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Keywords: Acetylsalicylic acid; molsidomine; synthesis; nitrogen oxide liberation; isolated heart; vasoactivity; coronary flow

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Abstract: Ischemic heart conditions are among the main causes of sudden cardiac death worldwide. One of the strategies for avoiding myocardial infarction is the low-dose, prophylactic use of acetylsalicylic acid (ASA), an inhibitor of platelet aggregation. To avoid the gastrointestinal damage, ASA prodrugs bearing nitric oxide (NO)-donating moiety covalently conjugated to ASA have been synthesized and evaluated extensively worldwide. Herein the synthesis of a new hybrid ASA ester covalently attached to the NO donor linsidomine, an active metabolite of molsidomine (MOL) is reported. Cell viability assay and hemolysis tests were performed in H9c2 cells and rat erythrocytes, respectively. Our new compound, the ERJ-500 not affected negatively the viability of living cells in the concentration range of 100 nM to 100 µM. Using the ex vivo Langendorff method on hearts originated from female rats, compound ERJ-500 displayed a dose-dependent, outwashable vasodilative effect in coronary arteries. Vasodilation was observed on isolated working heart model as well, with elevated stroke volume in hearts treated with ERJ-500. Furthermore, a decreased infarct size was also noticed in ERJ-500 treated hearts after ischemia/reperfusion. Based on these observations it can be expected that our new hybrid ASA may contribute to new pharmacological tool in the therapy of ischemic heart conditions and associated syndromes.

*Cover Letter

Dear Editor:

Please find attached a manuscript entitled "A NEW, VASOACTIVE HYBRID ASPIRIN CONTAINING NITROGEN MONOXIDE-RELEASING MOLSIDOMINE MOIETY" by Szoke *et al*., which we are here submitting for consideration as a full length article for publication in *European Journal of Medicinal Chemistry*.

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It is additionally here affirmed that all authors have made substantial contributions to creation of this manuscript in each of the following areas: (1) the conception and design of the study, or acquisition of data, or analysis and interpretation of data, (2) drafting the article or revising it critically for important intellectual content, (3) final approval of the version to be submitted.

The authors further affirm that no conflict of interest exists with respect to the topic material of the submitted manuscript.

Sincerely,

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Graphical Abstract (for review)

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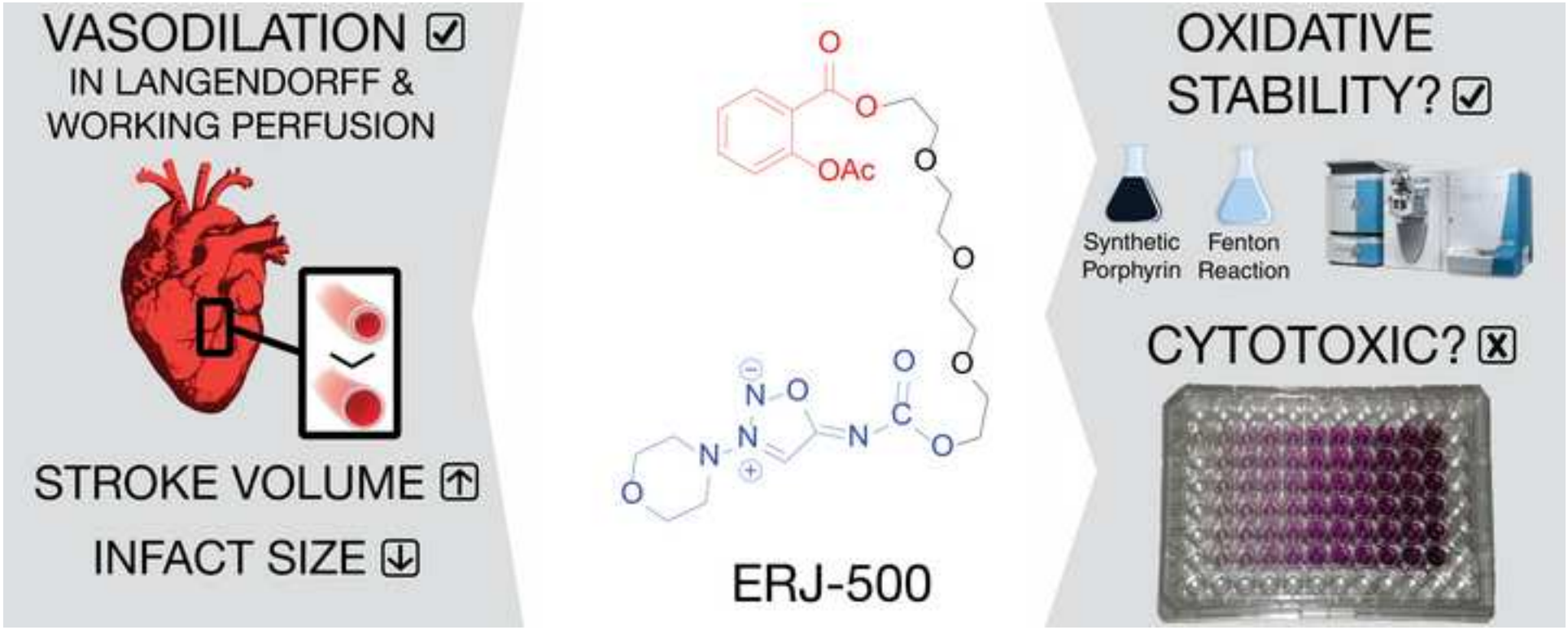

