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Short Communication

EX VIVO ANTICOAGULANT ACTIVITY OF 1, 3, 4-OXADIAZOLE DERIVATIVES

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ABSTRACT

Objective: The present medication for the management of arterial thromboembolism (ATE) disorders by anticoagulant therapy highlights its lacunae due to recurrent ATE episodes and indicates the need for better anticoagulant agents with clinical advantage.

Methods: 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}.

Results: The results of *ex vivo* anticoagulant evaluation revealed that the tested compounds 3a-3q did not exhibit a significant increase in PT with respect to acenocoumarol (1 mg kg^{-1}) employed as the reference drug for increase in PT. While the compounds, 3a-3q exhibited minimal increase in aPTT in comparison to unfractionated heparin (500 IU kg^{-1}) employed as the reference drug for increase in aPTT. Among all the tested compounds, only compound 3q exhibited moderate anticoagulant activity with an increase in PT (33 ± 0.4 s) to that of the reference drug acenocoumarol (48 ± 0.5 s).

Conclusion: The anticoagulant efficacy investigation highlights that the synthesized compound 3q could be considered for further clinical studies to ascertain its possible hit as anticoagulant agents.

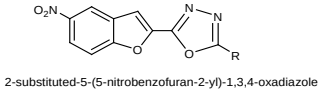
Keywords: 1,3,4-Oxadiazole, Activated Partial Thromboplastin Time, Benzofuran, Prothrombin Time.

Cardiovascular diseases (CVDs), majorly arterial thromboembolism (ATE) currently the leading cause of death and illness in developed countries, with more than 30% of all deaths in the world are from CVDs, as per American Heart Disease foundation, the study highlights that a person has greater chance of dying from heart disease than cancer, AIDS, diabetes and accidents combined [1]. ATE, thus is leading causes of morbidity and mortality world-wide. ATE is the most common cause of cardioembolic events including myocardial infarction, ischemic stroke, and limb gangrene. The treatment of ischemic heart diseases has changed during the past decade as newer approaches have become accessible as prevention of complications has been the cornerstones of treatment. 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].

4-Hydroxycoumarin derivatives mainly warfarin, acenocoumarol, and phenprocoumon have been the core of oral anticoagulation therapy for more than two decades [3]. These coumarin anticoagulants are vitamin K epoxide reductase (VKOR) inhibitors and are marked as antivitamin K. Vitamin K which is required for normal hemostasis as an essential cofactor for γ-carboxylation of glutamic acid residues in blood clotting proteins by vitamin K-dependent carboxylase. Wherein, 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]. Warfarin and other 4-hydroxycoumarin anticoagulants 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. These 4-hydroxycoumarin anticoagulants have numerous clinical drawbacks; these agents have a narrow therapeutic index and require continuous monitoring. Also, these agents have marked food-drug and drug-drug interactions which significantly affect the pharmacokinetics of these agents.

In our previous study, we had reported the synthesis, characterization and evaluation of antioxidant and anti-inflammatory activities of 1,3,4-oxadiaozle derivatives 3a-3q (fig. 1), from ethyl 5-nitrobenzofuran-2-carboxylate [5]. Wherein, 5-(5-nitrobenzofuran-2-yl)-2-substituted-1,3,4-oxadiazole; 3a-3p (table 1) were prepared from nucleophilic addition of aryl/heteroaryl/aliphatic carboxylic acids with 5-nitrobenzofuran-2-carbohydrazide in presence of phosphorous oxychloride. The acylhydrazide derivative was prepared by condensation of ethyl 5-nitrobenzofuran-2-carboxylate with hydrazine monohydrate. The ester derivative was prepared by condensation and followed by cyclization of 2-hydroxy-5-nitrobenzaldehyde with ethyl 2-chloroacetate in the presence of anhydrous potassium carbonate. The compound 5-(5-nitrobenzofuran-2-yl)-1,3,4-oxadiazole-2-thiol (3q) was prepared by condensation of the acylhydrazide derivative with carbon disulfide and potassium hydroxide. The synthesized compounds were evaluated for *in vitro* free radical scavenging activity by 2,2-diphenyl-1-picryl hydrazyl (DPPH) radical assay method using ascorbic acid as standard and *in vivo* anti-inflammatory activity at a dose of 50 mg kg^{-1} by carrageenan induced paw edema method using indomethacin as standard. The compounds 3a-3q exhibited significant antioxidant efficacy ranging from 34 to 86% and significant anti-inflammatory efficacy with edema reduction ranging from 9.1 to 72.5%. The results of anti-inflammatory evaluation revealed that compounds 3c, 3e and 3d exhibited potent anti-inflammatory activity of 72, 68 and 65% respectively.

