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Research paper

Synthesis and evaluation of dual antiplatelet activity of bispidine derivatives of N-substituted pyroglutamic acids

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ABSTRACT

N-aralkylpyroglutamides of substituted bispidine were prepared and evaluated for their ability to inhibit collagen induced platelet aggregation, both *in vivo* and *in vitro*. Some compounds showed high anti-platelet efficacy (*in vitro*) of which six inhibited both collagen as well as U46619 induced platelet aggregation with concentration dependent anti-platelet efficacy through dual mechanism. In particular, the compound **4j** offered significant protection against collagen epinephrine induced pulmonary thromboembolism as well as ferric chloride induced arterial thrombosis, without affecting bleeding tendency in mice. Therefore, the present study suggests that the compound **4j** displays a remarkable antithrombotic efficacy much better than aspirin and clopidogrel.

1. Introduction

Thrombotic disorders are an important cause of morbidity and mortality not only in the developed world but also in developing countries [1]. Platelets play a crucial role in haemostasis and thrombosis [2–6]. Selective inhibition of the pathways most relevant to the pathological aspects of atherothrombosis can be a logical approach for therapeutic interventions [2–6]. The prevalent anti-platelet therapy regimens that include aspirin and clopidogrel are fraught with increased bleeding risk implying thus the need for drugs with improved safety margins. A great deal of insight has been gained into the contribution of collagen, thromboxane A_2 (TxA_2) and their respective receptors & signaling mechanisms in promoting platelet adhesion, activation and subsequent thrombus growth and stability. Hence, targeting against the synergy between collagen and TxA_2 mediated platelet activation pathway could prove to be useful in terms of improving the outcome of high intensity antithrombotic therapy.

Building upon the previous findings of nipecotamide derivatives as inhibitors of platelet aggregation induced by ADP [7], collagen [8], thrombin [9], epinephrine [10] and stable TxA2 mimetics [11], we have recently reported *tert*-butyl ((*S*)-1-((*S*)-1-(4-methylbenzyl)-5-oxopyrrolidine-2-carbonyl) piperidin-3-yl)methylcarbamate **1** as a potent inhibitor of collagen induced platelet aggregation with very potent protection against *in vivo* pulmonary thromboembolism [12]. Moreover, the N-substituted oxazolidine and thiazolidine carboxamides exhibited inhibition of platelet aggregation by dual mechanism, collagen and thromboxane, but they significantly enhanced bleeding tendency by 50–70% [12]. Watson et al. have recently reported N-substituted bispidine (**2**) as a potent selective factor Xa inhibitor with a good anticoagulant activity [14]. Further, substituted bispidines [13] (7-diazabicyclo[3.3.1]nonane) (**3**) were reported to be useful in the treatment of cardiac arrhythmias [15]. In the present communication, we describe our work to introduce conformational rigidity on to the 3-aminomethylpiperidine substructure of **1** by its replacement with substituted bispidine, leading to synthesis of N-pyroglutamoylbispidines (**4**) wherein apart from collagen, we observe an additional

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inhibition of thromboxane A_2 induced platelet aggregation and thrombus formation, but without any evident impact on normal hemostasis or bleeding Fig. 1.

2. Chemistry

As reported previously N-alkylation of methyl (2*S*)-pyroglutamate (**5**) with various arylalkylhalides using LiHMDS [16] and subsequent hydrolysis of the ester group gave the corresponding N-substituted pyroglutamic acids **6**(*a-j*) [12].

3-(*t*-butyloxycarbonyl)-7-benzyl-3,7-diazabicyclo [3.3.1] nonane (**8**) was prepared using reported procedure [17]. The Boc group on **8** was removed using TFA in DCM to give **9**, while **10** was prepared by the N-debenzylation of **8** by catalytic hydrogenation over 20% $Pd(OH)_2$ – charcoal and the resulting amines were condensed with various N-substituted pyroglutamic acids **6**(***a-j***) using one-pot DCC-HOBt methodology to yield the corresponding pyroglutamoylbispidines **4**(***a-p***). Boc protection in pyroglutamoylbispidines ***4b*** and ***4c*** was removed and the resulting amine was converted to corresponding (N)-acyl, sulfonyl and substituted benzyl derivatives **4**(***q-w***) as shown in Scheme 1.

3. Biological results

All the synthesized compounds were primarily evaluated in a murine model of collagen-epinephrine induced pulmonary thromboembolism (*in vivo*) as well as collagen induced platelet aggregation (*in vitro*) to assess their antithrombotic efficacy. The compound 4j was found to be most efficacious in inhibiting collagen and U46619 induced platelet aggregation, and was further studied in depth.

3.1. Collagen epinephrine induced pulmonary thromboembolism

After 1 h of oral administration in mice, it was observed that 13 out of 23 compounds exhibited ~40–60% protection against collagen epinephrine induced thrombotic challenge at a dose of 30 μmol/kg (Table 1). Compounds with N-protected *tert*-butyloxycarbonyl (***4b***), benzyl (***4j***), benzoyl (***4q***) and tosyl (***4s***) substitution at the bicyclic end and with a bromine atom at the ortho position of phenyl ring were found to be very potent molecules, *in vivo*, with 50%, 45%, 40%, 60% and 55% protection respectively, while compound ***4d*** and ***4v*** exhibited 55% and 50% protection respectively in the same model (Table 1).

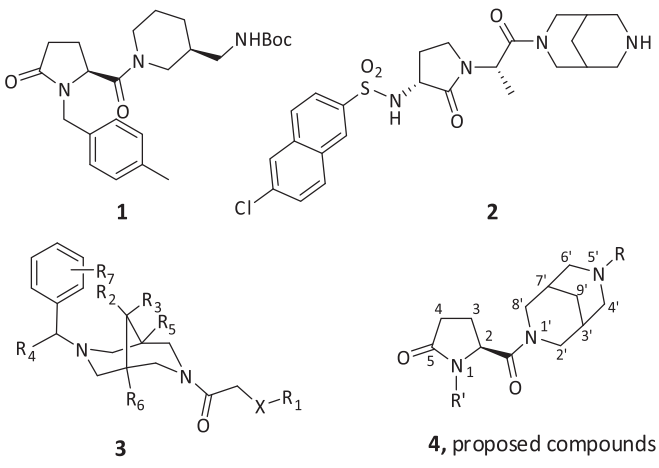


Fig. 1. Aminomethylpiperidine (**1**) and bispidine derivatives reported as anticoagulants (**2**) and for the treatment of cardiac arrhythmias (**3**); Structure of proposed prototype (**4**).

3.2. Platelet aggregation assay

Compounds were assessed for their inhibitory potential against platelet aggregation in order to identify their probable mechanism of antithrombotic action. Seven compounds were found to inhibit collagen induced platelet aggregation *in vitro*. In particular compounds ***4j*** (86 ± 3%), ***4l*** (68 ± 6%), ***4m*** (52 ± 8%), ***4o*** (57 ± 11%), ***4u*** (67 ± 10%), ***4v*** (61 ± 8%) and ***4w*** (67 ± 8%) exhibited significant anti-platelet efficacy at a concentration of 30 μM (Table 1).

3.3. Dual activity of compounds

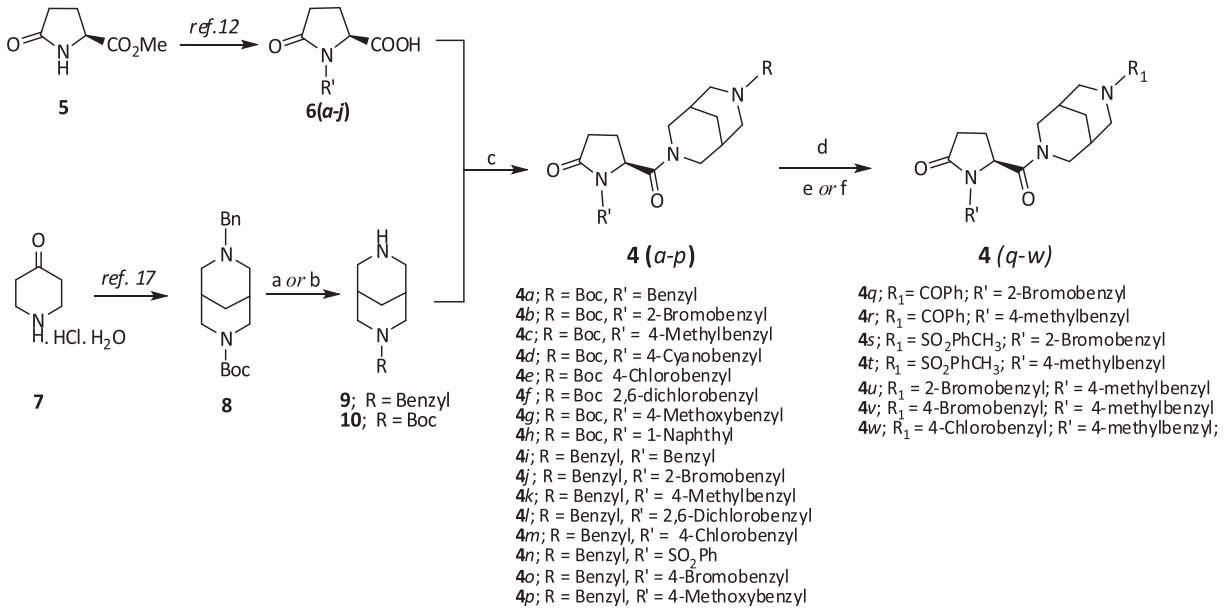
Further platelet aggregation studies showed that 6 of the compounds (***4j***, ***4l***, ***4m***, ***4u***, ***4v*** and ***4w***) inhibited both collagen as well as U46619 (thromboxane receptor agonist) induced platelet aggregation, thereby exhibiting dual mechanism of action (Table 1). The compound ***4j*** was observed to be the most potent among the entire series and exhibited an IC_{50} of 1.7 μM against collagen and 7.7 μM against U46619 induced platelet aggregation (Fig. 2ab). The compound ***4j***, even up to 300 μM, did not exhibit any significant inhibition against other platelet agonists like ADP, thrombin mimetic SFLLRN (TRAP), GPVI specific collagen related peptide (CRP-XL) and GP 1b-IX-V agonist Ristocetin induced platelet aggregation (Fig. 2c). It did not exhibit any inhibition of COX pathway via arachidonic acid induced platelet aggregation, but at higher concentration (300 μM and 500 μM) the compound ***4j*** attenuated platelet aggregation up to 50%. These findings indicate that the compound ***4j*** might exhibit its anti-platelet efficacy through dual mechanism, and hence warrants further confirmation regarding its mechanism of action.

3.4. Effect of 4j on pulmonary thromboembolism and bleeding time in mice

Interestingly the antithrombotic efficacy of ***4j*** was found to increase from 40% after 1 h to 60% after 4 h of oral administration (Fig. 3a). In comparison, aspirin (170 μmol/kg) showed only 40% protection which was effective up to 5 h. Clopidogrel, after 1 h of dosing, exhibited 70% protection which declined to 50% after 4 h and became almost similar to that of ***4j***. Moreover, both aspirin and clopidogrel exhibited significant increase in bleeding time in mice (Fig. 3b). However the bleeding time was not significantly altered from 1 h (3.4 ± 0.2 min) to 4 h (4.3 ± 0.1 min) in ***4j*** treated mice, and remained comparable to that of control mice (3.5 ± 0.3 min) but significantly less than aspirin (6.6 ± 0.2 min) and clopidogrel (9.0 ± 0.5 min) (Fig. 3b).

3.5. Effect of 4j on U46619 induced vasoconstriction

Results from the present study indicated that the maximal endothelial contraction evoked by U46619 was significantly reduced in the presence of ***4j*** at 30 μM concentration (14.2 ± 3.6%) and 15 μM (21.8 ± 3.8%) as compared to vehicle treated control (89.8 ± 8.5%) (Fig. 4a). Likewise pretreatment of TP receptor antagonist SQ29548 (8.4 ± 2.9%) significantly inhibited U46619 induced contraction. At lower concentrations (10 μM), U46619 shifted the concentration response curve towards right indicating competitive inhibition against U46619. At 5 μM and 3 μM concentration, ***4j*** did not show any significant effect. Moreover at the effective concentrations tested, ***4j*** did not exhibit any significant effect on KCl response (Fig. 4b).