-We have synthesized a new vasoactive, water soluble hybrid aspirin (ERJ-500).

-Our molecule has no toxic and/or hemolytic effects in vitro.

- ERJ-500 induced coronary flow increment and reduced infarct size.

A NEW, VASOACTIVE HYBRID ASPIRIN CONTAINING NITROGEN MONOXIDE-RELEASING MOLSIDOMINE MOIETY

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ABSTRACT

Ischemic heart conditions are among the main causes of sudden cardiac death worldwide. One of the strategies for avoiding myocardial infarction is the low-dose, prophylactic use of acetylsalicylic acid (ASA), an inhibitor of platelet aggregation. To avoid the gastrointestinal damage, ASA prodrugs bearing nitric oxide (NO)-donating moiety covalently conjugated to ASA have been synthesized and evaluated extensively worldwide. Herein the synthesis of a new hybrid ASA ester covalently attached to the NO donor linsidomine, an active metabolite of molsidomine (MOL) is reported. Cell viability assay and hemolysis tests were performed in H9c2 cells and rat erythrocytes, respectively. Our new compound, the **ERJ-500** not affected negatively the viability of living cells in the concentration range of 100 nM to 100 µM. Using the *ex vivo* Langendorff method on hearts originated from female rats, compound **ERJ-500** displayed a dose-dependent, outwashable vasodilative effect in coronary arteries. Vasodilation was observed on isolated working heart model as well, with elevated stroke volume in hearts treated with **ERJ-500.** Furthermore, a decreased infarct size was also noticed in ERJ-500 treated hearts after ischemia/reperfusion. Based on these observations it can be expected that our new hybrid ASA may contribute to new pharmacological tool in the therapy of ischemic heart conditions and associated syndromes.

Keywords

Acetylsalicylic acid, molsidomine, synthesis, nitrogen oxide liberation, isolated heart, vasoactivity, coronary flow.