In the continuation of the work, in our present investigation, we herein report the *ex vivo* anticoagulant efficacy of these seventeen 1,3,4-oxadiazole derivatives 3a-3q.



2-substituted-5-(5-nitrobenzofuran-2-yl)-1,3,4-oxadiazole
(3a-q)

R =H (3a)
=CH_3 (3b)
=C_2H_5(3c)
=C_3H_7(3d)
=$CH(OH)CH_3$(3e)
=CH_2NH_2(3f)
=C_6H_5(3g)
=2-$NO_2C_6H_4$(3h)
=3,5-$(NO_2)_2C_6H_3$(3i)
=4-$NO_2C_6H_4$(3j)
=4-$CH{=}CHC_6H_5$(3k)
=2-NH_2-C_6H_4(3l)
=4-NH_2-C_6H_4(3m)
=2-OH-C_6H_4(3n)
=4-OH-C_6H_4(3o)
=3-C_5H_4N(3p)
=SH(3q)

Fig. 1:2,5-disubstituted1,3,4-oxadiazole derivatives 3a-3q

Table 1: Synthesized 1,3,4-oxadiazole derivatives from benzofuran 3a-3q

S. No.	Compound
1.	2-(5-nitrobenzofuran-2-yl)-1,3,4-oxadiazole(3a)
2.	2-methyl-5-(5-nitrobenzofuran-2-yl)-1,3,4-oxadiazole(3b)
3.	2-ethyl-5-(5-nitrobenzofuran-2-yl)-1,3,4-oxadiazole (3c)
4.	5-(5-nitrobenzofuran-2-yl)-2-propyl-1,3,4-oxadiazole (3d)
5.	1-[5-(5-nitrobenzofuran-2-yl)-1,3,4-oxadiazol-2-yl]ethanol (3e)
6.	[5-(5-nitrobenzofuran-2-yl)-1,3,4-oxadiazol-2-yl]methanamine (3f)
7.	5-(5-nitrobenzofuran-2-yl)-2-phenyl-1,3,4-oxadiazole (3g)
8.	5-(5-nitrobenzofuran-2-yl)-2-(2-nitrophenyl)-1,3,4-oxadiazole (3h)
9.	2-(3,5-dinitrophenyl)-5-(5-nitrobenzofuran-2-yl)-1,3,4-oxadiazole (3i)
10.	5-(5-nitrobenzofuran-2-yl)-2-(4-nitrophenyl)-1,3,4-oxadiazole (3j)
11.	5-(5-nitrobenzofuran-2-yl)-2-styryl-1,3,4-oxadiazole (3k)
12.	2-(2-aminophenyl)-5-(5-nitrobenzofuran-2-yl)-1,3,4-oxadiazole (3l)
13.	2-(4-aminophenyl)-5-(5-nitrobenzofuran-2-yl)-1,3,4-oxadiazole (3m)
14.	2-(2-hydroxyphenyl)-5-(5-nitrobenzofuran-2-yl)-1,3,4-oxadiazole(3n)
15.	2-(4-hydroxyphenyl)-5-(5-nitrobenzofuran-2-yl)-1,3,4-oxadiazole (3o)
16.	5-(5-nitrobenzofuran-2-yl)-2-(pyridin-3-yl)-1,3,4-oxadiazole (3p)
17.	5-(5-nitrobenzofuran-2-yl)-1,3,4-oxadiazole-2-thiol (3q)

The chemical was procured from Sigma Aldrich and was used without further purification. PT and aPTT were determined in BCS XP Blood Coagulometer® Siemens. All the animal experimental procedures and protocols adapted in the study were reviewed and approved by the Institutional Animal Ethics Committee (Ethical approval number – 059/2010). 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 were performed to estimate the median lethal dose (LD50) value of the synthesized compounds 3a-3q 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 was determined as per the reported method [6].

Anticoagulant 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 the untreated control. The second and third groups were designated as the 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 an 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 an 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.