Scheme 1. Reagents and conditions: (a) TFA, dry DCM, 4h; (b) $Pd(OH)_2$–C, H_2, methanol, 120 psi, 10h; (c) HOBt, DCC, dry DCM, 0^0C-RT, 3h; (d) TFA, dry DCM, 5h; (e) R_1-Cl, TEA, dry DCM, 2h; (f) R_1-Br, an. K_2CO_3, dry acetone, reflux, 4 h.

3.6. Effect of 4j on coagulation parameters

To assess the effect of **4j** on coagulability of blood, thrombin time (TT), prothrombin time (PT) and activated partial thromboplastin ime (aPTT) were assessed in the human plasma (*in vitro*). No change was observed in TT, PT and aPTT when compared to their isovolumetric vehicle control (Fig. 4c). Hirudin was used as the standard anticoagulant for reference and when compared to vehicle control, it significantly prolonged the thrombin time (>120sec vs 20.03 ± 0.5 s), prothrombin time (18.7 ± 0.6 s vs 14.3 ± 0.03sec) and activated partial thromboplastin time (84.3 ± 0.4 s vs 29.6 ± 2.2 s) (Fig. 4c).

3.7. Platelet adhesion over collagen

4j (0–300 μM) exhibited a concentration-dependent inhibition

Table 1
Effect of bispidine derivatives of N-substituted pyroglutamic acid, 4 (a-w), aspirin and clopidogrel on collagen-epinephrine induced pulmonary thromboembolism (mice, *in vivo*) and platelet aggregation induced by collagen and U46619 (human, *in vitro*). Results are expressed as % Protection (*in vivo*) and % Inhibition (*in vitro*).

No.	Compound	R/R_1	R′	Protection (%)[a]	Inhibition (%)[b,d]	Inhibition (%)[c,d]
1	**4*a***	Boc	Benzyl	40	06.00 ± 14.00	
2	**4*b***	Boc	2-Bromobenzyl	50	02.00 ± 12.00	
3	**4*c***	Boc	4-Methylbenzyl	40	25.00 ± 9.00	
4	**4*d***	Boc	4-Cyanobenzyl	55	11.00 ± 3.00	
5	**4*e***	Boc	4-Chlorobenzyl	30	06.00 ± 11.00	
6	**4*f***	Boc	2,6-dichlorobenzyl	30	03.00 ± 3.00	
7	**4*g***	Boc	4-Methoxybenzyl	45	10.00 ± 3.00	
8	**4*h***	Boc	1-Naphthyl	30	11.00 ± 4.00	
9	**4*i***	Benzyl	Benzyl	30	44.00 ± 13.00	
10	**4*j***	Benzyl	2-Bromobenzyl	40	86.00 ± 3.41	79 ± 09
11	**4*k***	Benzyl	4-Methylbenzyl	25	07.00 ± 7.00	
12	**4l**	Benzyl	2,6-Dichlorobenzyl	40	68.00 ± 6.00	85 ± 03
13	**4 m**	Benzyl	4-Chlorobenzyl	30	52.00 ± 8.00	80 ± 03
14	**4n**	Benzyl	SO_2Ph	30	29.00 ± 1.00	
15	**4*o***	Benzyl	4-Bromobenzyl	40	57.00 ± 11.00	
16	**4*p***	Benzyl	4-Methoxybenzyl	30	10.00 ± 4.00	
17	**4*q***	Benzoyl	2-Bromobenzyl	60	25.60 ± 3.55	
18	**4*r***	Benzoyl	4-Methylbenzyl	40	20.50 ± 7.50	
19	**4*s***	Tosyl	2-Bromobenzyl	55	16.80 ± 5.66	
20	**4*t***	Tosyl	4-Methylbenzyl	40	37.50 ± 10.50	
21	**4*u***	2-Bromobenzyl	4-Methylbenzyl	30	67.00 ± 10.00	70 ± 08
22	**4*v***	4-Bromobenzyl	4-Methylbenzyl	50	61.00 ± 8.00	52 ± 03
23	**4*w***	2-Chlorobenzyl	4-Methylbenzyl	35	67.00 ± 8.00	81 ± 05
	Aspirin			40 (170 μmol/kg)		
	Clopidogrel			70 (70 μmol/kg)		
	DMSO				25.31 ± 2.59	

[a] Collagen-epinephrine induced pulmonary thromboembolism in mice (*in vivo*).
[b] Inhibition of collagen induced platelet aggregation in human platelets (*in vitro*).
[c] Inhibition of U46619 induced platelet aggregation in human platelets (*in vitro*).
[d] Compound dose/concentration used: *in vivo* = 30 μmol/kg; *in vitro*: 30 μM; n = 3.

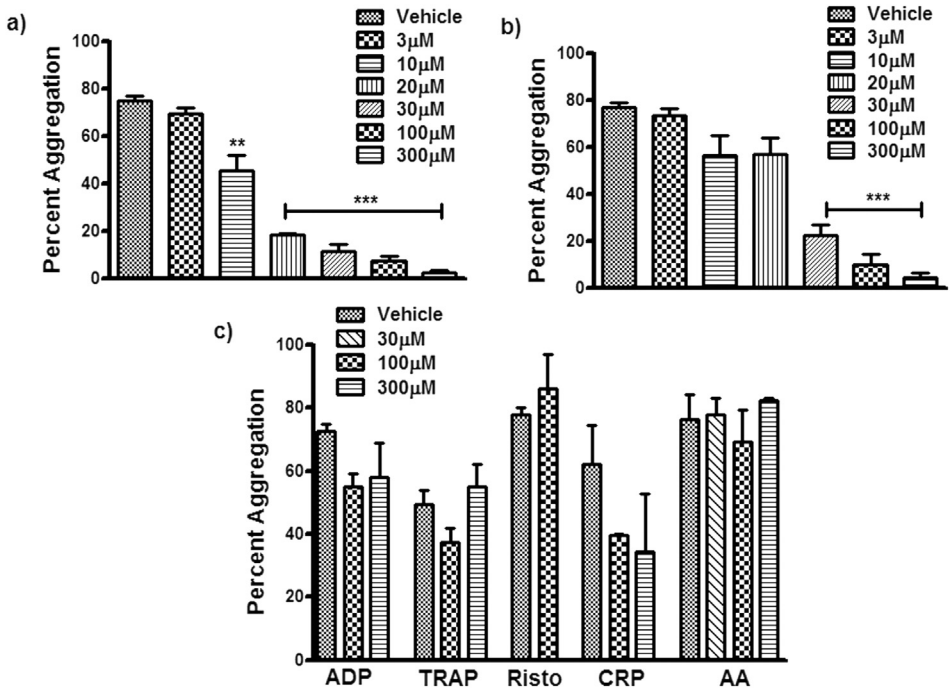


Fig. 2. Platelet aggregation assay (human, *in vitro*). Effect of compound 4j against (a) collagen; (b) U46619; (c) ADP, TRAP, Ristocetin, CRP-XL and arachidonic acid induced platelet aggregation in human platelet rich plasma. Results are expressed as Mean ± SEM (n = 3) ***P < 0.001, **P < 0.01, *P < 0.05 vs control.

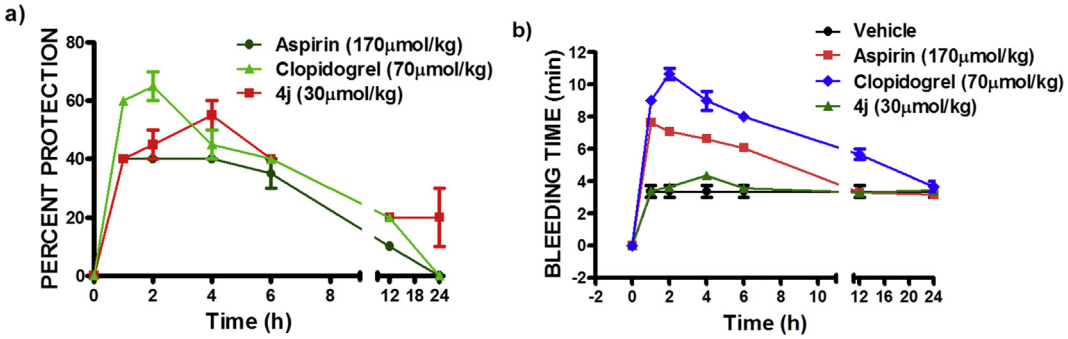


Fig. 3. Time dependent effect of 4j and standard antithrombotic drugs (aspirin and clopidogrel) after single dose administration on (a) collagen-epinephrine induced pulmonary thromboembolism; (b) bleeding time in mice. Results are expressed as Mean ± SEM (n = 5, 10 animals/group/experiment).

of platelet adhesion over collagen (Fig. 4d). Threshold inhibitory effects were evident at 30 μM (27 ± 8%), while maximal and almost complete abrogation of platelet adhesion occurred at 300 μM (Fig. 4d). The compound ***4j*** significantly inhibited both integrin dependent as well as integrin independent platelet adhesion over collagen in a concentration dependent manner.

3.8. Effect of compound 4j on collagen induced tyrosine phosphorylation

The activation of platelets by collagen or thromboxane receptor (TP) agonist U46619 contributes to the assembly and stabilization of various signaling complexes. Platelets pre-incubated with compound ***4j*** when stimulated with either collagen (Fig. 5a) or U46619 (Fig. 5b) showed reduced phosphorylation level of a number of proteins compared to the vehicle control. Further the effect of compound on phosphorylation of different proteins at tyrosine residues was found to be specific, since it exhibited a concentration dependent effect at various concentrations of compound ranging from 3 to 300 μM (Fig. 5a,b). The inhibitory effect was particularly evident on proteins of molecular weight around 120, 100, 70 and ~55–60 kDa. These findings suggest that ***4j*** probably obstructs both collagen and U46619 induced platelet activation thereby disrupting tyrosine kinase regulated signaling pathway.

3.9. Effect of 4j on ferric chloride induced arterial thrombosis

The efficacy of ***4j*** to combat pathologic and occlusive thrombus *in vivo* was further assessed in another robust model of ferric chloride induced arterial thrombosis in mice (Fig. 6). The injured carotid artery upon exposure to 10% $FeCl_3$ was occluded within 9.5 ± 0.4 min. The compound ***4j,*** 4 h after oral administration, significantly prolonged the time to occlusion (TTO) upto 13.3 ± 0.5 min and 20 ± 0.8 min at doses of 20 and 30 μmol/kg respectively (P < 0.01 vs control) (Fig. 6). The standard anti-platelet drug aspirin remained ineffective in this model even at 170 μmol/kg, while clopidogrel increased the TTO up to 23 ± 0.9 min at 70 μmol/kg (Fig. 6). The efficacy elicited in this model further substantiates the anti-thrombotic potential of this compound as the ***4j*** treated groups exhibited significant prolongation of artery occlusion time by 1.4 and 2.1 fold at 20 and 30 μmol/kg, respectively.