1. INTRODUCTION

Acetylsalicylic acid (ASA), also known as aspirin - the oldest non-steroidal anti-inflammatory drug (NSAID) - is extensively used for the treatment of pain and inflammation and because of its antithrombotic properties, it is also commonly used for the prophylaxis against myocardial infarction and stroke. The anti-inflammatory action of ASA is based on the inhibition of cyclooxygenase (COX1 and COX2) enzymes involved in prostaglandin (PG) biosynthesis [1]. However, ASA is a much more potent inhibitor of COX1 isoenzyme than that of COX2 [2]. Moreover, ASA irreversibly inhibits COX1 in platelets, consequently resulting in the inhibition of thromboxane A2 biosynthesis [3]. Since thromboxane A2 is a potent platelet aggregator and causes vasoconstriction, this inhibitory process affects the antiplatelet-aggregation property of ASA. Low-dose, long-term prophylactic use of ASA is limited by its strong local irritant effects and gastrotoxicity [4, 5] and ulcerative ability.

There is an increasing number of experimental data supporting basic physiological and protective roles of nitrogen monoxide, also called nitric oxide (NO), and nitrogen monoxide-releasing molecules (NMRMs) in injured tissues [6-9]. The main source of endogenous NO is nitrogen monoxide synthase (NOS). NOS/NO system was proved to play an important role in signaling mechanisms and several physiological processes, including the maintenance of neuronal [10], immune [11], and cardiovascular functions [12]. Moreover, NO acts as a crucial signaling molecule and an effector mediator to regulate the coronary artery function in the myocardium [13]. However, overexpression of inducible NOS and its consequence, an extensive increase in endogenous NO production may not be beneficial for the myocardium [14, 15]. On the other hand, molecules releasing NO including molsidomine (MOL) are used as antihypertensive and antianginal drugs.

The strategy for avoiding the systemic gastrointestinal damage, ASA prodrugs bearing nitrogen monoxide (NO)–donating moiety covalently attached to the carboxylic function of ASA were designed since locally released NO is able to trigger anti-inflammatory effects [16, 17]. Nitrate ester [18-20], furoxan [21], or diazenium-diolate derivatives [22-24] have been attached covalently to ASA to form ester-type prodrugs. The biological activity of these hybrid aspirins has been evaluated extensively. Thus, nitrate ester derivative NCX4016 prevented thromboembolism and restenosis and protected the heart from ischemia/reperfusion injury in animal models [25] displaying no gastrotoxicity in the stomach. Further beneficial effects include the inhibition of platelet COX1 activation and favorable influence on platelet-

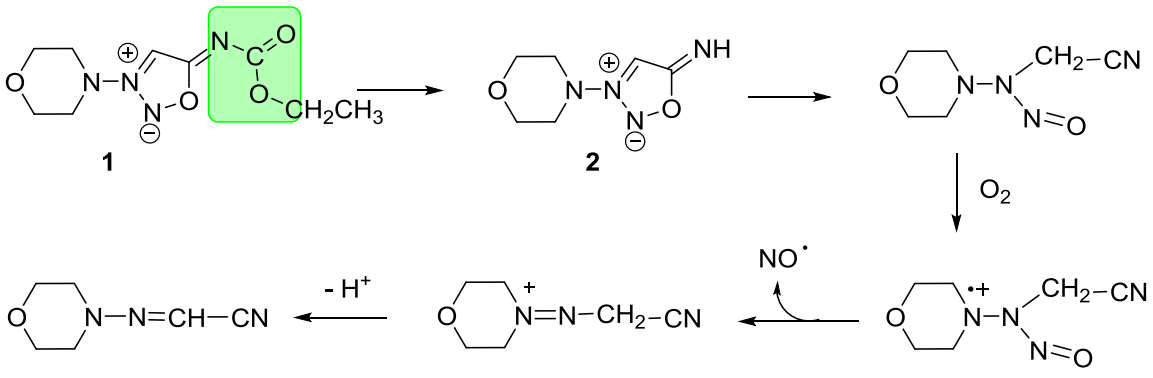
activation function in healthy volunteers [25]. Additional advantageous properties of ASA and NMRMs include anti-inflammatory and gastrosparing activities [19-24]. Furthermore, various NO donors have been developed as pharmacological tools to induce the protective effect of the ischemic myocardium [26]. The NMRMs release NO into biological systems for therapeutic purposes in a controlled and safe manner [27]. The cardiovascular effects of NMRMs are currently under intensive investigation and various classes of compounds are being developed with the goal of exploiting therapeutic potentials in the treatment of inflammatory and cardiovascular diseases [28, 29]. Thus, it is quite rational to hypothesize that an NMRM bearing ASA and molsidomine may have vasoactive, and COX inhibitor activity.

