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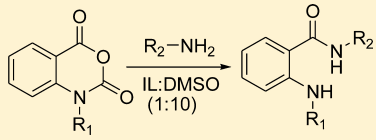
Novel 2-Aminobenzamides as Potential Orally Active Antithrombotic Agents

Amit Verma, Rajani Giridhar, Ashish Kanhed, Anshuman Sinha, Pratik Modh, and Mange R. Yadav*

Pharmacy Department, Faculty of Technology & Engineering, Kalabhavan, The M. S. University of Baroda, Vadodara, 390001 Gujarat, India

Supporting Information

ABSTRACT: In an effort to develop potent antithrombotic agents, a series of novel 2-aminobenzamide derivatives were synthesized and screened for their in vivo antithrombotic activity. Among the 23 compounds tested, compound (**8g**) showed the most promising antithrombotic activity, which was comparable with clinically used aspirin or warfarin, but at variance with these standard drugs, **8g** did not exhibit the increased bleeding time, suggesting its potential as a novel antithrombotic agent.



KEYWORDS: *2-aminobenzamide, antithrombotic agents, ionic liquid, thromboembolic disorders, antiplatelet*

Deep vein thrombosis, myocardial infarction, pulmonary embolism, and stroke have been the most frequent causes of morbidity and mortality due to cardiovascular disorders.[1] Although thrombosis plays a significant role in the development of cardiovascular diseases, the availability of safe antithrombotic drugs is limited. The unmet clinical need for a safe and orally active anticoagulant has resulted in widespread drug discovery efforts in this direction. Prevention of blood coagulation is a major target for new therapeutic agents. Factor Xa (fXa) is a trypsinlike serine protease that forms a prothrombinase complex with factor Va, Ca^{2+}, and phospholipids to produce thrombin. The key enzyme functions at the convergence of the intrinsic and extrinsic coagulation pathways in a process that involves signal amplification. One molecule of fXa activates many molecules of prothrombin to thrombin.[2] As inhibition of fXa prevents thrombin formation without affecting pre-existing thrombin, fXa inhibitors are predicted to cause less impairment of hemostasis than direct thrombin inhibitiors, leading to a wider therapeutic window.[3]

The discovery of orally active, small molecule competitive fXa inhibitors in preclinical animal thrombosis models in recent years has spurred the process for discovery of such molecules as antithrombotic drugs. The benzamidine derivative (**1**) exhibited potent oral anticoagulant activity prolonging prothrombin time (PT) more than 3-fold after oral administration to mice.[4] Thiophene/benzothiophene-substituted anthranilamides (**2**) have been reported as novel and potent inhibitors of human fXa.[5] Anthranilamide-based *N*,*N*-dialkylamidines have been reported to be orally available potent inhibitors of fXa.[6] Darexaban (YM150), a 1,4-diazepanylbenzamide (**3**), has been claimed to be a potent and orally bioavailable fXa inhibitor. The distinctive potent activity of the inhibitor (**3**) after oral dosing was explained by its unique pharmacokinetic profile and its favorable membrane permeability. Interestingly, some 2-substituted benzoylaminobenzoate analogues (**4**) have shown 200 times more potency as antiplatelet aggregating agents than aspirin.[7] A survey of the literature revealed carboxamides and anthranilamides to possess either fXa inhibitory activity or antiplatelet aggregating activity.[8] Betrixaban (**5**), an anthranilamide derivative, is a proven orally active fXa inhibitor useful in the prevention of thromboembolic events.[9] It has undergone human clinical trials for the prevention of embolism after knee surgery and prevention of stroke following atrial fibrillation,[10] with promising results.[11] Taking betrixaban (**5**) as the lead molecule, it was planned to substitute both of the nitrogens with various alkyl/aryl substituents to provide compounds of type **A** and to evaluate the synthesized compounds for their antithrombotic efficacy. It has been revealed earlier that some compounds exhibiting very high in vitro antithrombotic activity failed to produce the desired effect when administered orally. As the designed compounds (**A**) were expected to possess antiplatelet and/or anticoagulant activity, it would require a battery of in vitro tests to be performed to assess the compounds for these activities. For antiplatelet activity, the compounds need to be evaluated against a multitude of agonists like ADP, AA, PMA, A23187, collagen, and thrombin. Separate tests need to be performed for anticoagulant and thrombolytic activities as many proteases are involved in the activation of coagulation cascade. Moreover, it is difficult to test hydrophobic samples as methanol/DMSO can not be used for solubilization of these samples as both of the solvents are potent inhibitors of platelet activation. Realizing the complexity of the problem, it was planned to assess antithrombotic efficacy of the synthesized compounds after oral administration as that would exhibit the overall effect of the compounds on platelets as well as coagulation cascade after oral absorption. So, a simple, reliable, and reproducible animal model was used to assess the antithrombotic activity of the synthesized compounds in the current work. Bleeding time was used as a