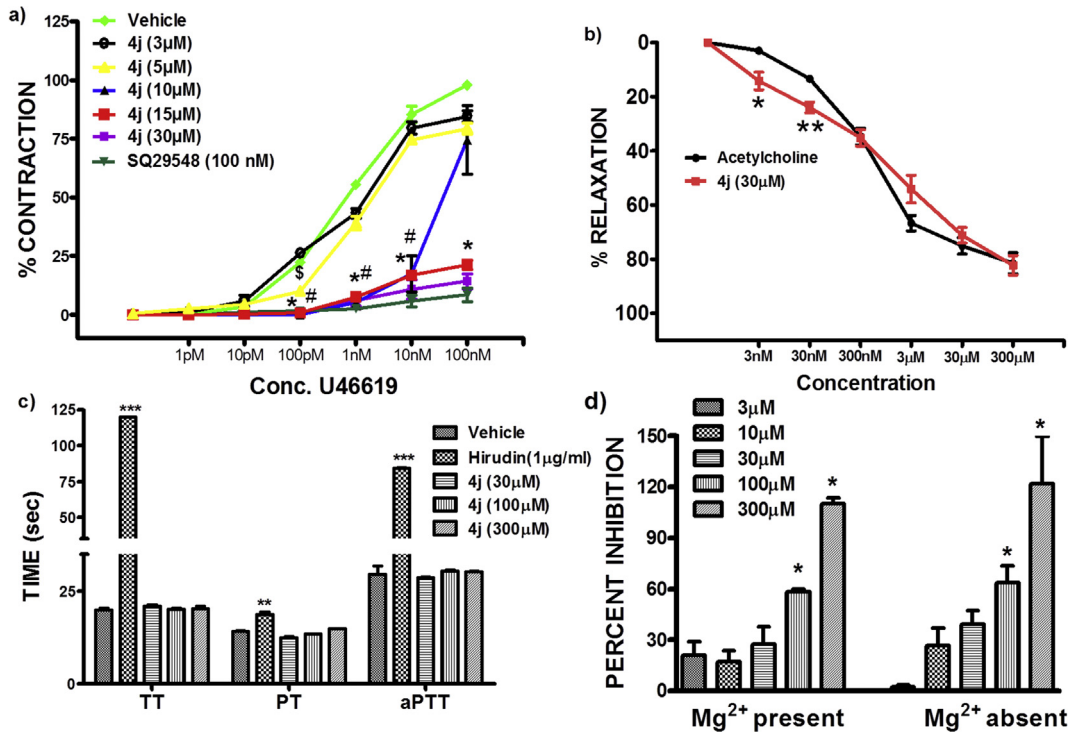


Fig. 4. Effect of 4j or TP receptor antagonist SQ29548 (100 nm) on (a) U46619 induced vasoconstriction (b) Acetylcholine induced vasorelaxation in rat aortic rings *in vitro*. Data shown as Mean + SEM (n = 5) *P < 0.05 [control vs 4j (15 μM, 30 μM and SQ 100 nM)]; #P < 0.001 [control vs 4j (10 μ M)]; $ P < 0.01 [(control vs 4j (5 μM)]. (c) Effect of compound 4j or Hirudin on (c) Thrombin time (TT), Prothrombin Time (PT) and activated partial thromboplastin time (aPTT) in human platelet poor plasma. Hirudin (1 μg/ml) was used as standard anticoagulant and 0.4% DMSO was used as vehicle. (d) Effect of compound 4j on human platelet adhesion on collagen coated surface (*in vitro*) in presence or absence of Mg^{+2} ions. Data shown as Mean + SEM. ***p < 0.001, **p < 0.01, *p < 0.05 vs control. vs control.

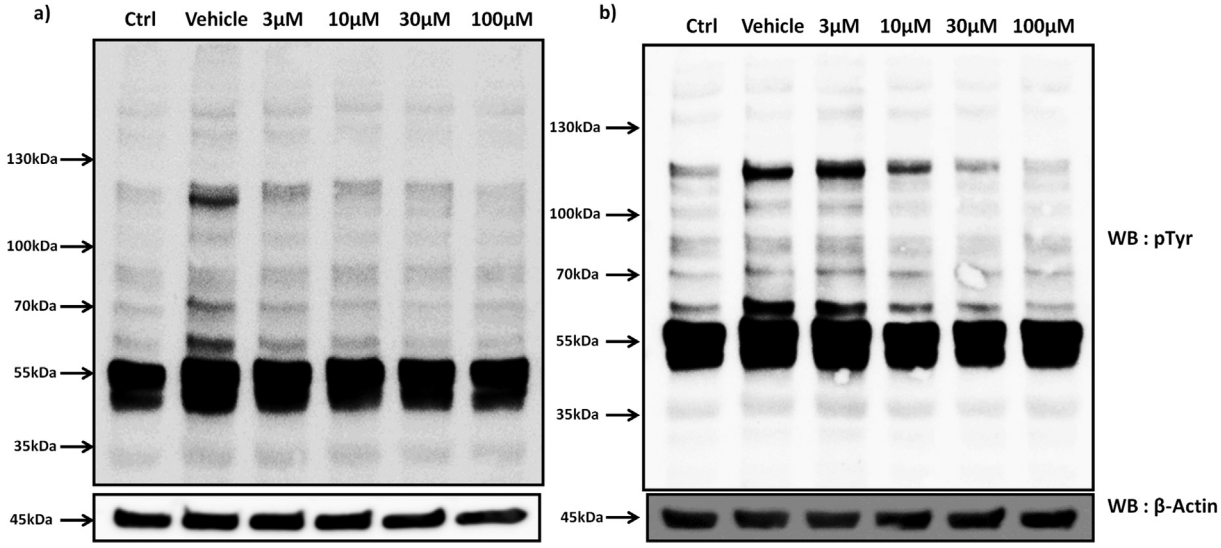


Fig. 5. Effect of compound 4j at different concentrations on tyrosine phosphorylation of platelet proteins following stimulation with (a) collagen, (b) U46619. Washed platelets pre-incubated at 37 °C with vehicle (0.4% DMSO) or various concentrations of compound 4j (3, 10, 30, 100 μM) for 5 min, and stimulated with either U46619 (0.25 μg/ml) or collagen (1 μg/ml). Blots are representative of three separate experiments.

4. Discussion

Twenty three pyroglutamide derivatives were synthesized and assessed for their anti-thrombotic activity against collagen-epinephrine induced pulmonary thromboembolism in mice as well as collagen induced platelet aggregation *in vitro*. However, in the collagen induced platelet aggregation assay only a moderate inhibition was observed at 30 μM with no apparent SAR. The in-vivo assay in pulmonary thromboembolism assay 12 compounds displayed 40–60% protection (asprin, 40% protection) with again no clear correlation hydro which Among active derivatives, ***4j*** exhibited significant inhibition against collagen induced platelet aggregation (86.00 ± 3.41%), and ~1.5 fold more antithrombotic protection than aspirin *in vivo*. Moreover, when compared to both

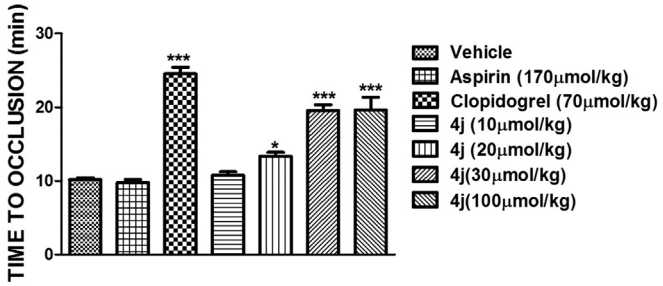


Fig. 6. Effect of compound 4j, aspirin and clopidogrel on total time to occlusion (TTO) in ferric chloride induced arterial thrombosis in mice (n = 6). Data shown as Mean + SEM. ***p < 0.001, **p < 0.01, *p < 0.05 vs control.

aspirin and clopidogrel, no significant effect of **4j** on bleeding time in mice implicated that **4j** might offer a new concept to develop novel anti-thrombotic, which may not only inhibit arterial thrombosis but may also escape the adverse effects of bleeding, a potentially disturbing issue that limits the clinical application of presently available antithrombotic drugs.

During arterial thrombosis, the cross-talk between endothelial dysfunction, platelet aggregation, and activation of the coagulation system plays a major role. Platelet adhesion on subendothelial collagen followed by aggregation at sites of vascular injury is recognized as critical aspect for hemostatic plug formation and thrombosis. Therefore, the effect of compound **4j** was assessed on all the three parameters to delineate its mechanism of action. The pyroglutamide derivative **4j** inhibited platelet aggregation induced by collagen and U46619, a thromboxane mimetic but exhibited no effect against ADP-, TRAP, arachidonic acid, CRP-XL- or Ristocetin-induced platelet aggregation. In fact the compound **4j** preferentially inhibited collagen-induced platelet aggregation as compared to that induced by U46619 (IC50, 1.7 vs. 7.7 μM, respectively). Since aspirin is already a clinically proven anti-platelet drug, well-recognized for its inhibitory effect on COX-1 and the subsequent production of thromboxane A_2, hence these compounds having a relatively potent efficacy to inhibit both collagen as well as thromboxane A_2 mediated platelet activation could serve as very useful antithrombotic agent with a distinct mechanism of action.

At the site of vascular injury, platelets adhere over subendothelial collagen and trigger thrombus formation. Thus blocking this initial step to prevent arterial thrombosis may be of critical therapeutic significance [23]. The present study shows that **4j** attenuated platelet adhesion over collagen surface in both integrin dependent and independent manner as distinguished by the presence of Mg^{+2} ions. However, the anti-platelet effect of **4j** against U46619 induced platelet aggregation further led us to speculate that the protection conferred by **4j** might be due to the integrated blockage of collagen and thromboxane A_2 signaling machinery. **4j** exhibited selective vasorelaxation and restoration of U46619 induced endothelial contraction. This further substantiates that **4j** might be producing its effect by inhibiting platelet collagen receptor and TP receptor. The antithrombotic effect of **4j** is platelet specific, since its presence did not alter the coagulability of blood as assessed by thrombin time, prothrombin time and activated partial thromboplastin time in human plasma.

Collagen activates platelets through a tyrosine kinase based signaling pathway and sequential activation of PLCγ2, leading to intracellular Ca^{2+} mobilization, as well as the release of thromboxane A2, which through autocrine and paracrine manner further amplifies the platelet activation response [23]. Hence, a well-established consequence of collagen and thromboxane induced platelet activation is assembly of numerous downstream kinases, including tyrosine and MAP kinases. The compound **4j** substantially inhibited both collagen and U46619-induced protein tyrosine phosphorylation, thereby indicating its participation in switching off critical signaling mechanisms during platelet activation.

Further investigation pertaining to the evaluation of antithrombotic as well as anti-platelet efficacy of **4j** was conducted in an experimental murine model of $FeCl_3$-induced arterial thrombosis. It is noteworthy that thrombus formation is inhibited by the anti-platelet drug clopidogrel while aspirin was observed to be devoid of any significant antithrombotic activity in this model. A similar lack of activity of aspirin has earlier been described in a rat model [24]. Thus **4j** significantly attenuated arterial thrombosis, with mild impact on bleeding time.

The present study therefore demonstrates that **4j**, a pyroglutamide derivative, possesses the ability to inhibit both thromboxane A_2 and collagen induced platelet activation and signaling. It does not affect coagulation cascade, and also exhibits *in vivo* protection against thrombus formation with little effect on hemostasis, suggesting that it might be a potential candidate for development as an antithrombotic agent.

5. Conclusion

Bispidine derivatives of N-arylalkyl pyroglutamides reported herein showed promising inhibitory effects on platelet activation, both *in vivo* and *in vitro.* However, from the data it is apparent that the platelet aggregation inhibition data for the compounds is not amenable to any SAR. This might be due to poor solubility of the compounds in the test medium. In an in-vivo collagen-epinephrine induced pulmonary thromboembolism assay, compounds **4a-4w** (Table 1) showed 25 to 60% protection. Among the tested compounds, six compounds exhibited significant anti-platelet effect, via dual action against both collagen as well as U46619 induced platelet activation, *in vitro.* The compound **4j** was found to exhibit significant antithrombotic efficacy and also escapes the unfavorable events of bleeding risk in mice model when compared to existing anti-platelet drugs aspirin and clopidogrel. The action of compound **4j** is platelet specific, since its presence did not alter the coagulability of blood as assessed by TT, PT and aPTT in human plasma. Moreover, the efficacy elicited in $FeCl_3$ induced thrombosis further substantiates the anti-thrombotic potential of this compound. These results indicate that substitution of N6 of 3,6-diaza-bicyclo [3.1.1]heptane-3-carboxyl moiety with groups (R/R1) which can lower the basicity/nucleophilicity are desirable for improved protection. All compounds, except **4q**, followed this trend. Also, an R′ substitution which can enhance electronegative character of the γ-lactam's nitrogen is advantageous for the activity.

