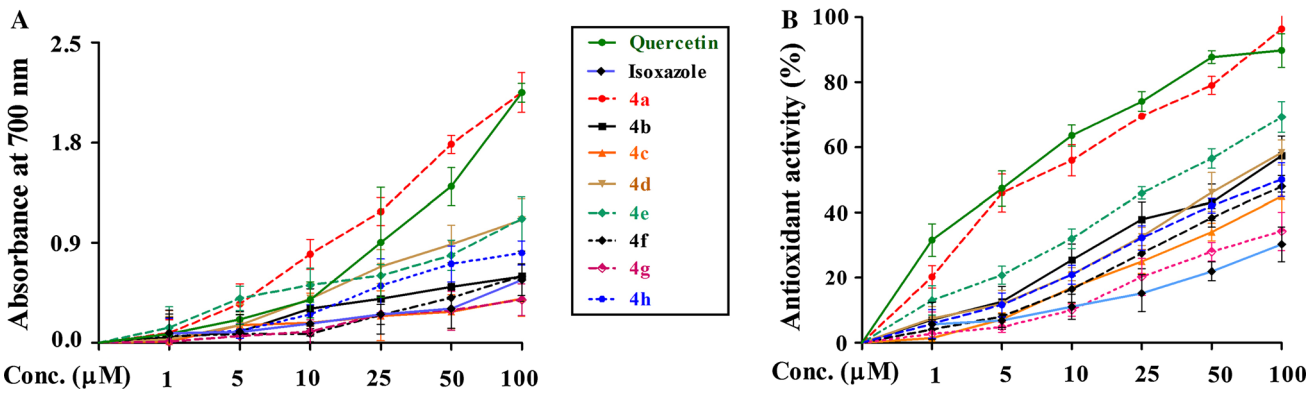


Fig. 2 Radical scavenging potentiality of isoxazole and its derivatives: activity measured in terms of **a** reducing ability toward Fe^{3+} to Fe^{2+} and **b** DPPH radical scavenging in comparison with quercetin. Values are presented as mean ± SEM ($n = 5$)

