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Original Article

Anticoagulant evaluation of 1,3,4-oxadiazole derivatives derived from benzimidazole

B. Vishwanathan* and B.M. Gurupadayya

Department of Pharmaceutical Chemistry, JSS College of Pharmacy, JSS University, Sri Shivarathreeshwara Nagar, Mysore, Karnataka-570 015, India

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Abstract

In the present study, a series of 1,3,4-oxadiazole derivatives (4a-4k) derived from benzimidazole were evaluated for *ex vivo* anticoagulant activity. The anticoagulant study was performed for increase in prothrombin time (PT) and activated partial thromboplastin time (aPTT) at a test dose of 25 mg kg^{-1}. The results of *ex vivo* anticoagulant evaluation revealed that the tested compounds 4a-4k exhibited moderate increase in PT with respect to acenocoumarol (1 mg kg^{-1}) employed as reference drug for increase in PT. While the compounds 4a-4k exhibited minimal increase in aPTT in comparison to unfractionated heparin (500 IU kg^{-1}) employed as reference drug for increase in aPTT. Compounds, 4c, 4b and 4k exhibited substantial anticoagulant activity with increase in PT 32 ± 0.7, 36 ± 0.5 and 41 ± 0.4 s, respectively to that of the reference drug acenocoumarol (48 ± 0.5 s).

Keywords: 1,3,4-Oxadiazole; Activated Partial Thromboplastin Time; Benzimidazole; Prothrombin Time.

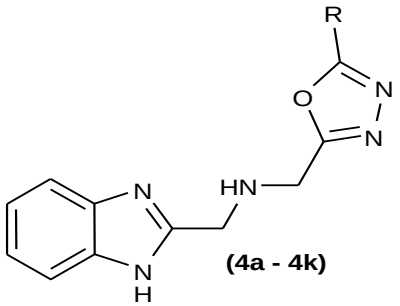
INTRODUCTION

The lacunae in present medication for management of arterial thromboembolism (ATE) disorders by anticoagulant therapy are having recurrent ATE episodes. ATE is the most common cause of cardioembolic events including myocardial infarction, ischemic stroke, and limb gangrene. ATE is currently the leading cause of death and illness in developed countries, principal causes of morbidity and mortality world-wide.[1] Clinical studies highlight that anticoagulants are the drugs of choice for the prevention and treatment of ATE disorders, and prophylaxis of thrombotic events in both pre- and post-surgery in clinical practices.[2] Warfarin, acenocoumarol and phenprocoumon which are the major 4-hydroxycoumarin derivatives presently available in clinical use as oral anticoagulants have been the core of anticoagulantion therapy for more than two decades.[3] Generally known as antivitamin K, pharmacological 4-hydroxycoumarin anticoagulants are vitamin K epoxide reductase (VKOR) inhibitors. Vitamin K is converted to its active form by the enzyme VKOR and recycled to vitamin K 2,3-epoxide to maintain the coagulation cycle.[4] 4-hydroxycoumarin derivatives like warfarin, acenocoumarol and phenprocoumon antagonize VKOR, thus preventing vitamin K recycling and resulting in an accumulation of abnormal form of coagulation protein, known as proteins induced by vitamin K antagonism or des-γ-carboxyprothrombin leading to inhibition of the coagulation process. The narrow therapeutic index and other major clinical drawbacks like drug-drug interaction, food-drug interaction, purple toe syndrome, eclampsia, risk of haemorrhage etc., call for continuous therapeutic drug monitoring for 4-hydroxycoumarin anticoagulants. These entire therapeutic shortcomings indicate the need for better anticoagulant agents with clinical advantage. In our previous study, we had reported the synthesis and characterization of 1,3,4-oxadiaozle derivatives **4a-4k** (Fig. 1), from (1*H*-benzo[*d*]imidazol-2-yl)methanamine.[5] Wherein, *N*-[(1*H*-benzo[*d*]imidazol-2-yl)methyl](5-substituted-1,3,4-oxadiazol-2-yl)methanamine; **4a-4j** (Table 1) were prepared by nucleophilic addition of aryl/heteroaryl/aliphatic carboxylic acids with 2-[(1*H*-benzo[*d*]imidazol-2-yl) methylamino] acetohydrazide in presence of phosphorous oxychloride. The acetohydrazide derivative was prepared by condensation of ethyl 2-[(1*H*-benzo[*d*]imidazol-2-yl)methylamino]acetate with hydrazine monohydrate. The ester derivative was prepared by *N*-alkylation of (1*H*-benzo[*d*]imidazol-2-yl)methanamine with ethyl 2-chloroacetate in the presence of anhydrous potassium carbonate. The