6. Experimental section

6.1. Chemistry

Reagents and other chemicals were used as purchased without further purification. All reactions with moisture/air sensitive reactants and solvents were carried out under nitrogen atmosphere using flame dried apparatus and all the solvents were distilled prior to use. THF was distilled under N_2 over potassium benzophenoneketyl radical prior to use. Dichloromethane was dried over P_2O_5 and methanol using magnesium cake. All the compounds were vacuum dried in Abderhalden. Reactions were monitored by thin layer chromatography over pre-coated silica gel plates ($60F_{254}$, E. Merck, Germany), using UV, iodine vapour, acidic and basic $KMnO_4$ or Dragendorff's reagent spray as the developing agents. Chromatographic separations were performed on flash column using silica gel (60–120 and 230–400 mesh). All melting points were taken in open tubes and are uncorrected. IR spectra were recorded

on a Perkin-Elmer 881 and FTIR 8201 PC Shimadzu spectrophotometers and values are expressed in cm^{-1}. ^1H NMR and ^{13}C NMR spectra were recorded on Bruker WM-200, WM-300 or WM-400 Spectrometer. The chemical shifts are expressed in δ using TMS as internal standard. Mass spectra were recorded on a JEOL JMS-D-3000 spectrometer with an ionization potential of ~70 eV and FAB on SX 102 instrument.

6.1.1. General synthesis of (2S)–N-arylalkyl-pyroglutamic acids [**6**(a-j)]

The compounds were prepared via following the same procedure using various substituted benzyl bromides [*Ref.* [12]].

6.1.2. 3-Benzyl-3,7-Diaza-bicyclo [3,3,1]nonane (**9**)

TFA (2.25 ml, 5eq, 30 mmol) was added to the stirring suspension of **8** (2.0 g, 1eq, 60 mmol) in DCM at 0 °C and allowed to stir at room temperature. Then reaction mixture was made alkaline by adding 20% aq. solution of Na_2CO_3 and resulting mixture was extracted with DCM (3 × 50 ml) and organics were washed with brine. The combined organics were dried with an. Na_2SO_4 and concentrated to obtain yellow oily liquid (1.641 g). Yield: 90%; IR(Neat): 3451.4, 2924.6, 1610.0, 1450.4 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$, ppm): δ 7.37–7.27 (m, 5H, Ph-H); 3.49 (s, 2H, $C\underline{H}_2Ph$), 3.36–3.41 (d, 2H, J = 12 Hz, 2 × NCH); 3.31–3.27 (d, J = 12 Hz, 2H, 2 × NCH); 3.19–3.11 (d, J = 12 Hz, 2H, 2 × NCH); 2.49–2.45 (d, J = 12 Hz, 2H, 2 × NCH); 2.12–2.07 (m, 2H, 2 × CH); 1.93–1.89 (d, J = 12 Hz, 1H, bridge CH); 1.79–1.75 (d, J = 12 Hz, 1H, bridge CH); MS (ESI): m/z = 217 $(M+1)^+$

6.1.3. 3,7-Diaza-bicyclo[3.3.1]nonane-3-carboxylic acid tert-butyl ester (**10**)

Palladium hydroxide (0.5 g) was added portion wise to a suspension of **8** (1.102 g, 36.7 mmol) in methanol (25 ml) in steel parr under nitrogen atmosphere. The reaction mixture was hydrogenated at 55 °C and 150 psi for about 10 h. Then it was allowed to cool and filtered over sintered funnel with the aid of vacuum and concentrated to get pale yellow solid. Yield = 93.33%; MP: 75 °C; IR (KBr): 2979.2, 2919.7, 2858.5, 1679.6, 1402.4, 1240.4, 1172.31131.9 cm^{-1}; 1H NMR (300 MHz, $CDCl_3$, ppm): δ 4.13–4.09 (d, J = 12 Hz, 2H, 2 × CONCH); 3.14–3.10 (m, 3H, 2 × CONCH, NCH); 3.01–2.96 (d, J = 15 Hz, 1H, NCH); 2.25 (s, 2H, 2 × NH); 1.92–1.88 (d, J = 12 Hz, 1H, CH); 1.80–1.76 (d, J = 12 Hz, 1H, CH); 1.67 (s, 2H, bridge CH_2); 1.48 (s, 9H, CMe_3); ^{13}C NMR (50 MHz, $CDCl_3$, ppm): δ155.49, 79.78, 51.50, 48.94, 31.39, 28.52, 28.15; MS (ESI): 227.1481$(M+H)^+$.

6.1.4. 7-(1-Benzyl-5-oxo-pyrrolidine-2-carbonyl)-3,7-diaza-bicyclo [3.3.1]nonane-3-carboxylic acid tert-butyl ester (**4a**)

DCC (0.308 g, 1.2 eq, 1.495 mmol) dissolved in dry DCM (5 ml) was added to the stirring reaction mixture containing N-benzyl pyroglutamic acid (0.273 g, 1 eq, 1.25 mmol) and HOBt (0.252 g, 1.5 eq, 1.86 mmol) dissolved in dry DCM (10 ml) at 0 °C and continued to stir for 15 min at same temperature. Then N-Boc bispidine (0.281 g, 1 eq, 1.25 mmol) dissolved in dry DCM (5 ml) was added drop wise to the stirring reaction mixture and continued to stir for a period of 2–3 h. The reaction mixture was then brought to room temperature and concentrated. The concentrated mass was then dissolved in diethyl ether and washed successively with dilute citric acid (1 × 20 ml), dilute $NaHCO_3$ (1 × 20 ml), brine and then extracted with ethyl acetate (3 × 20 ml). The combined organics were dried with anhydrous Na_2SO_4 and concentrated to obtain sticky oily product (0.534 g). Yield = 79%; $[\alpha]_D^{27^\circ C}$ = −15.1890 (Methanol, c = 0.3160); IR (Neat): 3017.0, 2366.7, 2337.8, 1678.9, 1432.0, 1217.9 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$, ppm): δ 7.36–7.14 (m, 5H, Ph-H); 5.15–5.10 (d, J = 15 Hz, 1H, $PhCH_A$); 4.59–4.54 (d, J = 12 Hz, 1H, $NC_{2'}H_A$); 4.09–4.06 (d, J = 15 Hz, 1H, $PhCH_B$; m, 2H, NC_2H); 3.81–3.76 (d, J = 15 Hz, 1H, $NC_{8'}H_A$); 3.51–3.47 (d, J = 12 Hz, 1H, $NC_{2'}H_B$); 3.08–2.86 (m, 4H, $PhCH_{B'}$, $NC_{8'}H_B$, $NC_{4'}H_A$, $C_{6'}H_2$); 2.51–2.40 (m, 2H, C_4H_A, $C_{4'}H_B$); 2.26–2.16 (m, 1H, C_4H_B); 2.16–1.89 (m, 4H, C_3H_2, $C_{3'}H$, $Ca_{7'}H$); 1.70 (s, 2H, $C_{9'}H$); 1.41 (s, 9H, CMe_3); ^{13}C NMR (50 MHz, $CDCl_3$, ppm): δ 176.16, 174.85, 135.40, 128.84, 128.53, 127.95, 49.48, 49.00, 45.36, 29.99, 34.49, 28.35, 27.70, 27.32, 22.88; MS (ESI): m/z = 427.9 (M^+).

Compound **4**(*b-p*) were obtained following the procedure described for **4***a*.

6.1.5. 7-[1-(2-Bromo-benzyl)-5-oxo-pyrrolidine-2-carbonyl]-3,7-diaza-bicyclo[3.3.1]nonane-3-carboxylic acid tert-butyl ester (**4b**)

Yield: 89%; MP: 138 °C; $[\alpha]_D^{27^\circ C}$: 3.7608 (Methanol, c = 0.2180); IR (KBr): 3458.8, 2927.9, 1679.6, 1434.4, 1241.5,1172.5, 1134.3 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$, ppm): δ 7.5–7.1 (m, 4H, Ph-H); 5.1–5.0 (d, J = 15 Hz, 1H, $PhCH_A$); 4.5 (d, 1H, $NC_{2'}H_A$); 4.1–4.0 (d, J = 15 Hz, 1H, $PhCH_B$; m, 1H, NC_2H); 3.5 (d, J = 15 Hz, 1H, $NC_{8'}H_B$); 3.1–2.8 (m, 4H, $NC_{2'}H_B$, $NC_{8'}H_B$, $NC_{4'}H$, $NC_{6'}H_A$); 2.4–2.3 (m, 3H, C_4H_A, $NC_{4'}H$, $NC_{6'}H_B$); 2.2–2.0 (m, 2H, C_4H_B, C_3H_A); 1.9 (m, 1H, C_3H_B); 1.8 (m, 2H, $C_{3'}H$, $C_{7'}H$); 1.7 (s, 2H, $C_{9'}H$); 1.4 (s, 9H, CMe_3); ^{13}C NMR (50 MHz, $CDCl_3$, ppm): δ 135.84, 132.75, 131.39, 129.38, 127.76, 124.13, 79.73, 56.85, 49.59, 46.51, 45.30, 34.64, 28.32, 28.13, 27.70, 27.29, 22.82; MS(ESI): m/z: 528.0 $(M+Na)^+$.

6.1.6. 7-[1-(4-Methylbenzyl)-5-oxo-pyrrolidine-2-carbonyl]-3,7-diaza-bicyclo[3.3.1]nonane-3-carboxylic acid tert butyl ester (**4c**)

Yield: 80%; $[\alpha]_D^{27^\circ C}$: −16.7970 (Methanol, c = 0.0980); IR (Neat): 3412.3, 2925.2, 1667.9, 1423.3, 1364.3, 1245.4, 1172.5, 1135.0 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$, ppm): δ 7.14–7.11 (d, J = 7.8 Hz, 2H, Ph-H), 7.07–7.04 (d, J = 7.8 Hz, 2H, Ph-H); 5.13–5.08 (d, J = 15 Hz, $PhCH_A$); 4.61–4.56 (m, 1H, $NC_{2'}H_A$); 4.15–4.07 (m, 1H, NC_2H); 3.76–3.71 (d, J = 15 Hz, 1H, $PhCH_B$); 3.54–3.50 (d, J = 12 Hz, 1H, $NC_{8'}H_A$); 3.04–2.94 (m, 4H, $NC_{2'}H_B$, $NC_{8'}H_B$, $NC_{4'}H_A$, $NC_{6'}H_A$); 2.34 (s, 1H, CH_3); 2.22–2.00 (m, 6H, C_4H_A, $C_{6'}H_B$, $C_{4'}H_B$, C_4H_B, C_3H_2); 1.91–1.90 (m, 2H, $C_{3'}H$, $C_{7'}H$); 1.80 (s, 2H, $C_{9'}H$); 1.42 (s, 9H, CMe_3); ^{13}C NMR (50 MHz, $CDCl_3$, ppm): δ 175.68, 168.33, 137.29, 129.28, 128.58, 49.52, 45.03, 34.64, 30.04, 28.35, 27.74, 27.35, 22.81, 21.08; MS (ESI): m/z = 441.9 (M^+).

6.1.7. tert-Butyl-7-((S)-1-(4-cyanobenzyl)-5-oxopyrrolidine-2-carbonyl)-3,7-diazabicyclo[3.3.1]nonane-3-carboxylate (**4d**)

Yield = 53%; MP: 160–165 °C; ^1H NMR (300 MHz, $CDCl_3$, ppm) δ7.64–7.61 (d, J = 7.8 Hz, 2H, Ph-H), 7.28 (d, J = 7.8 Hz, 2H, Ph-H) 5.17–5.12 (d, J = 15 Hz, 1H, $PhCH_A$), 4.59–4.55 (d, 1H, C(O) $NC_{2'}H_A$), 4.12–3.92 (d, 1H, $PhCH_B$), 3.87 (d, J = 15 Hz, 1H, NC_2H), 3.12 (m, 1H, $PhCH_A$), 3.10–3.01 (m, 2H, $PhCH_{B'}$, $C(O)NC_{4'}H_A$), 2.60–2.51 (m, 1H, $C(O)NC_{2'}H_B$), 2.40–2.50 (m, 2H, $C(O)NC_{8'}H_B$, $NC_{6'}H_A$), 2.12 (m, 1H, C_4H_A), 2.10–1.90 (m, 2H, $C_{4'}H_B$, $NC_{6'}H_B$, $NC_{8'}H_A$), 1.90–1.82 (m, 2H, C_4H_B, C_3H_A), 1.99–1.97 (bs, 4H, C_3H_B, $C_3'H$, $C_{7'}H$, $C_{9'}H$), 1.43 (s, 9H, $(CH_3)_3$); ^{13}C NMR (50 MHz,$CDCl_3$, ppm): δ 175.87, 169.33, 154.89, 142.20, 132.46, 128.87, 127.58, 111.52, 79.93, 56.99, 49.56, 46.54, 45.20, 30.27, 29.69, 29.52, 28.56, 28.38, 27.74, 27.32, 23.09; IR (KBr): 3896, 3744, 3700, 3576, 3456, 2924, 2859, 2361, 2228, 1679, 1418 cm^{-1}; MS (ESI): m/z = 452.5 (M^+).