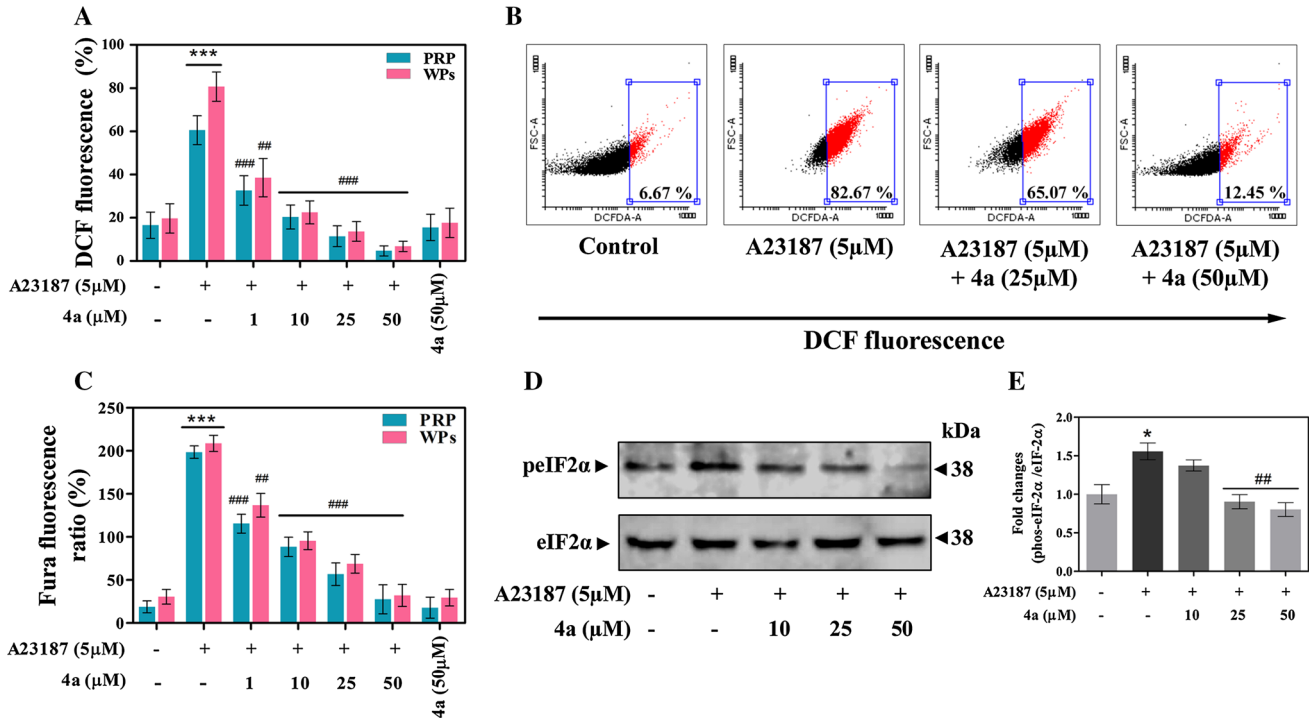


Fig. 3 Effect of compound 4a on A23187-induced **a** endogenous ROS generation as determined by conventional method in PRP and washed platelets and by **b** FACS in washed platelets. **c** Intracellular calcium level in PRP and washed platelets, and **d** immunoblot showing the expression of phospho-eIF2-α in platelets treated with A23187 in presence or absence of 4a. **e** Representative densitogram of immunoblot present in panel D. Values are presented as mean ± SEM ($n = 5$), expressed as percentage increase in DCF fluorescence, Fura-2/AM fluorescence, relative to control. *** $p < 0.001$ significant compared to control. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ significant compared to A23187

A23187 as an agonist. As anticipated, compound 4a showed significant inhibition of MPTP formation in A23187-treated platelets in a concentration-dependent manner (Fig. 4c). Further, compound 4a inhibited A23187-induced cytochrome c release from mitochondria to cytosol as demonstrated by immunoblot (Fig. 4d).

Inhibition of caspase activity and PS externalization by compound 4a

Caspases are critical mediators of apoptosis by their function in hydrolyzing many of the cellular substrates. Two specific fluorescent substrates AC-LEHD-AFC and AC-DEVD-AMC were used in order to determine the activities of caspase-9 and caspase-3, respectively. Treatment with compound 4a, dose dependently inhibited A23187-induced caspase-3 and caspase-9 activity (Fig. 5a, b). Inhibition of caspase-3 was confirmed by Western blot analysis wherein, there was significant reduction in the cleaved form of caspase-3 in platelets treated with compound 4a was observed (Fig. 4d). Further, PS externalization, which is considered as the hallmark of apoptosis was also evaluated in compound 4a-treated platelets. Platelets treated with A23187 showed significant increase in PS externalization. However, treatment with compound 4a showed concentration-dependent inhibition of PS externalization, and significant inhibition was found at 50 μM concentration of compound 4a (Fig. 5c).

Effect of compound 4a on G6PDH, GGT activity, and GSH/GSSG ratio

G6PDH activity is essential in restoring NADPH levels in cells and during oxidative stress its activity is observed to be decreased. Further, during oxidative stress, expression and activity of GGT is observed to be increased. Therefore, effect of compound 4a was evaluated for G6PDH and GGT activity in A23187-treated platelets. There was significantly decreased G6PDH, and increased GGT activity in platelets treated with A23187. However, compound 4a concentration dependently restored basal levels of both G6PDH and GGT in A23187-treated platelets (Fig. 6a, b). Further, pre-loaded platelets with agonist showed depleted GSH/GSSG level, while treatment with compound 4a restored levels of GSH/GSSG as compared to untreated platelets (Fig. 6c). The obtained results clearly demonstrate that compound 4a inhibited A23187-induced platelet apoptosis by mitigating oxidative stress.