Corresponding Author Address: B. Vishwanathan,** Department of Pharmaceutical Chemistry, JSS College of Pharmacy, JSS University, Sri Shivarathreeshwara Nagar, Mysore, Karnataka-570 015, India**; E-mail: vishwanathan_b@yahoo.co.in

compound 5-{[(1*H*-benzo[*d*]imidazol-2-yl)methyl amino] methyl}-1,3,4-oxadiazole-2-thiol **(4k)** was prepared by condensation of the acetohydrazide with carbon disulphide and potassium hydroxide.

In the present investigation an attempt has been made to find out the *ex vivo* anticoagulant efficacy of the synthesized 1,3,4-oxadiazole derivatives **4a-4k**.



(1*H*-benzo[*d*]imidazol-2-yl)-*N*-[(5-substituted-1,3,4-oxadiazol-2-yl) methyl]methanamine

R= C_6H_5 (4a)
= 2-NO_2-C_6H_4 (4b)
= 3,5-$(NO_2)_2$-C_6H_3 (4c)
= 4-NH_2-C_6H_4 (4d)
= 2-NH_2-C_6H_4 (4e)
= 4-OH-C_6H_4 (4f)
= 2-OH-C_6H_4 (4g)
= H (4h)
= CH_3 (4i)
= 4-C_5H_4N (4j)
= SH (4k)

Fig 1: Synthesized 1,3,4-oxadiazole derivative from benzimidazole 4a-4k.

Table 1: Synthesized 1,3,4-oxadiazole derivatives from benzimidazole 4a-4k.

Sl. No.	Compound
1.	*N*-[(1*H*-benzo[*d*]imidazol-2-yl)methyl](5-phenyl-1,3,4-oxadiazol-2-yl)methanamine (4a)
2.	(1*H*-benzo[*d*]imidazol-2-yl)-*N*-[(5-(2-nitrophenyl)-1,3,4-oxadiazol-2-yl)methyl]methanamine (4b)
3.	(1*H*-benzo[*d*]imidazol-2-yl)-*N*-[(5-(3,5-dinitrophenyl)-1,3,4-oxadiazol-2-yl)methyl]methanamine (4c)
4.	(1*H*-benzo[*d*]imidazol-2-yl)-*N*-[(5-(4-aminophenyl)-1,3,4-oxadiazol-2-yl)methyl]methanamine (4d)
5.	(1*H*-benzo[*d*]imidazol-2-yl)-*N*-[(5-(2-aminophenyl)-1,3,4-oxadiazol-2-yl)methyl]methanamine (4e)
6.	(1*H*-benzo[*d*]imidazol-2-yl)-*N*-[(5-(4-hydroxyphenyl)-1,3,4-oxadiazol-2-yl)methyl]methanamine (4f)
7.	(1*H*-benzo[*d*]imidazol-2-yl)-*N*-[(5-(2-hydroxyphenyl)-1,3,4-oxadiazol-2-yl)methyl]methanamine (4g)
8.	*N*-[(1,3,4-oxadiazol-2-yl)methyl](1*H*-benzo[*d*]imidazol-2-yl)methanamine (4h)
9.	(1*H*-benzo[*d*]imidazol-2-yl)-*N*-[(5-methyl-1,3,4-oxadiazol-2-yl)methyl]methanamine (4i)
10.	(1*H*-benzo[*d*]imidazol-2-yl)-*N*-[(5-(pyridin-4-yl)-1,3,4-oxadiazol-2-yl)methyl]methanamine (4j)
11.	5-{[(1*H*-benzo[*d*]imidazol-2-yl)methylamino]methyl}-1,3,4-oxadiazole-2-thiol (4k)

MATERIALS AND METHODS

The chemical were procured from Sigma Aldrich and were used without further purification. PT and aPTT were determined in BCS XP Blood Coagulometer® Siemens.