6.1.8. tert-butyl 7-((S)-1-(4-chlorobenzyl)-5-oxopyrrolidine-2-carbonyl)-3,7-diazabicyclo [3.3.1] nonane-3-carboxylate (**4e**)

Yield = 77%; ^1H NMR (300 MHz, $CDCl_3$, ppm): δ 7.30–7.28 (d, J = 7.8 Hz, 2H, Ph-H), 7.27–7.12 (d, J = 7.8 Hz, 2H, Ph-H) 5.11–5.04 (d, J = 15 Hz, 1H, $PhCH_A$), 4.59–4.54 (d, 1H, $C(O)NC_{2'}H_A$), 4.20–4.00 (d, J = 15 Hz, 1H, $PhCH_B$, $NC_{8'}H_A$), 3.79 (d, 12 Hz, 1H, NC_2H), 3.56 (d, J = 15 Hz, 1H, $PhCH_{A'}$), 3.09–3.04 (m, 2H, $PhCH_{B'}$, $C(O)NC_{4'}H_A$), 2.99–2.96 (m, 1H, $C(O)NC_{2'}H_B$), 2.91–2.86 (m, 2H, $C(O)NC_{8'}H_B$, $NC_{6'}H_A$), 2.50–2.48 (m, 1H, C_4H_A), 2.44–2.43 (m, 2H, $C_{4'}H_B$, $NC_{6'}H_B$),

2.41–2.40 (m, 2H, C_4H_B, C_3H_A), 1.94 (bs, 2H, C_3HB, $C_3'H$) 1.81(bs, 2H, $C_{7'}H$, $C_{9'}H$), 1.41 (s, 9H, $(CH_3)_3$); ^{13}C NMR (50 MHz,$CDCl_3$, ppm); δ 175.57, 169.53, 154.93, 134.94, 133.46, 129.87, 128.78, 79.58, 56.5, 56.49, 56.47, 49.54, 44.70, 29.81, 28.35, 27.74, 22.92; IR(KBr): 3869, 3759, 3496, 3010, 2926, 2860, 1679, 1423 cm^{-1}; MS(ESI): *m/z* = 461.9 (M^+).

6.1.9. tert-butyl 7-((S)-1-(2,6-dichlorobenzyl)-5-oxopyrrolidine-2-carbonyl)-3,7-diazabicyclo [3.3.1] nonane-3-carboxylate ***(4f)***

Yield = 63%; ^1H NMR (300 MHz, $CDCl_3$, ppm): δ 7.64–7.61 (m, 2H, Ph), 7.28 (m, 2H, Ph) 5.17–5.12 (d, *J* = 15 Hz, 1H, $PhCH_A$), 4.59–4.55 (d, 12 Hz, 1H, $C(O)NC_{2'}H_A$), 4.12–3.92 (d, *J* = 15 Hz, 1H, $PhCH_B$), 3.87 (d, 12 Hz, 1H, NC_2H), 3.12 (m, 1H, $PhCH_{A'}$), 3.10–3.01 (m, 2H, $PhCH_{B'}$, $C(O)NC_{4'}H_A$), 2.60–2.51 (m, 1H, $C(O)NC_{2'}H_B$), 2.40–2.50 (m, 2H, $C(O)NC_{8'}H_B$, $NC_{6'}H_A$), 2.12 (m, 1H, C_4H_A), 2.10–1.90 (m, 2H, $C_{4'}H_B$, $NC_{6'}H_B$, $NC_{8'}H_A$), 1.90–1.82 (m, 2H, C_4H_B, C_3H_A), 1.99–1.97 (bs, 4H, C_3HB, $C_3'H$, $C_{7'}H$, $C_{9'}H$), 1.43 (s, 9H, $(CH_3)_3$); IR (KBr): 3896, 3744, 3700, 3576, 3456, 2924, 2859, 2361, 2228, 1679, 1418 cm^{-1}; MS (ESI): *m/z* = 498.2 $(M+H)^+$.

6.1.10. tert-butyl 7-((S)-1-(4-methoxybenzyl)-5-oxopyrrolidine-2-carbonyl)-3,7-diazabicyclo[3.3.1] nonane-3-carboxylate ***(4g)***

Yield = 54%; ^1H NMR (300 MHz, $CDCl_3$, ppm): δ 7.28–7.23 (m, 2H, Ph), 6.83–6.72 (m, 2H, Ph), 5.14–5.10 (d, *J* = 15 Hz, 1H, $PhCH_A$), 4.61–4.56 (d, 12 Hz, 1H, $C(O)NC_{2'}H_A$), 4.20–4.04 (m, 2H, $PhCH_B$, NC_2H), 3.85–3.75 (s, 3H, OCH_3), 3.74–3.71 (m, 1H, $PhCH_{A'}$), 3.60–3.48 (m, 1H, $PhCH_{B'}$), 3.04–3.00 (m, 2H, $C(O)NC_{2'}H_B$, $C(O)NC_{4'}H_A$), 2.94–2.88 (m, 2H, $C(O)NC_{8'}H_B$, $NC_{8'}H_A$), 2.49–2.55 (m, 2H, C_4H_A, $NC_{6'}H_A$), 2.31–2.25 (m, 2H, $C_{4'}H_B$, $NC_{6'}H_B$), 2.20 (m, 2H, C_4H_B, C_3H_A), 1.80 (m, 4H, C_3HB, $C_3'H$, $C_{7'}H$, $C_{9'}H$), 1.42 (s, 9H, $(CH_3)_3$); ^{13}C NMR (50 MHz,$CDCl_3$, ppm): 172.17, 169.73, 159.90, 139.16, 137.77, 129.58, 120.78, 114.11, 114.03, 113.96, 113.22, 113.05, 79.74, 55.21, 55.18, 49.49, 45.31, 33.76, 29.63, 28.32, 22.81; IR (KBr): 3900, 3565, 3366, 3013, 2926, 2856, 2196, 1679, 1434, 1363, 1219 cm^{-1}; MS (ESI): *m/z* = 457.5 (M^+).

6.1.11. tert-butyl 7-((S)-1-(naphthalen-1-ylmethyl)-5-oxopyrrolidine-2-carbonyl)-3,7-diazabicyclo [3.3.1] nonane-3-carboxylate ***(4h)***

Yield = 47%; ^1H NMR (300 MHz, $CDCl_3$, ppm): δ 8.05–7.37 (m, 7H, Naphthyl), 5.62–5.67 (d, *J* = 15 Hz, 1H, $PhCH_A$),5.04–4.97 (d, *J* = 15 Hz, 1H, $PhCH_B$), 4.62–4.52 (d, 12 Hz, 1H, $C(O)NC_{2'}H_A$), 4.11–4.07 (m, 1H, NC_2H, $NC_{8'}H_A$), 3.77–3.76 (m, 1H, $PhCH_{A'}$), 3.24–3.20 (m, 1H, $PhCH_{B'}$), 3.01–2.91 (m, 3H, $C(O)NC_{4'}H_A$, $C(O)NC_{8'}H_B$, $C(O)NC_{2'}H_B$), 2.66–2.42 (m, 3H, $NC_{6'}H_A$1H, C_4H_A, $C_{4'}H_B$), 2.39 (m, 1H, $NC_{6'}H_B$), 2.07–2.03 (m, 5H, C_4H_B, C_3H_A, C_3H_B, $C_3'H$, $C_{7'}H$), 1.70 (bs, 2H, $C_{9'}H$), 1.42 (s, 9H, $(CH_3)_3$); IR (KBr): 3947, 3675, 3484, 3421, 3287, 2923, 2853, 2361, 1674, 1452, 1365 cm^{-1}; MS (ESI): *m/z* = 447.5 (M^+).

6.1.12. 1-Benzyl-5-(7-benzyl-3,7-diaza-bicyclo[3.3.1]nonane-3-carbonyl)-pyrrolidin-2-one ***(4i)***

Yield = 70%; $[\alpha]_D^{27^\circ C}$ = +3.44 (Methanol, c = 0.2120); MP: 144 °C; IR (KBr): 3424.2, 3010.2, 2924.5, 1680.5, 1642.6, 1449.8, 1218.9 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$, ppm): δ 7.29–7.22 (m, 10H, 2 × Ph); 5.23–5.18 (d, *J* = 15 Hz, 1H, $PhCH_A$); 4.60–4.45 (d, *J* = 12 Hz, 1H, $NC_{2'}H_A$); 4.17–4.15 (m, 1H, NC_2H); 3.85–3.79 (d, *J* = 15 Hz, 1H, $PhCH_B$); 3.50–3.45 (m, 2H, $PhCH_{A'}$, $NC_{8'}H_A$); 3.26–3.22 (d, *J* = 15 Hz, 1H, $PhCH_{B'}$); 3.08–2.99 (m, 3H, $NC_{2'}H_B$, $NC_{8'}H_B$, $NC_{4'}H_A$); 2.85 (m, 1H, $NC_{6'}H_A$); 2.57 (m, 1H, C_4H_A); 2.38–2.33 (m, 1H, $C_{4'}H_B$, $NC_{6'}H_B$); 2.10–2.06 (m, 2H, C_4H_B, C_3H_A); 1.97 (m, 1H, C_3H_B); 1.88 (m, 2H, $C_3'H$, $C_{7'}H$); 1.70 (s, 2H, $C_{9'}H$); ^{13}C NMR (50 MHz,$CDCl_3$, ppm): δ 175.67, 168.41, 128.64, 128.54, 128.35, 128.07, 127.58, 127.05, 63.52, 59.35, 58.38, 56.54, 46.44, 45.40, 31.04, 29.98, 29.19, 28.54, 21.56; MS (ESI): *m/z* = 418.2 $(M+1)^+$.

6.1.13. 5-(7-Benzyl-3,7-diaza-bicyclo[3.3.1]nonane-3-carbonyl)-1-(2-bromo-benzyl)-pyrrolidin-2-one ***(4j)***

Yield = 74%; MP: 131 °C; $[\alpha]_D^{27^\circ C}$: +25.5130 (Methanol, c = 0.2040); IR (KBr): 3464.8, 3354.4, 2911.5, 2802.9, 1690.2, 1638.9, 1442.4, 1342.4, 1285.8, 1254.6 cm^{-1}; 1H NMR (300 MHz, CDCl3, ppm): δ 7.29–7.22 (m, 9H, 2 × Ph); 5.14–5.09 (d, *J* = 15 Hz, 1H, $PhCH_A$); 4.57–4.53 (d, *J* = 12 Hz, 1H, $NC2'H_A$); 4.21–4.29 (t, 1H, NC_2H) 4.14–4.09 (d, *J* = 15 Hz, 1H, $PhCH_B$); 3.50–3.43 (m, 2H, $PhCH_{A'}$, $NC8'H_A$); 3.25–3.16 (m, 2H, $PhCH_{B'}$, $NC2'H_B$); 3.0–2.84 (m, 3H, $NC8'H_B$, $NC4'H_A$, $C6'H_A$); 2.61–2.49 (m, 1H, $C4H_A$); 2.35–2.27 (m, 2H, $NC4'H_B$, $C6'H_B$); 2.05–1.93 (m, 1H, $C3H_B$); 1.89 (m, 2H, $C3'H$, $C7'H$); 1.68 (s, 2H, $C_{9'}H$); 13C NMR (50 MHz, CDCl3, ppm): δ 175.72, 168.34, 137.90, 136.149, 132.84, 128.61, 128.33, 127.77, 127.01, 124.43, 115.35, 63.83, 59.73, 58.56, 56.97, 49.29, 45.67, 34.64, 31.33, 29.20, 21.96; MS (ESI): *m/z* = 496.2 $(M+H)^+$.

