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Synthesis, antiplatelet and antithrombotic activities of resveratrol derivatives with NO-donor properties

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ABSTRACT

Resveratrol (RVT) is a stilbene with a protective effect on the cardiovascular system; however, drawbacks including low bioavailability and fast metabolism limit its efficacy. In this work we described new resveratrol derivatives with nitric oxide (NO) release properties, ability to inhibit platelet aggregation and *in vivo* antithrombotic effect. Compounds (**4a**–**f**) were able to release NO *in vitro*, at levels ranging from 24.1% to 27.4%. All compounds (**2a**–**f** and **4a**–**f**) have exhibited platelet aggregation inhibition using as agonists ADP, collagen and arachidonic acid. The most active compound (**4f**) showed reduced bleeding time compared to acetylsalicylic acid (ASA) and protected up to 80% against *in vivo* thromboembolic events. These findings suggest that hybrid resveratrol-furoxan (**4f**) is a novel lead compound able to prevent platelet aggregation and thromboembolic events.

Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) (RVT) is a natural non-flavonoid polyphenolic compound found in several sources including grapes, peanuts, apples, plums, a variety of berries and products derived therefrom.[1] RVT has received worldwide attention after studies suggesting an inverse correlation between wine consumption and reduced incidence of coronary heart disease.[2] Moreover, animal studies have shown effects of RVT against several diseases including cancer, cardiovascular disorders, inflammation and neurodegenerative diseases.[3,4]

For the cardiovascular diseases, RVT has exhibited anti-hypertensive activity and *in vivo* protective effects against stroke, myocardial ischemia and heart failure.[5] Multiple targets are implicated in these effects, including sirtuins (e.g. SIRT-1), nuclear factor-kappa B (NF-κB), AMP-activated protein kinase (AMPK) and nuclear factor (erythroid-derived 2)-like 2 (Nrf2).[5,6] RVT also reduces *in vitro* platelet aggregation induced by ADP, collagen and thrombin[7,8] and upregulates eNOS, enhancing nitric oxide-mediated vasodilation.[9] *In vivo* studies have demonstrated that resveratrol exhibits protective effect on rats that have undergone portal vein system thrombosis.[10]

Interestingly, it was reported that RVT acts synergically with hypocholesterolemiant drugs (e.g. pravastatin) by down-regulating the enzyme 3-hydroxy-3-methyl-glutaryl-CoAreductase (HMG-CoA reductase) involved in cholesterol biosynthesis.[11] Hypercholesterolemic mice have exhibited a reduced atherosclerotic area after systemic administration of this stilbene.[12] RVT also increases the expression of LDL receptors in hepatocytes contributing to the prevention of atherosclerosis.[13] All of these effects, associated with antioxidant/anti-inflammatory activities, reduction of expression of adhesion molecules (e.g. ICAM, VCAM) and ability to inhibit smooth cell migration to atherogenic plate make this natural product an interesting prototype for drug design.

However, despite the beneficial effects, RVT exhibits unfavorable pharmacokinetic properties with reduced bioavailability and fast pre-systemic metabolism.[14] Several clinical trials have failed to prove the efficacy of RVT in humans. Therefore, molecular modifications are necessary to improve its pharmacokinetic and pharmacodynamic profiles.

Nitric oxide (NO) is one of the crucial components that maintain the vasculature homeostasis and regulates intracellular signaling pathways.[15] NO decreases endothelial cell adhesion, inhibits platelet aggregation and thereby limits the thrombotic process.[16] The platelet aggregation inhibition effect of NO is attributed to upreg-

Abbreviations: ASA, acetylsalicylic acid; ADP, adenosine diphosphate; AA, arachidonic acid (AA); NO, nitric oxide; DNS, isosorbide dinitrate; RVT, resveratrol.