2. RESULTS AND DISCUSSION

2.1 Chemistry

Design and synthesis of ERJ-500

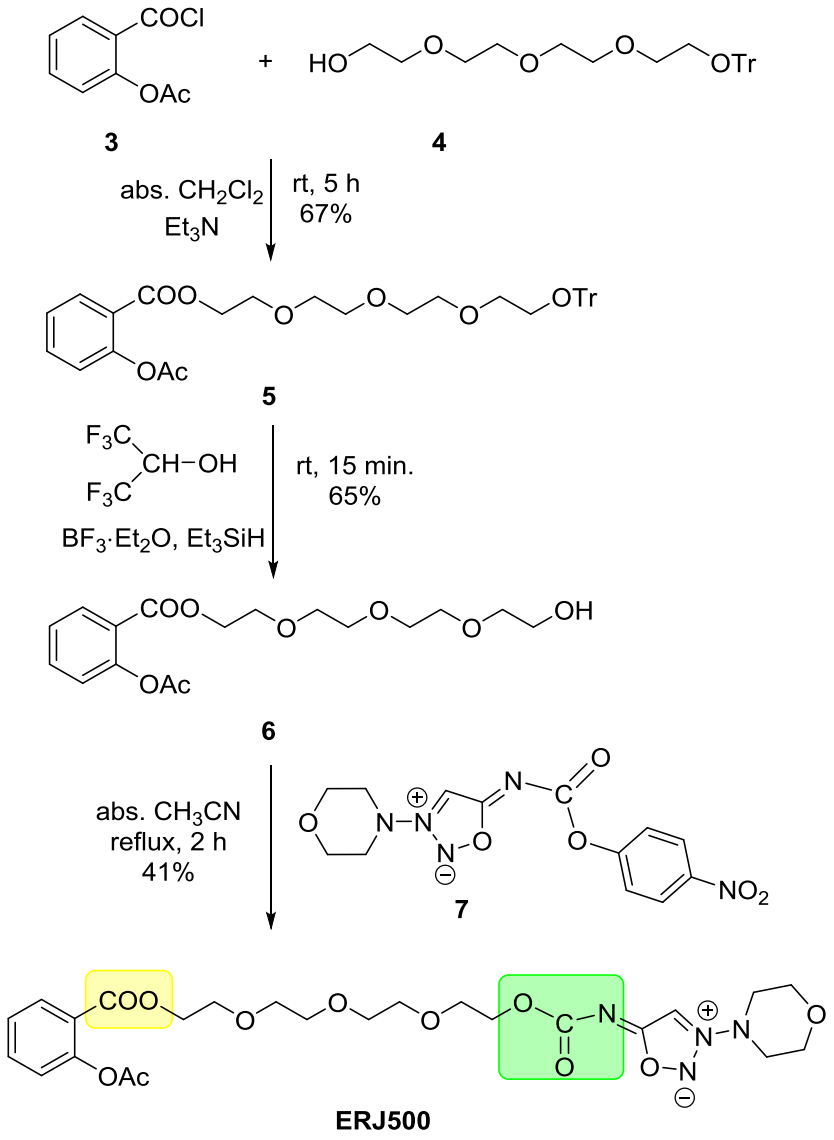
Starting with a research program for the synthesis of new hybrid ASA derivatives we turned our attention to molsidomine (**1**) (3-morpholino-*N*-ethoxycarbonyl sydnonimine), which is a NO donor and used as a coronary vasodilator [30] in patients suffering from coronary artery diseases. Compound **1** displayed protective effect on indomethacin and ASA-induced gastric injury in rats [31]. Moreover, molsidomine (MOL) has a significant platelet antiaggregatory activity *in vitro* [32]. We postulated that a hybrid derivative of ASA and MOL would exhibit advantageous and synergistic effects of the two drugs, i. e. diminished side effects of ASA and improved inhibition of platelet aggregation. The mesoionic MOL is metabolized in the following way (Scheme 1) [33].