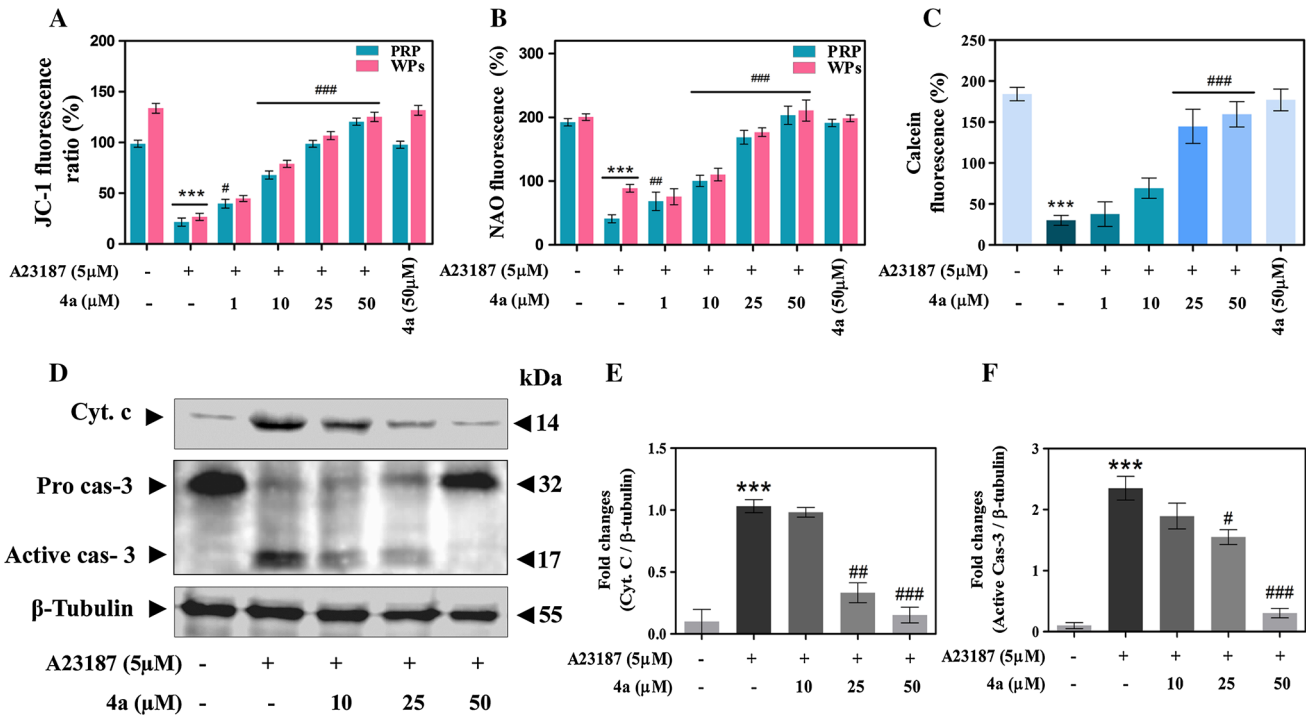


Fig. 4 Effect of compound 4a on A23187-induced **a** mitochondrial membrane depolarization **b** peroxidation of cardiolipin in PRP/washed platelets, **c** mitochondrial permeability transition pore (MPTP) formation in washed platelets, and **d** immunoblot showing the expression level of cytosolic cytochrome c and active caspase-3. Representative densitograms of immunoblots of **e** cytosolic cytochrome c and **f** active caspase-3. β-tubulin is used as loading control. Values are presented as mean ± SEM ($n = 5$), expressed as percentage decrease in JC-1 fluorescence ratio, NAO fluorescence, and calcein fluorescence, relative to control. *** $p < 0.001$ significant compared to control. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ significant compared to A23187

Platelet protective effect of compound 4a

In order to determine the cytoprotective nature of compound 4a, LDH leakage was measured. Treatment of platelets with A23187, induced the release of LDH into medium whereas, compound 4a was able to significantly inhibit LDH release owing its cytoprotective property (Fig. 7a). Further, lysosomal alkalization assay was performed as healthy cells show intact lysosomes and during apoptosis, alkalization of lysosomes occurs. Compound 4a treatment showed concentration-dependent inhibition of lysosomal alkalization (Fig. 7b). Similarly in order to determine viability of platelets, MTT assay was performed. Treatment of platelets with A23187 (5 μM) significantly reduced the viability up to 30.58 %. However, treatment of platelets with 4a restored the viability in a concentration-dependent manner, and at 50 μM, 66.9 % viable platelets were observed (Fig. 7c).