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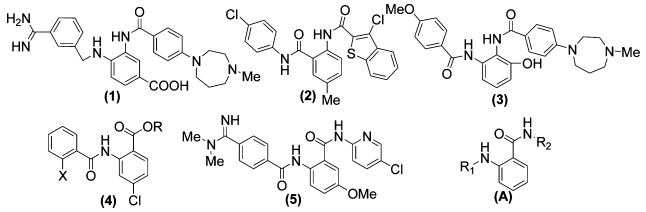
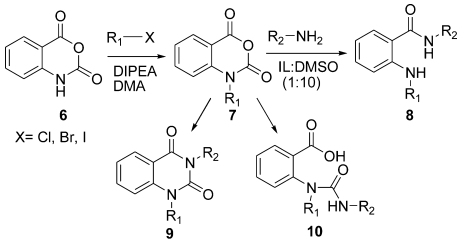
parameter to have an idea of their effect on hemostasis at a particular dose.



In the present study, it was planned to synthesize a new series of 2-aminobenzamides by substituting the 2-amino position and the amidic nitrogen with hydrophobic alkyl and aryl moieties. The route followed for the preparation of such derivatives (**8**) is outlined in Scheme 1.

Scheme 1. Synthesis of 2-Aminobenzamide Derivatives



Nucleophilic substitution of the isatoic anhydride (**6**) in diisopropylethylamine (DIPEA) and dimethylacetamide (DMA) with suitable alkyl halides yielded N-substituted isatoic anhydride (**7a–d**). The N-alkylated derivatives (**7**) on treatment with primary amines in DMF and anhydrous potassium carbonate at 80 °C yielded the desired product (**8a–w**), but it took 8–12 h to complete the reaction. It was observed earlier that heating the reaction mixture at 110–120 °C in toluene provided the cyclic quinazolinone derivatives (**9**) predominantly. So, increasing the temperature of the reaction mixture as a means of decreasing the reaction time was ruled out. Previously,[12] we have used a mixture of ionic liquid:-dimethylsulfoxide (IL:DMSO) (1:10) successfully as the solvent for nucleophilic substitution at an activated carbon. We thought of exploring this green and simple protocol for the above said reaction on substituted isatoic anhydrides (**7**). To our pleasant surprise, the protocol worked very well at room temperature to yield the products quantitatively. Although there was a possibility of formation of products (**9** or **10**), none of these undesired side products were formed. Dibutylimidazolium bromide ([bbim]$^+$[Br]$^-$) was used as IL in these protocols. All of the synthesized compounds were characterized using IR, ^{1}H NMR, and MS. The purity of the compounds was established by TLC and elemental analysis. Experiments were conducted to evaluate the in vivo effects of the synthesized compounds on antithrombotic activity,[13] bleeding time,[14] and PT[15] in mice. The compounds were suspended in 0.5% aqueous CMC (sodium carboxymethyl cellulose) and administered orally. Warfarin as a reference compound was tested under the same conditions, and the results are presented in Table 1. Out of all of the compounds evaluated for activity, 12 compounds showed 40% or higher antithrombotic activity, while the remaining compounds exhibited activity between 20 and 40%. The most promising one was compound **8g**, showing antithrombotic protection of 48% (warfarin under similar