6.1.14. 5-(7-Benzyl-3,7-diaza-bicyclo[3.3.1]nonane-3-carbonyl)-1-(4-methylbenzyl)-pyrrolidin -2- one ***(4k)***

Yield = 79%; MP: 133 °C; $[\alpha]_D^{27^\circ C}$: +0.9200 (Methanol, c = 0.1260); IR (KBr): 3445.7, 2362.3, 1637.4, 1466.5, 1219.1 cm^{-1}; ^1H NMR (300 MHz,$CDCl_3$, ppm): δ 7.30–7.07 (m, 9H, 2 × Ph); 5.18–5.14 (d, *J* = 15 Hz, 1H, $PhCH_A$); 4.59–4.45 (m, 1H, $NC_{2'}H_A$); 4.17–4.15 (t, 1H, NC_2H); 3.79–3.74 (d, *J* = 15 Hz, 1H, $PhCH_B$); 3.51–3.47 (m, 2H, $PhCH_{A'}$, $NC_{8'}H_A$); 3.27–3.10 (d, *J* = 15 Hz, 1H, $PhCH_{B'}$); 3.02–2.86 (m, 4H, $NC_{2'}H_B$, $NC_{8'}H_B$, $NC_{4'}H_A$, $NC_{6'}H_A$), 2.55 (m, 2H, C_4H_A), 2.33 (s, 3H, CH_3); 2.33 (m, 2H, $NC_{4'}H_B$, $NC_{6'}H_B$); 2.09–2.06 (m, 2H, C_4H_B, C_3H_A); 1.97 (m, 1H, C_3H_B); 1.89 (m, 2H, $C_3'H$, $C_{7'}H$); 1.70 (s, 2H, $C_{9'}H$); MS (ESI): *m/z* = 432.2$(M+1)^+$.

6.1.15. (5S)-5-(7-benzyl-3,7-diazabicyclo[3.3.1]nonane-3-carbonyl)-1-(2,6-dichlorobenzyl)pyrrolidin-2-one ***(4l)***

Yield = 76%; MP: 50–55 °C; $[\alpha]_D^{27^\circ C}$ = +51.6304 (Chloroform, c = 0.10); ^1H NMR (300 MHz, $CDCl_3$, ppm); δ 7.35–7.24 (m, 8H, 2 × Ph), 5.31–5.26 (d, *J* = 15 Hz, 1H, $PhCH_A$), 4.60–4.56 (d, 1H, $C(O)NC_{2'}H_A$), 4.46–4.41 (d, *J* = 15 Hz, 1H, $PhCH_B$), 4.05–4.02 (dd, 12 Hz, 1H, NC_2H), 3.50–3.46 (m, 2H, $PhCH_{A'}$, $NC_{8'}H_A$), 3.28–3.24 (m, 2H, $PhCH_{B'}$, $C(O)NC_{4'}H_A$), 3.05–3.01 (m, 1H, $C(O)NC_{2'}H_B$), 2.90–2.87 (m, 2H, $C(O)NC_{8'}H_B$, $NC_{6'}H_A$), 2.56 (m, 1H, C_4H_A), 2.36 (m, 2H, $C_{4'}H_B$, $NC_{6'}H_B$), 2.11–2.01 (m, 2H, C_4H_B, C_3H_A), 1.99–1.97 (m, 1H, C_3H_B), 1.93 (m, 2H, $C_3'H$, $C_{7'}H$), 1.73 (s, 2H, $C_{9'}H$); ^{13}C NMR (50 MHz,$CDCl_3$, ppm): δ 175.10, 168.25, 151.52, 137.89, 129.57, 128.58, 128.45, 128.33, 127.26, 127.01, 63.54, 59.53, 58.47, 56.20, 46.45, 31.08, 29.62, 29.30, 28.55, 21.81; IR (KBr): 3639.2, 3400, 2955, 2800, 1690, 1645, 1439, 1362, 1228, 1154, 1119 cm^{-1}; MS (ESI): *m/z* = 486.3 (M^+).

6.1.16. (5S)-5-(7-benzyl-3,7-diazabicyclo[3.3.1]nonane-3-carbonyl)-1-(4-chlorobenzyl)pyrrolidin-2-one ***(4m)***

Yield = 70%; MP: 131 °C; $[\alpha]_D^{27^\circ C}$: +25.5130 (Methanol, c = 0.2040); IR (KBr): 3464.8, 3354.4, 2911.5, 2802.9, 1690.2, 1638.9, 1442.4, 1342.4, 1285.8, 1254.6 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$, ppm): δ 7.29–7.22 (m, 9H, 2 × Ph); 5.14–5.09 (d, *J* = 15 Hz, 1H, $PhCH_A$); 4.57–4.53 (d, *J* = 12 Hz, 1H, $NC_{2'}H_A$); 4.21–4.09 (d, *J* = 15 Hz, 1H, $PhCH_B$; m, 1H, NC_2H); 3.50–3.43 (m, 2H, $PhCH_{A'}$, $NC_{8'}H_A$); 3.25–3.16 (m, 2H, $PhCH_{B'}$, $NC_{2'}H_B$); 3.0–2.84 (m, 3H, $NC_{8'}H_B$, $NC_{4'}H_A$,$C_{6'}H_A$); 2.61–2.49 (m, 1H, C_4H_A); 2.35–2.27 (m, 2H, $NC_{4'}H_B$, $C_{6'}H_B$); 2.05–1.93 (m, 1H, C_3H_B); 1.89 (m, 2H, $C_3'H$, $C_{7'}H$); 1.68 (s, 2H, $C_{9'}H$); ^{13}C NMR (50 MHz, $CDCl_3$, ppm): δ 175.72, 168.34, 137.90, 136.149, 132.84, 128.61, 128.33, 127.77, 127.01, 124.43, 115.35, 63.83, 59.73, 58.56, 56.97, 49.29, 45.67, 34.64, 31.33, 29.20, 21.96; MS (ESI): *m/z* = 452.2 $(M+H)^+$.

6.1.17. (7-Benzyl-3,7-diazabicyclo[3.3.1]nonan-3-yl)((S)-1-(phenylsulfonyl)pyrrolidin-2-yl) methanone ***(4n)***

Yield = 83%; ^1H NMR (300 MHz, $CDCl_3$, ppm): δ 7.81–7.79 (d, *J* = 7.8 Hz, 2H, SO_2Ph) 7.31–7.21 (m, 7H, SO_2Ph, Ph), 4.84–4.81 (d,

12 Hz, 1H, C(O)NC$_{2'}$H$_A$), 3.86–3.82 (d, J = 15 Hz, 1H, PhCH$_{A'}$), 3.56–3.32 (m, 4H, PhCH$_{B'}$, C(O)NC$_{4'}$H$_A$, NC$_{8'}$H$_A$, NC$_2$H), 3.01–2.89 (m, 3H, C(O)NC$_{2'}$H$_B$, C(O)NC$_{8'}$H$_B$, NC$_{6'}$H$_A$), 2.43 (s, 3H, CH$_3$), 2.34–2.31 (d, 12 Hz, 1H, C$_3$H$_A$), 2.17–1.69 (m, 10H, C$_{4'}$H$_B$, NC$_{6'}$H$_B$, C$_{3'}$H, C$_{7'}$H, C$_3$H$_B$, C$_4$H, C$_5$H, C$_{9'}$H); ^{13}C NMR (200 MHz,CDCl$_3$, ppm): δ 175.61, 168.19, 163.33, 139.25, 129.83, 128.77, 128.62, 128.33, 127.05, 63.53, 59.34, 58.36, 56.57, 49.22, 46.46, 31.91, 29.79, 29.14, 22.67, 21.58; IR (KBr): 3783, 3448, 3374, 2923, 2361, 2135, 1817, 1640, 1446, 1337, 1224, 1098 cm^{-1}; MS (ESI): m/z = 468.3 (M$^+$).

6.1.18. (5S)-5-(7-benzyl-3,7-diazabicyclo[3.3.1]nonane-3-carbonyl)-1-(4-bromobenzyl)pyrrolidin-2-one (**4o**)

Yield = 65%; ^{1}H NMR (300 MHz, CDCl$_3$, ppm): δ 7.45–7.04 (m, 8H, Ph-H) 5.20–5.15 (d, J = 15 Hz, 1H, PhCH$_A$), 4.61–4.56 (d, J = 15 Hz, 1H, C(O)NC$_{2'}$H$_A$), 4.20–4.12 (dd, 12 Hz, 1H, NC$_2$H), 3.83–3.71 (d, J = 15 Hz, 1H, PhCH$_B$), 3.52–3.41 (m, 2H, PhCH$_{A'}$, NC$_{8'}$H$_A$), 3.19–3.15 (m, 2H, PhCH$_{B'}$, C(O)NC$_{4'}$H$_A$), 2.95 (m, 2H, C(O) NC$_{2'}$H$_B$, NC$_{8'}$H$_B$), 2.65 (m, 1H, NC$_{6'}$H$_A$), 2.15–1.88 (m, 4H, C$_{4'}$H$_B$, NC$_{6'}$H$_B$ C$_4$H$_B$, C$_3$H$_A$), 1.83–1.79 (m, 1H, C$_3$H$_B$), 1.74 (m, 2H, C$_{3'}$H, C$_{7'}$H, bs, 2H, C$_{9'}$H); ^{13}C NMR (50 MHz,CDCl$_3$, ppm); 175.33, 168.44, 137.22, 136.79, 133.38, 131.46, 130.16, 129.28, 128.54, 127.89, 120.79, 62.72, 59.36, 58.16, 49.20, 46.33, 45.04, 31.05, 29.96, 29.68, 29.40, 29.19, 28.48, 21.10.; IR (KBr): 3870,3777, 3588, 3526, 2924, 2276, 1680, 1451, 1220 cm^{-1}; MS (ESI): m/z = 495.2 (M$^+$).

6.1.19. (5S)-5-(7-benzyl-3,7-diazabicyclo[3.3.1]nonane-3-carbonyl)-1-(4-methoxybenzyl)pyrrolidin-2-one, (**4p**)

Yield = 61%; ^{1}H NMR (300 MHz,CDCl$_3$, ppm): δ 7.29–7.22 (m, 9H, 2 × Ph); 5.14–5.09 (m, 1H, PhCH$_A$); 4.57–4.53 (d, J = 12 Hz, 1H, NC$_{2'}$H$_A$); 4.21–4.09 (m, 2H, NC$_2$H, PhCH$_B$); 3.79 (s, 3H, OCH$_3$) 3.50–3.43 (m, 2H, PhCH$_{A'}$, NC$_{8'}$H$_A$); 3.25–3.16 (m, 2H, PhCH$_{B'}$, NC$_{2'}$H$_B$); 3.0–2.84 (m, 3H, NC$_{8'}$H$_B$, NC$_{4'}$H$_A$,C$_{6'}$H$_A$); 2.61–2.49 (m, 1H, C$_4$H$_A$); 2.35–2.27 (m, 2H, NC$_{4'}$H$_B$, C$_{6'}$H$_B$); 2.05–1.93 (m, 1H, C$_3$H$_B$); 1.89 (m, 2H, C$_{3'}$H, C$_{7'}$H); 1.68 (s, 2H, C$_{9'}$H); ^{13}C NMR (50 MHz, CDCl$_3$, ppm): δ 175.72, 168.34, 137.90, 136.149, 132.84, 128.61, 128.33, 127.77, 127.01, 124.43, 115.35, 63.83, 59.73, 58.56, 56.97, 49.29, 45.67, 34.64, 31.33, 29.20, 21.96; IR (KBr): 3872, 3778, 3578, 3526, 2928, 2276, 1680, 1449, 1220 cm^{-1}; MS (ESI): m/z = 448.2 (M+H)$^+$.