Effect of compound 4a on collagen-induced platelet aggregation

Collagen was used as an agonist to stimulate platelet aggregation. Pre-treatment of PRP with compound 4a at different concentrations prior to collagen treatment resulted in a dose-dependent inhibition of platelet aggregation. At a concentration of 50 μM, compound 4a was able to inhibit platelet aggregation by 99 %, (Fig. 8a, b). However, compound 4a alone did not induce platelet aggregation.

Discussion

Thrombocytopenia is a major life threatening clinical condition, in which a victim's blood has an unusually low level of platelets around 50,000/μL of blood beyond the normal count, which is 1.5×10^5 to 4×10^5 platelets/μL of blood. The contributing factors for the development of thrombocytopenia are several leading to multiple formats of health issues which is often a neglected issue in the treatment regime [41–43]. Our continuing efforts in developing new synthetic routes for the synthesis of various bioactive heterocycles [44–46], prompted us to develop a simple approach to produce structurally diversified 3, 5-disubstituted isoxazole-4-hydrazide derivatives through adaptable step-wise reactions. Isoxazole is the basic structure in numerous pharmaceutical drugs including Cloxacillin and Valdecoxib which are found in wide

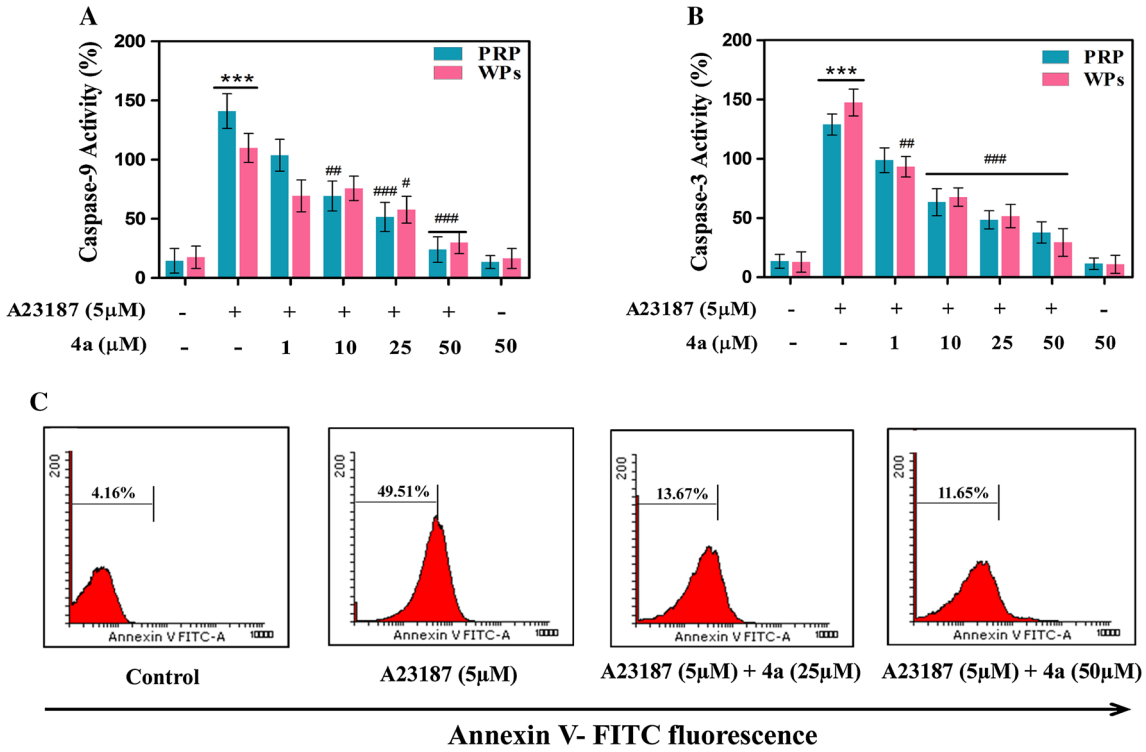


Fig. 5 Effect of compound 4a on A23187 induced **a** caspase-9, **b** caspase-3 activity in PRP/washed platelets, and **c** FACS analysis of PS externalization in washed platelets. Values are presented as mean ± SEM ($n = 5$), expressed as percentage increase in (**a**, **b**) caspase activity relative to control. *** $p < 0.001$ significant compared to control. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ significant compared to A23187

variety of natural products such as ibotenic acid. In addition, isoxazole substituted chromanes are previously reported to protect from stress-induced neuronal damage [47].