Table 1: The results of PT and aPTT measure values of the test compounds 3a-3q.

S. No.	Compound (25 mg kg^{-1})	Anticoagulant efficacy[a,b]	
		PT (s)	aPTT (s)
1.	3a	12.67 ± 0.27	28.37 ± 0.42
2.	3b	13.10 ± 0.25	29.33 ± 0.23***
3.	3c	13.40 ± 0.60	28.70 ± 0.44*
4.	3d	12.43 ± 0.38	27.80 ± 0.32
5.	3e	12.77 ± 0.35	29.33 ± 0.29***
6.	3f	13.70 ± 0.55	27.87 ± 0.15
7.	3g	13.47 ± 0.35	29.13 ± 0.23***
8.	3h	14.13 ± 0.46	28.43 ± 0.38
9.	3i	14.10 ± 0.38	29.07 ± 0.20***
10.	3j	13.23 ± 0.64	27.37 ± 0.18
11.	3k	14.03 ± 0.38	28.43 ± 0.09
12.	3l	12.80 ± 0.44	29.40 ± 0.12***
13.	3m	13.90 ± 0.44	29.73 ± 0.09***
14.	3n	13.93 ± 0.35	28.10 ± 0.25
15.	3o	14.00 ± 0.45	28.23 ± 0.24
16.	3p	12.23 ± 0.18	28.10 ± 0.25
17.	3q	32.50 ± 0.38***	29.40 ± 0.21***
18.	Control	13.00 ± 0.12	27.13 ± 0.54
19.	Acenocoumarol (1 mg kg^{-1})	48.17 ± 0.47***	---
20.	Unfractionated Heparin (500 IU kg^{-1})	---	72.20 ± 0.56***

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

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 was performed using ''GraphPad Prism version 4.0'' software. The data obtained is expressed as mean ± SEM.

0.1 ml* of citrated plasma was incubated for 1 min at 37°C. Then 0.2 ml* 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.

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

The 1,3,4-oxadiazole derivatives 3a-3q were evaluated for *ex vivo* blood coagulation activity at a dose of 25 mg kg^{-1} by measuring an increase in PT and aPTT. Wherein, acenocoumarol at 1mg kg^{-1} was used as the reference standard for PT and unfractioned heparin was used as the reference standard for aPTT. CMC was employed as the negative control. The result of the anticoagulant activity at the end of six hours after the administration of test compounds were not significant or promising since all the tested compounds (except the mercapto derivatives 3q) did not produce a significant increase in PT values. The PT measure values of the test compounds 3a-3p were in normal range (13 ± 1 s) as that of negative control (13 ± 0.1 s), in comparison to acenocoumarol (48 ± 0.5 s) employed as reference standard drug for PT measure. While, the mercapto derivative of the 1,3,4-oxadiazole derivative; 3q exhibited a moderate increase in prothrombin time (32.5 ± 0.4 s). The increase in PT by the mercapto derivatives highlights the possible inhibition of vitamin K epoxide reductase enzyme by the tautomeric intermediate.

All the tested compounds exhibited minimal increase in aPTT values. The aPTT measure value of the tested compounds were in normal range (28 ± 2 s) as that of negative control (27 ± 0.6 s), in comparison with unfractionated heparin (72 ± 0.6 s) employed as reference standard drug for aPTT measure. The result of the anticoagulant activity is given in table 2.

The results highlight that compounds 3b (methyl derivative), 3e (ethanol derivative), 3g (phenyl derivative), 3i (3,5-dinitrophenyl derivative), 3l (2-aminophenyl derivative), 3m (4-aminophenyl derivative) and 3q (mercapto derivative) exhibited a significant increase in aPTT measure value in comparison to negative control CMC. The aPTT measure values are not substantial in comparison to unfractionated heparin (72 ± 0.6 s) employed as reference standard drug for aPTT measure. The other 1,3,4-oxadiazole derivatives exhibited a minimal increase in aPTT measure value.

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 minimal increase in aPTT and failed to produce any increase in PT when compared to standard drug acenocoumarol and unfractionated heparin for PT and aPTT, respectively. Compound 3q exhibited moderate increase in PT (33 ± 0.4 s) and minimal increase in aPTT (29 ± 0.2 s). The increase in PT by the compound can be attributed to its antagonizing action on VKOR enzyme.

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