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ulation of cyclic guanosine monophosphate, leading to a reduced dimerization of integrin αIIbβ3 and inhibition of von Willebrand factor (VWF)-mediated platelet adhesion.[17,18] Furoxan (1,2,5-oxadiazole-2-*N*-oxide) is a member of a class of heterocyclic compounds known for their ability to release nitric oxide[19–21] with platelet aggregation inhibition and antithrombotic effects.[22–24]

In this study, molecular modification was used to design new hybrid furoxan-resveratrol derivatives design as platelet antiaggregant and antithrombotic. First, bioisosteric replacement of the alkene subunit presents in the resveratrol to *N*-acylhydrazone subunit leads to compounds (**2a**–**f**) (Fig. 1). We have reported bioisosteric replacement of the double bond present in RVT to the *N*-acylhydrazone scaffold.[25] Resveratrol derivatives **2a**–**f** were optimized aiming to embed the phenylsulphonyl subunit as a NO release scaffold resulting in resveratrol derivatives-furoxan hybrid (**4a**–**f**).[26] The combination of resveratrol derivatives effects with NO activity might enhance both platelet aggregation inhibition and antithrombotic effects.

Compounds **2a**–**f** were prepared through a condensation reaction involving benzhydrazides and aldehydes functionalized in ethanol using acidic catalysis.[22,25] (Supplementary material) (Scheme 1). All these compounds were obtained in excellent yields ranging from 70 to 90%. Compounds **4a**–**f** were synthesized through reactions of hydroxybenzaldehydes and bis-arylsulfonylfuroxan (Scheme 2). The intermediates containing the aldehyde function were reacted with functionalized benzhydrazides in order to obtain *N*-acylhydrazones.[21] The structures of all compounds were established by infrared spectroscopy, and ^{1}H- and ^{13}C-nuclear magnetic resonance (^{1}H- and ^{13}C NMR). The compounds were identified by ^{1}H NMR and their spectra showed chemical shifts ranging from 8.32 to 8.36 ppm and 9.62 to 9.98 ppm for H—C=N and N—H, respectively. All of the compounds were analyzed by high-performance liquid chromatography and their purities were >98.5%.

The ability of all compounds to release nitric oxide in the presence of cysteine was evaluated through *in vitro* assays using Griess reaction.[24] In solution, nitric oxide is quickly oxidized to nitrite, which is measured in the medium. Table 1 summarizes the results expressed as percentage of nitrite (NO_2^-, mol/mol). All furoxan derivatives (**4a**–**f**) were capable of inducing nitrite formation at levels ranging from 24.1 to 27.4%. Compounds (**2a**–**f**), as well as aspirin, did not generate nitrite in the medium. Isosorbide dinitrate (DNS), used as control, was able to generate nitrite at levels of 12.1% (not shown).

The inhibition of platelet aggregation for all compounds were evaluated *in vitro* using platelet-rich plasma in the presence of ADP (10 μM), arachidonic acid (100 μM) and collagen (5 μg/mL).[27] All compounds (**2a**–**f** and **4a**–**f**) at 150 μM were able to inhibit platelet aggregation induced by these agonists. For ADP, for example, the inhibition ranged from 31.3% to 91.9%. Compound (**3**) inhibits around 89.8% platelet aggregation induced by ADP, however, this compound have shown weak inhibition in the presence of the agonists arachidonic acid or collagen. The presence of phenylsulfonyl-furoxan subunit increased the antiaggregant effect for compounds **4a**–**f** compared to its parent (**2a**–**f**). For compound **4f**, for example, the presence of furoxan subunit increased by 2.8 times the platelet aggregation inhibitory effect compared to **2f** (Table 1). For arachidonic acid (AA) used as agonist, the platelet aggregation inhibition ranged from 6.7% to 79.6%. Interestingly, the presence of the phenylsulfonyl-furoxan subunit for compounds