Based on the above observations and biological activities of isoxazole scaffolds, we have synthesized 3,4,5 trisubstitued isoxazole derivatives (4a-h) and screened for their radical scavenging ability. Among the derivatives, compound 4a was found to be potent antioxidant as demonstrated by DPPH radical scavenging and ferric reducing assays. Therefore, compound 4a was selected to determine its platelet protective potential during oxidative stress. In many pathological conditions, the production of ROS is the hallmark feature in oxidative stress-induced cell death [48]. In addition, alteration in Ca^{2+} ion transport mechanism brings the change in the second messenger system leading to mitochondrial membrane depolarization, caspase activation, PS externalization, and finally increased platelet destruction [49]. In parallel, increased Ca^{2+} concentration results in the phosphorylation of eukaryotic initiation factor 2 alpha (eIF2-α), which is a hallmark of ER stress [50], cell under ER stress undergoes apoptosis mediated by caspase-12 and caspase-3. As platelets lack caspase-12, analysis of caspase-12 was eliminated from the study. Therefore, in the present study, A23187 (5 μM) was used as an agonist to induce the ROS production and to increasing intracellular Ca^{2+} level in platelets. Compound 4a was able to alleviate ROS and restore calcium homeostasis with subsequent inhibition of eIF2-α phosphorylation there by reducing ER stress.

It is a well-known fact that GSH is a ubiquitous master antioxidant in all cells, decreased reduced GSH and increased oxidized GSH (GSSG) content seems to be an obvious situation in increased ROS level in the platelets. Alteration in GSH/GSSG homeostasis during oxidative stress leads to the elevated activity of certain enzymes for example, GGT, which is required to metabolize GSH allowing the precursor amino acids to be reused for intracellular GSH synthesis. Compound 4a which had scavenged ROS in platelets also showed restored GSH/GSSG ratio and GGT activity.

Unremitting oxidative stress and increased calcium levels also affect mitochondria by depolarizing the membrane potential and peroxidizing the cardiolipin [51]. Further peroxidized cardiolipin aids in the formation of MPTP, through which cytochrome c is released into cytosol. Release of cytochrome c into the cytosol serves a regulatory function as it precedes morphological alterations associated with apoptosis. The released cytochrome c binds with apoptotic protease activating factor-1 and ATP, which