Table 1. Ex Vivo Assay of Novel 2-Aminobenzamide Derivatives for Antithrombotic Activity, Bleeding Time, and Prothrombin Time after 3 Days of Treatment at a Dose of 30 μM/kg*

Entry	R1	R2	AA % protection	BT % increase	PT sec.
Control	--	--	--	--	12.2
8a	Me	Cl	40	100	9.5
8b	Me	F	40	55	11.0
8c	Me	Me, Me	23	100	11.4
8d	Ph	Ph	40	82	8.7
8e	Ph	Cyclohexyl	30	109	9.7
8f	Ph	Cyclopropyl	26	36	10.6
8g	Ph	OMe	48	45	11.2
8h	Ph	Cl	44	65	10.5
8i	Ph	Cl	40	68	10.3
8j	Ph	F	46	55	11.1
8k	Ph	Me, Me	34	100	9.9
8l[a]	Ph	Ph	38	62	10.2
8m	Ph	Ph	32	91	11.9
8n	Allyl	Ph	30	63	9.3
8o	Allyl	Cyclohexyl	26	55	10.2
8p	Allyl	Me, Me	20	65	11.6
8q	Allyl	Me	23	68	9.4
8r	Allyl	Cyclopropyl	20	44	10.2
8s	Allyl	Cl	42	82	10.0
8t	Allyl	Cl	40	70	10.2
8u	Allyl	Cl	42	68	8.5
8v	Allyl	F	44	55	11.4
8w	Allyl	Me, Me	26	50	11.0
8x	Me	Cl	42	100	9.5
Warfarin	--	--	50	45	118.2

*AA, antithrombotic activity; BT, bleeding time; PT, prothrombin time; and control, 0.5% aqueous CMC. [a]Compound (**8l**) is known (ref 18).

conditions showed 50% protection), but at variance with warfarin, **8g** exhibited a low prothrombin time of 11.2 min as against 118.2 min for warfarin, suggesting that the mechanism of the newly synthesized compounds might be different. Next, we evaluated these compounds using aspirin as the standard drug, and the results are presented in Table 2. Nine of the compounds (**8g**, **8h**, **8i**, **8j**, **8s**, **8t**, **8u**, **8v**, and **8x**) showed comparable antithrombotic activity to the standard drug aspirin. The tolerability of the subacute treatment with the compounds was examined by evaluating their adverse prohemorrhagic effect on tail bleeding time. Again, compound **8g** emerged as the most promising one, because in addition to showing 42% protection, it did not increase the bleeding tendency substantially in comparison to aspirin at its optimal

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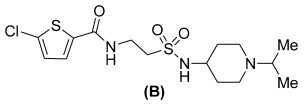
Table 2. Ex Vivo Assay of Novel 2-Aminobenzamides for Antithrombotic Activity as Well as Bleeding Time after 1 h of Treatment at a Dose of 30 μM/kg

entry	AA % protection	BT % increase	entry	AA % protection	BT % increase
8a	35	48	**8n**	20	17
8b	30	45	**8o**	20	58
8c	20	17	**8p**	20	50
8d	30	58	**8q**	20	34
8e	20	34	**8r**	10	25
8f	10	32	**8s**	40	100
8g	40	23	**8t**	30	36
8h	40	59	**8u**	30	20
8i	40	60	**8v**	30	44
8j	40	75	**8w**	20	25
8k	30	33	**8x**	30	83
8l	30	52	aspirin	37 ± 3	100 ± 20
8m	20	38			

antithrombotic dose. Although the other active compounds exhibited good protection, they also caused increase in bleeding time, which was a drawback.

The in vivo toxicity study was also performed for compounds **8g**, **8h**, **8j**, and **8v**. For all of the test compounds (**8g**, **8h**, **8j**, and **8v**), the LD_{50} was found to be >2000 mg/kg.