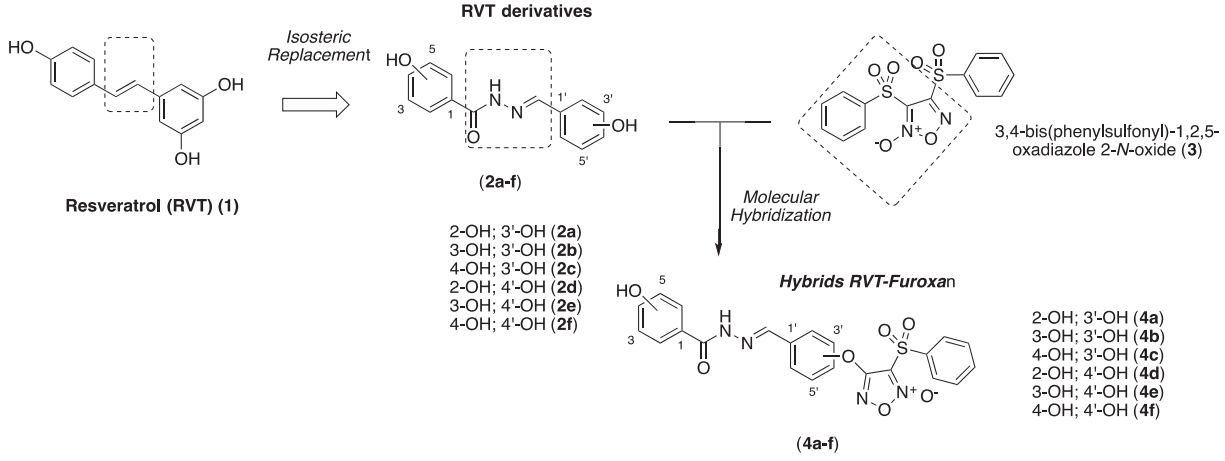
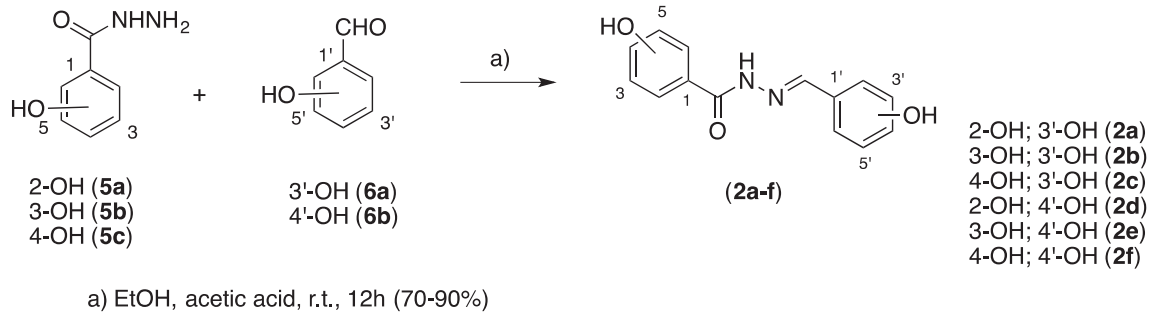
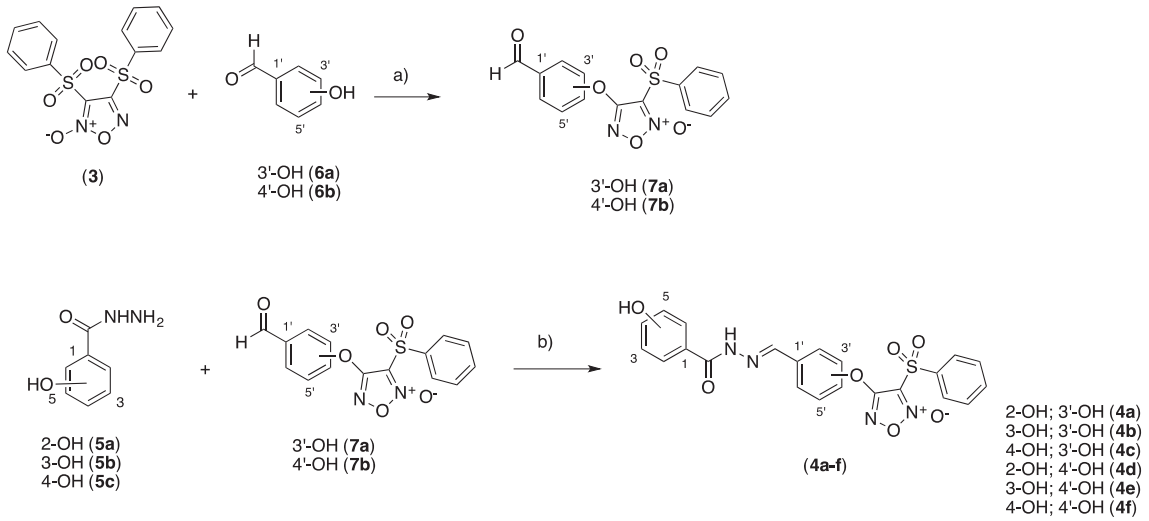