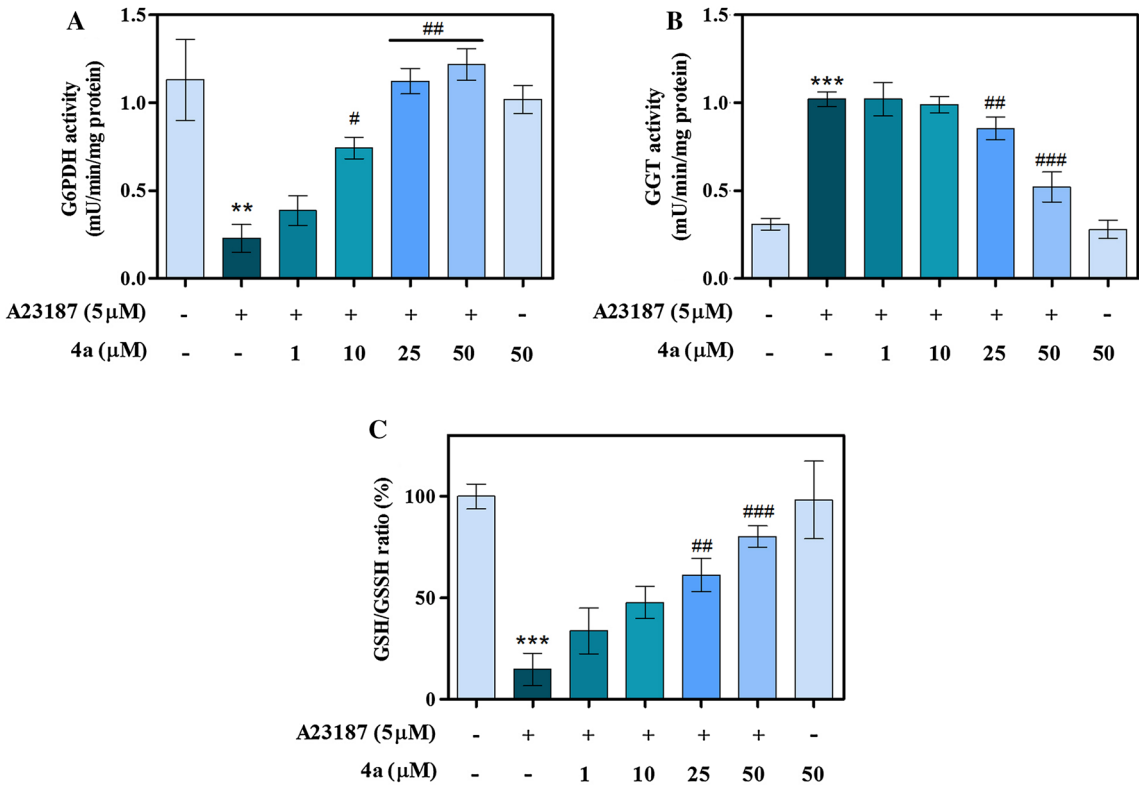


Fig. 6 Effect of compound 4a on A23187 induced **a** glucose-6-phosphate dehydrogenase (G6PDH) activity, **b** γ-glutamyltransferase (GGT) activity, and **c** GSH/GSSG ratio. Values are presented as mean ± SEM ($n = 5$). *** $p < 0.001$ significant compared to control. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ significant compared to A23187

then binds to pro-caspase-9 to create a protein complex known as apoptosome. The apoptosome cleaves the pro-caspase-9 to its active form, which in turn activates the effector caspase-3. Hence, cytochrome c release can be regarded as a crucial event in the intrinsic pathway of apoptosis [52]. Caspases are the cysteine-dependent proteases that cleave proteins at aspartic acid residues and play a crucial role in apoptosis. Generally, caspases are expressed in an inactive pro-enzyme form as pro-caspases, during apoptosis caspases gets activated and inturn activates other pro-caspases, allowing initiation of a protease cascade. This proteolytic cascade amplifies the apoptotic signaling pathway and thus ends up in PS externalization. The expression of PS results in the early phagocytic recognition of apoptotic cells, permitting quick phagocytosis.

Considering these facts, compound 4a was evaluated for its abrogation of oxidative stress-induced downstream apoptotic markers. The compound 4a effectively inhibited the A23187-induced membrane potential depolarization, cardiolipin peroxidation, MPTP formation, cytochrome c release, caspase activation, and PS externalization. Thus, we demonstrate that compound 4a could effectively block oxidative stress-induced mitochondrial damage and subsequent caspase activation in platelets and rescued from their premature death. Further, decreased levels of LDH release and increased cell viabilty in A23187 treated platelets confirms the cytoprotective effect of compound 4a on platelets.

To further highlight compound 4a role as a cardio protective compound, its influence on platelet aggregation was scrutinized. Platelets are considered to play a vital role in the progression of atherothrombotic diseases. During vascular injury, they lead up the hemostasis plug formation and mediate thrombosis, which in turn promulgates a multiplicity of CVDs. Platelet aggregation and adhesion are engaged in the instigation of intraluminal thrombosis and thus hasten heart attack, peripheral vascular occlusions, and stroke [53]. Compound 4a by inhibiting collagen-stimulated platelets aggregation demonstrated its ability as an anti-platelet molecule.