As fXa is responsible for thrombotic activity ultimately, it was thought of studying the binding interactions of the active compounds using the crystallographic 3D structure of fXa. fXa has a well-identified active site with mainly four recognized regions. It has S1, S2, S4, and an ester binding pocket (EBP) in the active binding space. Among these, S1 and S4 sites are more important for ligand binding and exhibiting the biological activity. S2 is a small pocket separated from S4 by Tyr 99.[16] Extra precision (XP) docking studies were performed for the compounds under investigation. To validate the docking studies under the Glide tool of Schrödinger 2009[17] environment, the cocrystallized molecule (**B**) present in the 3D structure of fXa (PDB Code: 4A7I) was first knocked out of the binding site. The molecule was constructed again, energy-minimized, and redocked into the active site of the enzyme. Very similar interactions were observed between the redocked molecule and the enzyme, as was observed in the original cocrystallized structure, that is, similar orientations of the groups in S1 and S4 binding pockets and binding interactions with Tyr-228, Gln-192, and Gly-216 were observed. The root-mean-square deviation between the predicted conformation and the original conformation of compound **B** as it existed in the X-ray crystallographic structure was found to be 0.26 Å.



(B)

The intermolecular docking interactions of the most active compound (**8g**) are shown in Figure 1. It is clearly observed that occupation of S1 and S4 sites by specific lipophilic functionalities is very important for greater binding affinity of the ligands with the enzyme. To establish the probable mechanistic orientation of the synthesized molecules, the intermolecular interactions of the lead molecule betrixaban were compared with the docking interactions of the most active compound (**8g**). In compound **8g**, the phenyl ring of the *N*-benzyl group fits in the S1 pocket, indicating good lipophilic

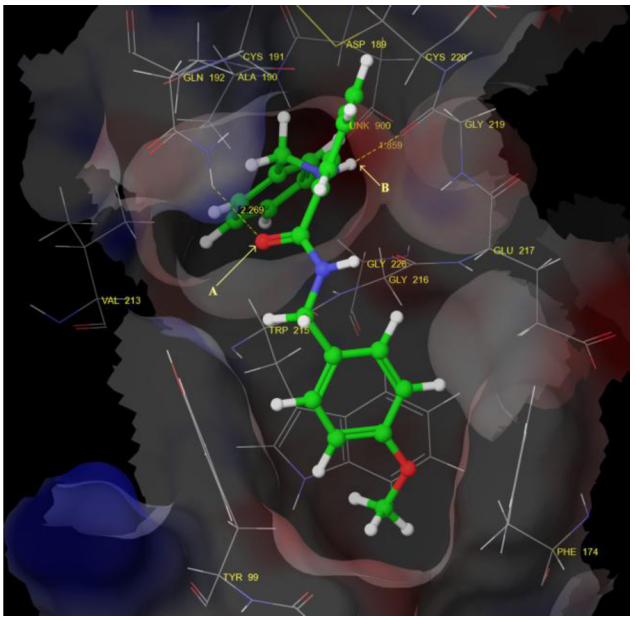


Figure 1. Compound **8g** docked into the active site of fXa (pointers A and B showing H-bonding with Gln-192 and Gly-219, respectively).

interaction, while in betrixaban, the pyridine ring shows a similar interaction in addition to the noncovalent lipophilic interaction between −Cl of pyridine and π-system of Tyr-228 of S1 pocket. Here, −Cl is placed 3.8 Å away from centroid of Tyr-228 aromatic ring. The amino NH of compound **8g** stabilizes the complex by forming hydrogen bond with −C═O of Gly-219 (1.8 Å), whereas in betrixaban, the pyridine amide NH stabilizes the complex by hydrogen bonding with −C═O of Gly-219 (2.1 Å) and with disulfide linkage of Cys-191 and Cys-220 (2.4 Å). The methoxyphenyl component of compound **8g** fits into the S4 binding pocket with centroid to centroid distances of 4.6, 4.3, and 5.1 Å from the phenyl ring of methoxybenzyl moiety to the aromatic rings of Tyr-99, Trp-215, and Phe-174, respectively. The amide −C═O of our ligand (**8g**) interacts with NH of Gln-192 by forming a hydrogen bond (2.26 Å), while in the case of betrixaban, the *N*,*N*-dimethylcarbamimidoyl benzoyl part fits in the S4 binding pocket with centroid to centroid distances of 4.47, 5.60, and 6.35 Å between the aromatic ring of this part and the aromatic rings of Tyr-99, Trp-215, and Phe-174, respectively. From this part of betrixaban, the −C═O and NH of amide shows a hydrogen-bonding interaction with NH of Gln-192 (2.35 Å) and −C═O of Gly-216 (1.90 Å), respectively.