6.1.20. (Boc deprotection)

To a stirred solution **4b** or **4c** (1.0 eq.) in dry DCM (10 ml), TFA (5.0 eq.) was added slowly at a temperature of 0 °C and allowed to stir for 1 h. Reaction mixture was made alkaline by 20% Na_2CO_3 solution and the resulting mixture was extracted with DCM. It was washed with water and brine. Organic layer was collected and the combined fractions were dried over anhydrous sodium sulphate and concentrated to get yellow oily liquid.

6.1.21. 5-(7-Benzoyl-3,7-diaza-bicyclo[3.3.1]nonane-3-carbonyl)-1-(2-bromobenzyl)-pyrrolid in-2-one (**4q**)

Benzoyl chloride (0.104 ml, 1.2 eq, 0.741 mmol) was added drop wise to the stirring solution of deprotected product (0.25 g, 1eq, 0.617 mmol) and triethylamine (0.198 ml, 2.3 eq, 1.42 mmol) in dry dichloromethane at 0 °C and allowed to stir for half h. The reaction mixture was washed with 1 N HCl (1 × 25 ml), 20% $NaHCO_3$ (1 × 25 ml). The combined organics were washed with anhydrous sodium sulphate and concentrated to obtain yellow oily liquid and the crude product was purified by column chromatography on silica to obtain the pure product. Yield: 88%; MP: 85 °C; $[\alpha]_D^{27°C}$: +13.73 (Methanol, c = 0.1000); IR (KBr): 3404.0, 2929.5, 2365.0, 1629.8, 1429.4, 1351.7, 1246.7, 1085.7 cm^{-1}; ^{1}H NMR (300 MHz,CDCl$_3$, ppm): δ 7.57–7.54 (d, J = 7.8 Hz, 1H, Ph-H), 7.42–7.17 (m, 8H, Ph-H); 5.15–5.10 (d, J = 15 Hz, 1H, PhCH$_A$); 4.80–4.75 (d, J = 15 Hz, 1H, NC$_{2'}$H$_A$); 4.62–4.57 (d, J = 15 Hz, 1H, PhCH$_B$); 4.22–4.11 (t, 1H, NC$_2$H); 3.88–3.83 (d, J = 12 Hz, 1H, NC$_{8'}$H$_A$); 3.71–3.66 (d, J = 12 Hz, 1H, NC$_{2'}$H$_B$); 3.24–3.12 (m, 3H, NC$_{8'}$H$_B$, NC$_{4'}$H$_A$,,NC$_{6'}$H$_A$); 2.90–2.86 (d, J = 12 Hz, NC$_{6'}$H$_B$); 2.52–2.49 (m, 1H, C$_4$H$_A$); 2.44–2.40 (m, 1H, C$_{4'}$H$_B$); 2.34–2.24 (m, 1H, C$_4$H$_B$); 2.22–2.07 (m, 1H, C$_3$H$_A$); 1.95–1.91 (m, 3H, C$_3$H$_B$, C$_{3'}$H, C$_{7'}$H); 1.83 (m, 2H, C$_{9'}$H); ^{13}C NMR (75 MHz, CDCl$_3$, ppm): δ 175.83, 171.35, 170.21, 135.87, 132.80, 131.37, 129.43, 128.74, 127.84, 126.70, 124.14, 56.95, 52.47, 49.53, 46.56, 46.08, 45.30, 30.85, 29.87, 27.66, 23.39; MS (ESI): m/z = 512 (M+3)$^+$.

6.1.22. 5-(7-Benzoyl-3,7-diaza-bicyclo[3.3.1]nonane-3-carbonyl)-1-(4-methylbenzyl)-pyrrolidin-2-one (**4r**)

Yield = 89%; $[\alpha]_D^{27°C}$: +2.1583 (Methanol, c = 0.1000); IR (Neat): 3420.3, 2958.5, 1678.4, 1632.11, 1438.3, 1220.2 cm^{-1}; ^{1}H NMR (300 MHz,CDCl$_3$, ppm): δ 7.42–7.00 (m, 9H, 2 × Ph); 5.15–5.04 (d, J = 15 Hz, 1H, PhCH$_A$); 4.81–4.55 (d, J = 12 Hz, 1H, NC$_{2'}$H$_A$); 4.16 (t, 1H, NC$_2$H); 3.89–3.77 (d, J = 12 Hz, 1H, NC$_{8'}$H$_A$); 3.67–33.64 (d, J = 15 Hz, 1H, PhCH$_B$); 3.33–3.19 (m, 2H, NC$_{2'}$H$_B$, NC$_{8'}$H$_B$); 3.04–3.02 (m, 1H, NC$_{4'}$H$_A$); 2.93–2.89 (m, 7H, C$_4$H$_A$, NC$_{6'}$H$_B$, C$_4$H$_B$, C$_{4'}$H$_B$, CH$_3$); 2.22–2.18 (m, 2H, C$_3$H$_2$); 1.95–1.81 (m, 2H, C$_{3'}$H, C$_{7'}$H); 1.27–1.26 (m, 2H, C$_{9'}$H); ^{13}C NMR (75 MHz, CDCl$_3$, ppm): δ 175.83, 171.28, 170.20, 137.4, 136.08, 136.00, 129.40, 129.22, 128.87, 128.72, 128.65, 128.56, 128.20, 127.22, 126.76, 57.49, 49.47, 46.49, 46.16, 45.92, 45.13, 34.27, 30.40, 27.61, 23.40, 21.29, 21.17; MS (ESI): m/z = 446.1 (M+H)$^+$.

6.1.23. 1-(2-Bromo-benzyl)-5-[7-(toluene-4-sulphonyl)-3,7-diaza-bicyclo[3.3.1]nonane-3-carbonyl]-pyrrolidin-2-one (**4s**)

p-Toluene sulphonyl chloride (0.140 g, 1.2 eq, 0.739 mmol) was added drop wise to the stirring solution of deprotected product (0.250 g, 1eq, 0.616) and triethyl amine (0.197 ml, 2.3 eq, 1.41 mmol) in dry DCM at 0 °C and allowed to stir for half h. The reaction mixture was washed with 1 N HCl (1 × 25 ml), 20% $NaHCO_3$ (1 × 25 ml). The combined organics were washed with anhydrous sodium sulphate and concentrated to obtain yellow oily liquid (0.488 g). The crude product was purified by column chromatography on silica (Chloroform/Methanol = 7.5/2.5) to obtain the pure product (0.266 m). Yield = 85%; $[\alpha]_D^{27°C}$: −2.8263 (Methanol, c = 0.1000); MP: 203–205 °C; IR (KBr): 3451.8, 1638.4 cm^{-1}; ^{1}H NMR (300 MHz, CDCl$_3$, ppm): δ 7.58–7.15 (m, 8H, 2 × Ph); 5.13–5.08 (d, J = 15 Hz, 1H, PhCH$_A$); 4.66–4.62 (d, J = 12 Hz, 1H, NC$_{2'}$H$_A$); 4.27–4.23 (m, 1H, NC$_2$H); 4.15–4.10 (d, J = 15 Hz, 1H, PhCH$_B$); 3.79–3.76 (d, J = 9 Hz, 2H, NC$_{8'}$H$_A$, NC$_{2'}$H$_B$); 3.66–3.62 (d, J = 12 z, 1H, NC$_{4'}$H$_A$); 3.20–3.15 (m, 1H, NC$_{8'}$H$_B$); 2.96–2.91 (m, 1H, NC$_{6'}$H$_A$); 2.71–2.62 (m, 1H, NC$_4$H$_A$); 2.47–2.43 (m, 6H, NC$_{4'}$H$_B$, NC$_{6'}$H$_B$, C$_4$H$_B$, CH$_3$); 2.34–2.31 (m, 2H, C$_3$H$_2$); 2.28–2.05 (C$_{3'}$H, C$_{7'}$H); 1.98 ppm (m, 2H, C$_{9'}$H); ^{13}C NMR (75 MHz,CDCl$_3$, ppm): δ 176.05, 169.32, 143.74, 135.97, 132.80, 131.50, 131.12, 129.67, 129.33, 127.79, 127.75, 124.06, 56.80, 50.59, 48.65, 46.13, 45.38, 29.74, 27.73, 27.28, 22.57, 21.51 ppm; MS (ESI): m/z: 562.0 (M+1)$^+$.

6.1.24. 1-(4-Methylbenzyl)-5-[7-(toluene-4-sulphonyl)-3,7-diaza-bicyclo[3.3.1]nonane-3-carbonyl]-pyrrolidin-2-one (**4t**)

Yield = 91.5%; $[\alpha]_D^{27°C}$: −0.9313 (Methanol, c = 0.1000); IR (Neat): 3449.8, 2953.7, 1641.5, 1443.2, 1220.9 cm^{-1}; ^{1}H NMR (300 MHz, CDCl$_3$, ppm): δ 7.60–7.00 (m, 8H, 2 × Ph), 5.20–5.03 (d, J = 15 Hz, 1H, PhCH$_A$), 4.68–4.65 (d, J = 12 Hz, 1H, NC$_{2'}$H$_A$), 4.24–4.23 (t, 1H, NC$_2$H), 3.94–3.64 (m, 3H, PhCH$_B$, NC$_{2'}$H$_B$, NC$_{8'}$H$_A$), 3.13–2.97 (m, 2H, NC$_{8'}$H$_B$, NC$_{4'}$H$_A$), 2.77–2.76 (m, 1H, NC$_{6'}$H$_B$), 2.72–2.66 (m, 3H, C$_4$H$_A$, NC$_{6'}$H$_B$, C$_{4'}$H$_B$), 2,29 (s, 6H, 2 × CH$_3$), 2.45–2.07 (m, 2H, C$_4$H$_A$, C$_3$H), 1.92 (m, 2H, C$_{3'}$H), 1.66 (m, 1H, C$_{7'}$H), 1.28–1.25 (m, 2H, C$_{9'}$H); ^{13}C NMR (50 MHz, CDCl$_3$, ppm): δ 151.53, 135.80, 129.68, 129.22, 128.51, 128.25, 128.65, 128.56, 128.20, 127.22, 126.76, 46.10, 45.01, 34.20, 34.23, 30.34, 27.80, 27.36, 22.64, 21.53, 21.20; MS (ESI): m/z: 496.0 (M+H)$^+$.

*6.1.25. (5S)-5-(2-bromobenzyl-3,7-diazabicyclo[3.3.1]nonane-3-carbonyl)-1-(4-methylbenzyl)pyrrolidin-2-one (**4u**)*

Yield = 64%; MP: 85–90 °C; ^{1}H NMR (300 MHz, $CDCl_3$, ppm) δ7.29 (m, 9H, 2 × Ph), 5.18–5.14 (d, J = 15 Hz, 1H, $PhCH_A$), 4.94–4.90 (m, 1H, $C(O)NC_{2'}H_A$), 4.61–4.56 (d, J = 15 Hz, 1H, $PhCH_B$), 4.14 (t, 1H, NC_2H), 3.85–3.80 (m, 2H, $PhCH_{A'}$, $NC_{8'}H_A$), 3.51–3.47 (m, 2H, $PhCH_{B'}$, $C(O)NC_{4'}H_A$), 3.29–3.15 (m, 2H, $C(O)NC_{2'}H_B$, $C(O)NC_{8'}H_B$), 3.04–2.89 (m, 1H, $NC_{6'}H_A$), 2.57 (m, 1H, C_4H_A), 2.37 (m, 2H, $C_{4'}H_B$, $NC_{6'}H_B$), 2.33 (s, 3H, CH_3), 2.09–2.08 (m, 2H, C_4H_B, C_3H_A), 1.98 (m, 1H, C_3H_B), 1.93 (m, 2H, $C_{3'}H$, $C_{7'}H$), 1.72 (m, 2H, $C_{9'}H$); ^{13}C NMR (50 MHz,$CDCl_3$, ppm): δ 181.36, 180.12, 169.59, 143.12, 129.42, 128.74, 128.17, 127.64, 114.05, 63.58, 59.23, 58.80, 49.77, 48.27, 46.64, 30.88, 29.83, 29.68, 29.33, 21.52; IR (KBr): 3444, 2925,2857, 2372, 2338, 2141, 1638, 1447, 1355, 1225, 1093 cm^{-1}; MS (ESI): m/z = 452.3 (M^+).