Fig. 1. Design of hybrids RVT derivatives-Furoxan using molecular modifications.



Scheme 1. Synthesis of resveratrol derivatives (**2a**–**f**).



a) hydroxybenzaldehyde (**6a** or **6b**), DBU, CH_2Cl_2, 25°C, 2 to 4 h (46 - 57%); b) aminobenzohydrazide derivative (**5a-c**), ethanol, acetic acid, 25°C, 12 h (60-90%).

Scheme 2. Synthesis of hybrids resveratrol derivatives- furoxan (**4a–f**).

Table 1
Inhibition of platelet aggregation induced by collagen (5 μg/mL), arachidonic acid (100 μM) and ADP (10 μM) for resveratrol derivatives.

Compounds	Collagen (5 μg/mL)[a] Inhibition (%)	Arachidonic acid (100 μM)[a] Inhibition (%)	ADP (10 μM)[a] Inhibition (%)	% NO_2^- (mol/mol^{-1})[b] L-Cys 50 × 10^{-4} M
ASA	63.4 ± 4.0	28.9 ± 8.0	27.7 ± 1.8	–
3	29.7 ± 4.8*	6.7 ± 4.0*	89.8 ± 1.5*	32.1 ± 1.7
2a	27.5 ± 3.5*	60.8 ± 4.8*	56.0 ± 0.4*	–
2b	29.1 ± 7.5*	48.2 ± 3.0*	46.1 ± 0.6*	–
2c	33.1 ± 10.2*	74.1 ± 4.0*	42.4 ± 0.4*	–
2d	35.9 ± 3.8*	42.5 ± 2.0*	58.2 ± 0.5*	–
2e	19.1 ± 10.0*	72.8 ± 3.0*	60.3 ± 0.4*	–
2f	44.0 ± 10.0*	79.6 ± 3.8*	31.3 ± 0.6	–
4a	25.5 ± 5.2*	17.0 ± 1.4*	84.2 ± 0.5*	25.3 ± 1.3
4b	44.8 ± 9.3*	25.0 ± 3.5	88.4 ± 0.6*	24.1 ± 2.6
4c	39.9 ± 9.2*	14.3 ± 6.3*	91.9 ± 0.6*	27.4 ± 3.1
4d	34.4 ± 10.2*	8.5 ± 3.3*	74.3 ± 0.3*	24.5 ± 2.3
4e	54.3 ± 3.3*	37.3 ± 9.3	82.2 ± 0.8*	26.2 ± 2.7
4f	72.2 ± 3.5*	20.3 ± 0.5	87.1 ± 0.6*	25.8 ± 2.4

[a] Results are expressed as the mean for n = 3 independent experiments performed in triplicate. All compounds were evaluated at 150 μM.
[b] All values are the mean ± SEM. Determined by Griess reaction, after incubation for 1 h at 37 °C in pH 7.4 buffered water, in the presence of 1:50 M excess of L-cysteine).
* $P < 0.01$ versus the control group ASA (ANOVA followed by Tukey test).