Considering today's life style which includes frequent administration of medications, it is not surprising that DIT is an increasingly major cause of early platelet death [13]. Along with DIT, platelets are stimulated by various agonist including thrombin, von Willebrand factor, anti-platelet antibodies, and intrinsic oxidative stress, which eventually leads to the generation of platelet-derived microparticles. The released microparticles worsen the condition by triggering inflammatory cells for the production of cytokines,

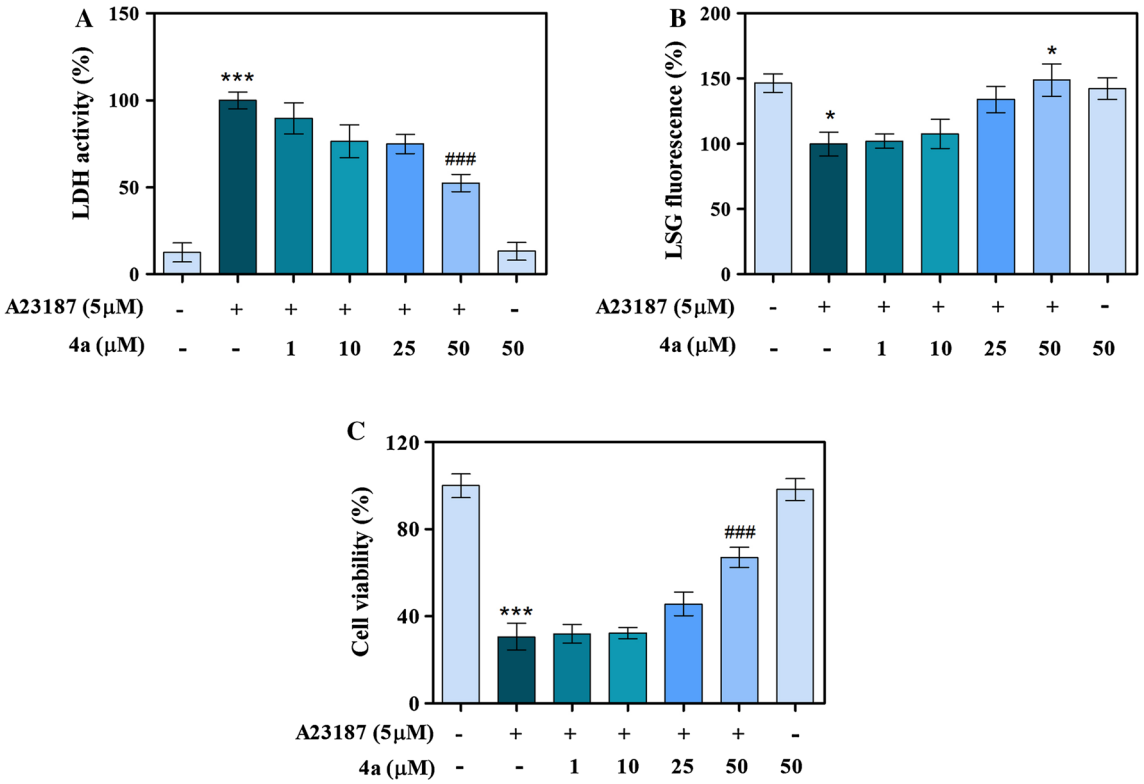


Fig. 7 Effect of compound 4a on A23187 induced cytotoxicity in platelets: **a** LDH leakage, **b** lysosomal stability, and **c** MTT cell viability assay. Values are presented as mean ± SEM ($n = 5$), expressed as percentage decrease in LSG fluorescence relative to control. *** $p < 0.001$ significant compared to control. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ significant compared to A23187