Pharmacology: All the animal experimental procedures and protocols adapted in the study were reviewed and approved by the Institutional Animal Ethics Committee. The experimental procedures and protocols were in accordance with the guidelines of the CPCSEA, Ministry of Forests and Environment, Govt. of India. The animals were obtained from the JSS Medical college, Mysore, India, and were maintained in colony cages at 25 ± 2°C, relative humidity of 45-55%, under a 12 h light and dark cycle; they were fed standard animal feed. All the animals were acclimatized for a week before use. Animals were deprived of food 12 h prior to experiment and only water was allowed *ad libitum*.

Acute toxicity studies: Acute toxicity studies were performed to estimate the median lethal dose (LD50) value of the synthesized compounds 4a-4k

as per the OECD guidelines (TG 420) and the testing dose for the newly synthesized compounds on the animal model for the *ex vivo* anticoagulant activity was fixed. The LD50 of the 1,3,4-oxadiazoles were determined as per the reported method. [6]

***Ex vivo* blood coagulation test:** Anti-coagulant activity was evaluated by blood coagulation test method with modification from the reported method [7,8] using groups of albino rats weighing 100-120 g each and 6 rats per group. The first group was given 0.5% carboxy methyl cellulose orally and served as untreated control. The second and third groups were designated as positive control, and the second group received 1 mg kg^{-1} acenocoumarol (Acitrom® Piramal Healthcare) orally as reference standard anticoagulant as a positive control for increase in PT. The third group received 500 IU kg^{-1} unfractionated heparin (Declot® Zydus Cadila) intraperitoneal as reference standard anticoagulant as a positive control for increase in aPTT. The test compounds were suspended in 0.5% carboxy methyl cellulose (CMC) and given to the rats orally at a dose of 25 mg kg^{-1}. At the end of six hours after the administration of the test compounds and reference standard, the animals were anesthetized by intravenous injection of 60 mg kg^{-1} Thiopental sodium (Intraval sodium® Piramal Healthcare) and the caudal caval vein was exposed by a mid-line incision and 1.8 mL blood are collected into a plastic syringe containing 0.2 mL 100 mM citrate buffer pH 4.5. The blood sample was immediately agitated and centrifuged in a plastic tube at 1500 *g* for 10 min. The plasma so obtained was transferred to another plastic tube and the coagulation tests for the determination of PT and aPTT were performed within 3 h. The citrated plasma was coagulated by the addition coagulating agent and the time for the clot formation was determined in the coagulometer, which highlights the time in sec required for the coagulation of the treated and the untreated plasma sample. The mean increase in PT and aPTT of reference drugs; acenocoumarol (1 mg kg^{-1}) and unfractionated heparin (500 IU kg^{-1}), respectively and the tested compounds at 25 mg kg^{-1} concentrations was compared with control using the repeated measures ANOVA with Dunnet's test. Mean, standard error of mean (SEM) calculations and ANOVA test were performed using ''GraphPad Prism version 4.0'' software. The data obtained is expressed as mean ± SEM.

Prothrombin Time (PT): 0.1mL of citrated plasma was incubated for 1 min at 37°C. Then 0.2mL of human thromboplastin (Thromborel S® Siemens) was added and the coagulometer was started. The time to clot formation is determined. The PT highlights effects on the exogenous pathway of coagulation.

Activated Partial Thromboplastin Time (aPTT): To 0.1mL of citrated plasma 0.1mL of human placenta (Dade Actin® Siemens) is added and the mixture is incubated for 2min at 37°C. The coagulation process is initiated by the addition of 0.1mL 25 mM calcium chloride solution. The coagulometer was started and the time for the clot formation was determined. The aPTT measures effects on the endogenous pathway of coagulation.