4a–f reduced dramatically the capacity to inhibit platelet aggregation compared to resveratrol derivatives (**2a–f**). For collagen at 5 μg/mL, the platelet aggregation inhibition ranged from 19.1% to 72.2%. The presence of the phenylsulfonyl-furoxan subunit increased the platelet aggregation inhibition for compounds **4b**, **4c**, **4e** and **4f** compared to their respective parental compounds.

Bleeding time is an assay used to evaluate platelet function and the capacity of our body to form a clot. Increased bleeding time is a common adverse effect for all platelets antiaggregant drugs that limits their clinical use. In this work, the most promising compound **4f** and its parent **2f** were evaluated in order to characterize its ability to alter the bleeding time.[28] Both **2f** and **4f** have exhibited bleeding times of 200 s and 380 s, respectively. For compound (**2f**), the values are similar to those found in the negative control (CMC 0.5%). Acetyl salicylic acid (ASA) has exhibited bleeding time of around 800 s (Fig. 2).

In vivo antithrombotic activity was evaluated using mouse pulmonary thromboembolism model after oral administration in a single dose.[29–32] Compound **3** did not exhibit protective effect

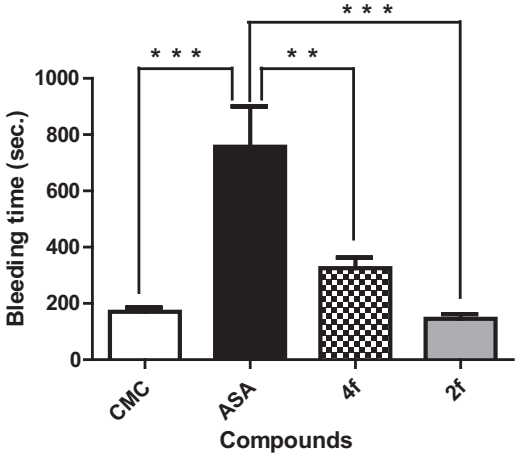


Fig. 2. *In vivo* bleeding time effect from compounds (**2f**), (**4f**) and (ASA). ** $P < 0.001$; *** $P < 0.0001$, ANOVA followed by Tukey test.

Table 2
In vivo antithrombotic effect of compounds (**2f**), (**4f**) and acetylsalicylic acid (ASA).

Groups	Paralysed[a] animals/total	Death	% Protection
ASA	6/10	4	40
2f	6/10	4	40
4f	2/10*	1*	80*

Compounds were orally administered at a dose of 100 μmol/10 g body weight (0.1 mL/10 g body weight). The χ^2 test was used to compare the survival rate between the control and treated groups. *$P < 0.05$ compared with ASA. The vehicle did not exhibit protective effect and no death was observed in this group.
[a] The loss of the righting reflex was considered to indicate paralysis.

(not shown). Compounds **2f** and **4f** have demonstrated protection against thrombus formation at levels of 40% and 80%, respectively (Table 2). ASA, used as control, has protected 40% against these events. For compounds **2f** and ASA the survival rate was 60%, while for **4f** this rate was 90%.

In conclusion, we synthesized new resveratrol (**2a**–**f**) and resveratrol-furoxan hybrids (**4a**–**f**) and evaluated their capacity to prevent thromboembolic events. Compounds **4a**–**f** have shown NO-released properties due to the presence of phenylsulfonyl-furoxan. Among the series, **4f** has inhibited platelet aggregation induced by ADP, arachidonic acid and collagen. This compound has shown bleeding time 4-fold less than ASA. Moreover, compound **4f** demonstrated potent *in vivo* antithrombotic effect using mouse pulmonary thromboembolism model, protecting up to 80% of mice against thromboembolic events while ASA protection was up to 40%. Based on these results, compound **4f** has emerged as a novel lead compound able to prevent platelet aggregation and thromboembolic events.

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2017.04.007.

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