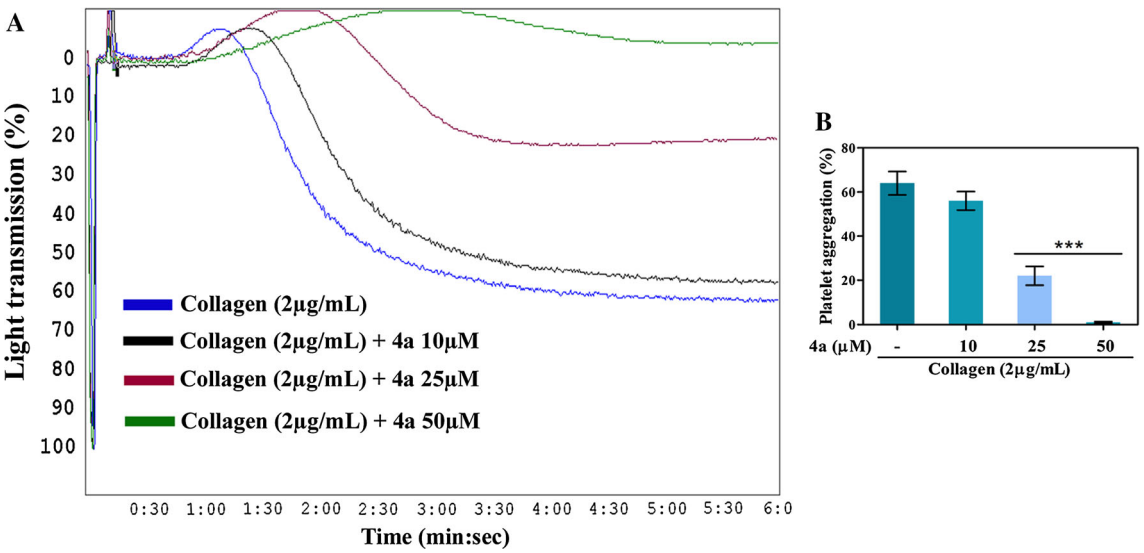


Fig. 8 Effect of compound 4a on **a** platelet aggregation induced by collagen and its inhibition by compound 4a, **b** graphical representation of the data showing the percentage platelet aggregation. Values are presented as mean ± SEM ($n = 5$), expressed as percentage decrease in platelet aggregation. ** $p < 0.01$, *** $p < 0.001$ significant compared to collagen

elevating leukocytes adhesion to endothelial cells and thereby altering vascular and endothelial function which contributes in the development of CVD's [54]. In this scenario, there is a need and scope for novel synthetic molecules, which can protect platelets from oxidative stress-induced cell death. In addition, it could receive

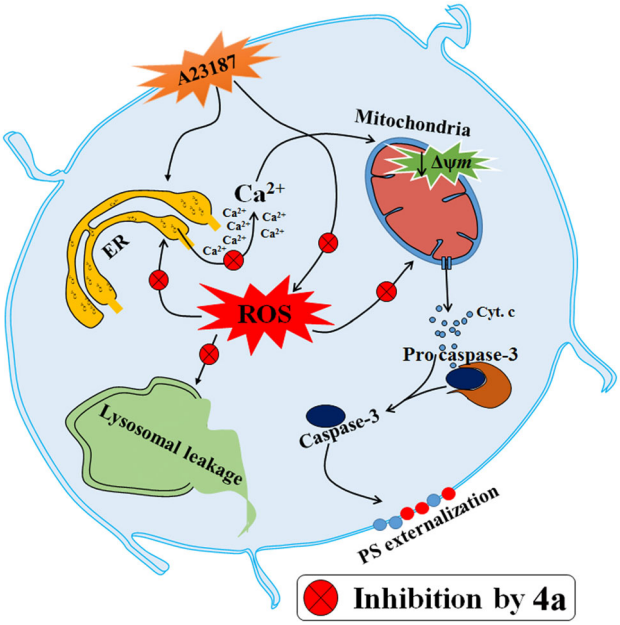


Fig. 9 Schematic representation of protective mechanism of 4a in A23187-induced platelet apoptosis

greater attention by the clinicians in order to treat thrombocytopenia, which is a commonly occurring secondary complication in the chemotherapy treatments. From the current study, out of a series of novel 3,4,5 trisubstituted isoxazole derivatives (4a-h), compound 4a was found to be potent molecule with platelet protective efficacy by inhibiting collagen-induced platelet aggregation and A23187-induced apoptosis (Fig. 9).

Conclusion

In conclusion, it can be summarized that the compound 4a is a promising molecule, which could be used in the treatment of oxidative stress-induced thrombocytopenia and associated pathologies. Altogether, the inhibitory property of compound 4a on platelet apoptosis and aggregation could be employed in the treatment strategy of vascular pathophysiology and CVDs. Therefore, compound 4a could be a potential candidate in the field of vascular pharmacology and hemostasis. Together, compound 4a has a scope to be tested in vivo models and clinical trials for its anti-platelet properties before its development as a new drug to treat thrombocytopenia on the bed side.

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Compliance with ethical standards

Conflict of interest The authors have declared that there is no conflict of interest.

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