*6.1.26. (5S)-5-(7-(4-bromobenzyl)-3,7-diazabicyclo[3.3.1]nonane-3-carbonyl)-1-(4-methylbenzyl)pyrrolidin-2-one (**4v**)*

Yield = 67%; ^{1}H NMR (300 MHz, $CDCl_3$, ppm): δ 7.45–7.04 (m, 8H, Ph-H) 5.20–5.15 (d, J = 15 Hz, 1H, $PhCH_A$), 4.61–4.56 (d, J = 15 Hz, 1H, $C(O)NC_{2'}H_A$), 4.20–4.12 (t, 1H, NC_2H), 3.83–3.71 (d, J = 15 Hz, 1H, $PhCH_B$), 3.52–3.41 (m, 2H, $PhCH_{A'}$, $NC_{8'}H_A$), 3.19–3.15 (m, 2H, $PhCH_{B'}$, $C(O)NC_{4'}H_A$), 2.95 (m, 2H, $C(O)NC_{2'}H_B$, $NC_{8'}H_B$), 2.65 (m, 1H, $NC_{6'}H_A$), 2.34 (s, 3H, CH_3), 2.15–1.88 (m, 4H, $C_{4'}H_B$, $NC_{6'}H_B$ C_4H_B, C_3H_A), 1.83–1.79 (m, 1H, C_3H_B), 1.74 (m, 2H, $C_{3'}H$, $C_{7'}H$, bs, 2H, $C_{9'}H$); ^{13}C NMR (50 MHz,$CDCl_3$, ppm): δ 180.33, 173.40, 142.20, 138.34, 137.69, 134.78, 134.30, 133.53, 132.90, 67.65, 64.34, 63.14, 61.36, 59.96, 54.18, 51.32, 50.03, 36.06, 34.68, 34.17, 26.47; IR (KBr): 3891, 3806, 3708, 3625, 3585, 3446, 2924, 2411, 1676, 1452, 1363, 1221, 1091 cm^{-1}; MS (ESI): m/z = 510.4 (M^+).

*6.1.27. (5S)-5-(7-(4-chlorobenzyl)-3,7-diazabicyclo[3.3.1]nonane-3-carbonyl)-1-(4-methylbenzyl)pyrrolidin-2-one (**4w**)*

(5*S*)-5-(3,7-diazabicyclo[3.3.1]nonane-3-carbonyl)-1-(4-methylbenzyl)pyrrolidin-2-one (0.250 g, 1.0 eq., 0.76 mmol) was weighed and taken in round bottom flask, dissolved in dry acetone (2 ml). 2 g of anhydrous potassium carbonate (K_2CO_3) was added. 2-chlorobenzyl chloride (0.182 g, 1.5 eq., 1.14 mmol) was added to the reaction mixture and refluxed in an oil bath at 60 °C for 2 h with stirring. The reaction was monitored for completion by TLC. After the completion of reaction, reaction mixture was filtered to remove K_2CO_3 and concentrated in vacuum. The desired product was isolated from the crude reaction mixture by column chromatography. Yield = 62%; ^{1}H NMR (300 MHz, $CDCl_3$, ppm) δ: 7.52–7.49 (m, 1H, Ph-H) 7.35–7.28 (m, 2H, Ph-H), 7.11–7.00 (m, 4H, Ph-H) 6.85–6.82 (d, 1H, J = 12 Hz, Ph-H) 5.18–5.13 (d, J = 15 Hz, 1H, $PhCH_A$), 4.64–4.59 (d, 12 Hz, 1H, $C(O)NC_{2'}H_A$), 4.16–4.12 (t, 12 Hz, 1H, NC_2H), 3.79–3.74 (d, J = 15 Hz, 1H, $PhCH_B$), 3.60–3.41 (m, 2H, $PhCH_{A'}$, $NC_{8'}H_A$), 3.16–3.00 (m, 2H, $PhCH_{B'}$, $C(O)NC_{4'}H_A$), 2.91–2.88 (m, 1H, $C(O)NC_{2'}H_B$), 2.33 (s, 3H, CH_3), 1.98–1.97 (m, 2H, $C(O)NC_{8'}H_B$, $NC_{6'}H_A$), 1.95–1.93 (m, 2H, $C_{4'}H_B$, $NC_{6'}H_B$), 1.85 (m, 2H, C_4H_B, C_3H_A), 1.44–1.43 (m, 1H, C_3H_B), 1.28 (m, 2H, $C_{3'}H$, $C_{7'}H$, bs, 2H, $C_{9'}H$); ^{13}C NMR (50 MHz,$CDCl_3$, ppm): δ 175.50, 168.55, 137.20, 136.98, 133.44, 132.65, 129.27, 128.54, 62.45, 59.62, 58.58, 56.41, 54.93, 49.15, 46.36, 45.04, 29.90, 29.90, 29.26, 28.51, 21.10; IR (KBr): 3869, 3441, 3013, 2365, 1679, 1515, 1450, 1218 cm^{-1}; MS (ESI): m/z = 466.01 (M^+).

6.2. Biology (materials and method)

6.2.1. Animal studies

Male Swiss albino mice (18–22 g) and Sprague Dawley rats (160–180 g) were obtained from the National Laboratory Animal Centre of the Institute. The animals were grouped into vehicle (0.5% carboxymethyl cellulose in water), test compounds or standard drugs (asprin and clopidogrel). Before experimental procedure, vehicle, drugs or test compounds were administered to animals orally for either 1 h or as indicated.

6.2.2. Collagen-epinephrine induced pulmonary thromboembolism

Pulmonary thromboembolism in mice, following intravenous administration of collagen epinephrine suspension, was assessed as described previously [12]. Briefly, the compounds to be tested, standard drugs (aspirin and clopidogrel) or the vehicle were administered by oral route at different time points (1, 2, 4, 6, 12, 24 h) prior to the thrombotic challenge. Ten mice were used for evaluating the effect of test compound, aspirin or clopidogrel, while a group of 5 mice was used to evaluate the effect of vehicle. A mixture of collagen (150 μg/ml) and adrenaline (50 μg/ml) was injected into the tail vein to induce hind limb paralysis or death. The number of dead or paralyzed mice was counted for 15 min and the results are reported as % protection [12].

6.2.3. Bleeding time in mice

Mice tail tip (approximately 2 mm) was incised and the blood oozed was soaked on a filter paper, which was monitored at an interval of 15 s till the bleeding stops. The time elapsed from the tail incision to the stoppage of bleeding was determined as the bleeding time as described previously [12].

6.2.4. Vasoreactivity studies

Thoracic aortic rings from male Sprague Dawley rats (160–180 g) were prepared and equilibrated in KCl Kreb's buffer followed by assessment of their functions as described previously [18]. Rings were subsequently incubated with or without vehicle (0.2%) or test compounds and concentration response curves of acetylcholine (Ach), U46619 (1 pM–100 nM) or phenylephrine (PE) (3nM–300μM) was generated as described previously [18].

6.2.5. Ferric chloride induced arterial thrombosis in mice

Swiss albino mice were pretreated with test compound, clopidogrel or vehicle for 60 min and were anesthetized by urethane (1.25 g/kg, i.p.). The carotid artery was carefully dissected and a pulsed Doppler flow probe (DBF-120A-CPx) was placed around it to record the blood flow using Biopac Data Acquisition System (CBI-8000; Crystal Biotech, USA). Thrombosis was monitored as the time taken for the cessation of carotid artery blood flow, time to occlusion (TTO) after the application of 10% ferric chloride-soaked filter paper as described earlier [19,24].

6.2.6. Platelet isolation

Blood from healthy volunteers was centrifuged at 1000 rpm for 10 min and platelet-rich plasma (PRP) was obtained. The remaining blood was further centrifuged at 2500 rpm for 10 min to obtain platelet poor plasma (PPP). For washed platelets, ACD, apyrase type VII (0.5 U/mL) and PGI_2 (0.5 μM) were added to platelet rich plasma and centrifuged at 2200 rpm for 10 min. Platelets were washed and finally resuspended in Tyrode's HEPES buffer (134 mM NaCl, 2.9 mM KCl, 1.0 mM $MgCl_2$, 10.0 mM HEPES, 5.0 mM glucose, 12.0 mM $NaHCO_3$,0.34 mM Na_2HPO_4, 5 mM glucose, pH 7.4) as described previously [20].

6.2.7. Platelet aggregation assay

A turbidimetric method was applied to measure platelet aggregation, using a four channel-Aggregometer (Chronolog-corp, Havertown, USA). Platelet rich plasma (1×10^8 platelets/ml, 0.45 ml) was pre-treated with vehicle (DMSO, 0.4%) or test compounds for 5 min, followed by stimulation with platelet agonists (collagen, collagen related peptide [CRP-XL] ADP, thrombin receptor activating peptide [TRAP], arachidonic acid [AA], ristocetin and

U46619) at 37 °C. The reactions were allowed to proceed for at least 5–10 min. The percentage of aggregation was calculated by using Aggrolink Software [20].

6.2.8. Coagulation parameters

Coagulation parameters namely thrombin time (TT), prothrombin time (PT), activated partial thromboplastin time (aPTT) were assayed in human plasma samples using commercial kits (Stago, France) as per manufacturer's instructions and measured by using a Coagulometer (Start4 Semi automated, Young Instruments, Stago, France) [19].

6.2.9. Platelet adhesion assay

96-well micro titer plates were coated with fibrillar type I collagen (Chrono-log Corp., USA) overnight, and blocked with BSA (0.005 g/ml) for 1 h. Washed platelets (1×10^8 cells/mL) were preincubated with test compounds or vehicle (DMSO) for 15 min at 37 °C and added to the wells (10^7/well) for 1 h at room temperature. Divalent cation-free adhesion buffer was made by replacing Mg^{2+} (1 mM) in the Tyrode-HEPES buffer with 50 μM EDTA. After three washes in Tyrode's buffer, the number of adherent platelets was evaluated colorimetrically as described by Bellavite et al. [21]. Briefly, 150 μl of a 100 mM citrate buffer (pH 5.4), containing 5 mM *p*-nitrophenyl phosphate and 0.1% TritonX-100 was added to the wells after washing. After incubation for 60 min at 25 °C in the absence of ambient light, colour was developed by the addition of 100 μl of 2 N NaOH and the absorbance at 405 nm was read using a microplate reader (Powerware XS, Biotek, USA). After washing with PBS, the number of adherent platelets was evaluated colorimetrically by a method described previously [21].

6.2.10. Immunoblotting

Washed platelets, pre-incubated with vehicle or test compounds, were stimulated with collagen or U46619 for 10 min. The reaction was stopped by the addition of boiling 4X Laemmli sample loading buffer and the protein samples were subjected to SDS-PAGE and Western blotting as described previously [20,22].

6.2.11. Materials

Fibrillar type I collagen from equine tendon, Arachidonic acid, ristocetin and ADP (Chronolog Corp, Havertown, USA); bovine serum albumin (BSA), Apyrase Type VII, phenylmethyl sulfonylfluoride, Phenylephrine hydrochloride (PE), Acetylcholine chloride (ACh), were purchased from Sigma–Aldrich (St. Louis, MO USA); anti phospho-Tyrosine antibody 4G10 (Upstate Biotechnology, USA), U46619 and SQ29548 from Cayman Chemicals (USA).

6.2.12. Animal welfare and ethical statements

All the animal experiments were conducted according to the ethical guidelines of the IAEC. Human Blood was collected in citrate-phosphate-dextrose (CPD) in the ratio of 1:7 from healthy volunteers after a written consent from the donors at the blood donation centre of Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow. A detailed medical history and physical examination was carried out before phlebotomy.

6.2.13. Data analysis and statistical procedures

All data are presented as mean ± SEM and *P*-values of 0.05 or less were considered to be statistically significant. One-way ANOVA followed by either Newman-Keuls' Multiple Test or Dunnett's Comparison Test was used for statistical analysis, using Graph pad Prism software version 5.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2016.01.019.

Conflicts of interest

The authors declare that there is no conflict of interests.

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