RESULTS AND DISCUSSION

Anticoagulant activity: The 1,3,4-oxadiazole derivatives 4a-4k were evaluated for *ex vivo* blood coagulation activity at a dose of 25 mg kg^{-1} by measuring increase in PT and aPTT. Wherein, acenocoumarol at 1mg kg^{-1} was used as reference standard for PT and unfractioned heparin was used as reference standard for aPTT. CMC was employed as negative control. The results of the anticoagulant activity at the end of six hours after the administration of test compounds were promising and the obtained data is given in Table 2.

Table 2: The results of PT and aPTT measure values of the test compounds 4a-4k.

Sl. No.	Compound (25 mg kg^{-1})	Anticoagulant efficacy[a,b]	
		PT (s)	aPTT (s)
1.	4a	15.67 ± 0.27***	29.40 ± 0.21***
2.	4b	36.17 ± 0.45***	29.33 ± 0.23***
3.	4c	31.54 ± 0.68***	28.70 ± 0.44*
4.	4d	14.43 ± 0.38***	29.73 ± 0.09***
5.	4e	15.77 ± 0.35***	29.33 ± 0.29***
6.	4f	17.70 ± 0.55***	29.40 ± 0.12***
7.	4g	17.47 ± 0.35***	29.13 ± 0.23***

8.	4h	16.13 ± 0.46***	28.43 ± 0.38
9.	4i	16.10 ± 0.38***	29.07 ± 0.20***
10.	4j	14.23 ± 0.64	27.37 ± 0.18
11.	4k	40.82 ± 0.36***	28.43 ± 0.09
12.	Control	13.00 ± 0.12	27.13 ± 0.54
13.	Acenocoumarol (1 mg kg^{-1})	48.17 ± 0.47***	---
14.	Unfractionated Heparin (500 IU kg^{-1})	---	72.20 ± 0.56***

[a] Results are expressed as the mean values from three independent experiments ± SEM.
[b] Data was analyzed by Dunnet's test. n = 3; (***) equals $P<0.001$, (**) equals $P<0.01$, (*) equals $P<0.05$.

The results highlight that compound 4c (3,5-dinitrophenyl), 4b (2-nitrophenyl) and 4k (mercapto) exhibited a PT measure value of 32 ± 0.7, 36 ± 0.2 and 41 ± 0.4 s respectively, in comparison to that of the reference drug acenocoumarol, which exhibited a PT measure value of 48 ± 0.5 s. The increase in the PT by the compound 4b and 4c indicates the prerequisite of electron withdrawing substituent on the phenyl moiety at C2 position of the oxadiazole series for the possible interaction with VKOR enzyme and inhibiting the same. The other 1,3,4-oxadiazole derivatives 4a and 4d-4j exhibited a minimal increase in PT measure value (16 ± 2 s) due to the lack of an electron withdrawing substituent on the phenyl moiety at C2 position of the oxadiazole series.

Compound 4k (mercapto derivative) exhibited a PT measure value of 41 ± 0.4 s, and the increase in PT can be attributed to the tautomeric thione intermediate as the possible cause for the interaction with VKOR enzyme.
The aPTT measure value of the tested compounds were in the range (28 ± 2 s) as that of negative control (27 ± 0.6 s), in comparison to unfractionated heparin (72 ± 0.6 s) employed as reference standard drug for aPTT measure.

Conclusion

A series of benzofuran encompassing 1,3,4-oxadiazole derivatives were evaluated for *ex vivo* anticoagulant activity by measuring increase in PT and aPTT. Result of present study highlights that, the tested 1,3,4-oxadiazole derivatives exhibited moderate increase in PT and minimal increase in aPTT when compared to standard drug acenocoumarol and unfractionated heparin for PT and aPTT, respectively. Compound 4c, 4b and 4k exhibited a substantial increase in PT measure values and a minimal increase in aPTT measure values.

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