Furthermore, to correlate the structural affinities of cocrystallized compound **B**, betrixaban, and **8g**, the ligand efficiencies (LEs) were calculated. The LEs of **B**, betrixaban, and **8g** were found to be 0.22, 0.29, and 0.29, respectively, which further strengthened the efficacy of **8g** for fXa, as LE values of betrixaban and **8g** are exactly same.

A look at Table 1 reveals that alkyl substituents at both of the nitrogens [amine NH (R1) and amidic NH (R2)] offer compounds (**8o**, **8p**, **8q**, and **8r**) having increased bleeding time but poor antithrombotic activity. This is in consonance with the docking studies wherein poor interactions of both of the alkyl groups in S1 and S4 pockets were observed. Replacement of the alkyl groups at the amidic "N" or the amine "N" by benzyl/substituted benzyl (**8a**, **8b**, **8d**, **8e**, **8f**, **8k**, **8m**, **8s**, **8t**, **8u**, **8v**, and **8x**) led to an increase in antithrombotic

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activity with moderate to high enhancement in bleeding time. Substitution of the alkyl groups at both of the nitrogens with benzyl/substituted benzyl groups offered compounds (**8g**, **8h**, **8i**, and **8j**) with improved antithrombotic activity. Both of these observations are supported by the docking studies. Simultaneous substitution of both of the nitrogens with benzyl/substituted benzyl group yields compounds having much better interactions with the enzyme in both of the vital binding pockets S1 and S4. 4-Methoxybenzyl substituent (**8g**) at amide nitrogen with a benzyl group (R1) at amine nitrogen is responsible for an increase in the antithrombotic activity with a negligible effect on PT. Replacement of 4-methoxyphenyl with halo-substituted phenyl ring maintained the antithrombotic activity in the resulting compounds (**8h**, **8i**, and **8j**), but it led to increase in the bleeding time also. Among the halo derivatives, the fluoro derivative (**8j**) offered the best biological activity profile. For the purpose of comparative study, compound **8l**[18] having benzyl substituents at both of the nitrogens was synthesized and biologically evaluated. In the docking studies also, it was observed that a lipophilic substituent at 4-position of amidic benzyl ring was essential for lipophilic interaction in the S4 pocket of the enzyme with the ligand. It seems substitution of benzyl/substituted benzyl in place of alkyl/cycloalkyl groups at amidic "N" (**8a**, **8b**, **8s**, **8t**, **8u**, **8v**, and **8x**) has a more profound impact on antithrombotic activity than the substitution of benzyl group on amino "N" (**8e**, **8f**, and **8k**). It is interesting to note that the newly reported compound **8g** did not cause an increase in bleeding time or PT at variance with aspirin or warfarin at antithrombotic doses, while maintaining almost the same antithrombotic activity as warfarin. These observations point out that derivative **8g** has the potential to provide a better therapeutic window over warfarin and aspirin.

In summary, a series of novel 2-aminobenzamide derivatives have been synthesized and evaluated orally for antithrombotic activity using warfarin and aspirin as the standard drugs. Docking studies have been performed with fXa to establish a prospective mechanism of action of the synthesized compounds as potential inhibitors of fXa. Some of the compounds exhibited better activity than the reference drugs. Interestingly, the most potent compound **8g** seems to possess a potentially high benefit−risk profile, as it was found to be equipotent to the standard drugs warfarin and aspirin as antithrombotic agents with lesser hemorrhagic tendency.

■ ASSOCIATED CONTENT

Supporting Information

Synthetic procedures, analytical data, procedures for pharmacological activities, details of toxicity studies, and intermolecular interactions in docking studies. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +91-265-2434187. Fax: +91-265-2418927. E-mail: mryadav11@yahoo.co.in.

Author Contributions

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Notes

The authors declare no competing financial interest.

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