Scheme 1. Metabolism of molsidomine (The carbamate moiety is highlighted in green)

The active metabolite is **2** (linsidomine, SIN-1), therefore, we hypothesized that its covalent conjugation to acetylsalicylic acid would result in a NO donor hybrid ASA. For the linkage between ASA and compound **2**, we designed a tetraethyleneglycol chain to improve the water solubility of the product and a carbamate group, similar to that in compound **1**. It is assumed that the planned ASA-MOL conjugate could serve as a NO donor with a similar mechanism to MOL, a drug already used for pharmacotherapy. Our goals practically in the present study were (i) to produce a new NO donor hybrid aspirin and (ii) to study its toxic and vasodilator effects, in the highlight of coronary artery dilation in the myocardium.

For the synthesis of **ERJ-500**, acetylsalicylic acid chloride **3** [34] was reacted with mono-triphenylmethyl tetraethyleneglycol **4** [35], obtaining the **5** ester. The trityl group was removed using a reagent cocktail [36] resulting in compound **6**, which was allowed to react with linsidomine active carbamate ester **7** [37] to give **ERJ-500**, the desired hybrid ASA derivative (Scheme 2).



Scheme 2. Synthesis of ASA-molsidomine hybrid with a hydrolysable ester linkage (highlighted in yellow) and the metabolically labile carbamate moiety (highlighted in green)

It is important to note that **ERJ-500** proved to be stable after one-year long storage at room temperature (NMR analysis showed no degradation).

Oxidative stability assays

The oxidative stability of the **ERJ-500** compound was assessed utilizing two novel biomimetic model systems. In the first set of experiments a synthetic porphyrin, Fe(III) meso-tetra(4-sulfonatophenyl)porphine chloride was applied. The total ion chromatograms of the control and **ERJ-500** after oxidation by synthetic porphyrin were almost identical, therefore the **ERJ-500** molecule was resistant against simple oxidative conditions, which could possibly change the structure of the molecule in another case.

The oxidation of the **ERJ-500** was done by the classical Fenton reaction as well. The reaction mixtures were analyzed by HPLC-MS/MS. Based on the recorded spectra of the control and test samples it can be concluded that the compound was stable under the applied conditions, as the peaks on the chromatogram were not changed notably after 1 hour of oxidation compared to the control chromatogram. The obtained results were identical to the ones achieved by the synthetic porphyrin oxidation, further confirming the stability of the new molecule under simple oxidative conditions.

A sample chromatogram of each oxidative stability models mentioned above can be found in the supplementary material (Fig. S1 and S2).

2.2 Biological studies

Safety evaluation of ERJ-500

To assess the direct cytotoxic effects of **ERJ-500**, we carried out MTT assays at different concentrations of the studied molecule, and its two constituents, ASA and MOL in H9c2 cells. A slight decrement can be seen in all treated groups compared to the control, but all treated groups resulted in a significantly higher cell viability compared to the positive control group, which was treated with 1% H_2O_2. No significant differences can be observed between the groups treated by **ERJ-500** or other molecules studied (Figure 1.A.), therefore, we may conclude that **ERJ-500** is an equally safe compound as the MOL or ASA.

To confirm our previously demonstrated cytotoxicity results, we performed hemolytic activity studies in blood cells isolated from Sprague Dawley rats. The hemolytic activity in rat erythrocytes at different concentrations of **ERJ-500**, ASA and MOL were significantly lower compared to the positive control group (Figure 1.B.). Samples of the latter group received sterile water, which induced 100% hemolysis. No significant differences can be observed among the groups treated by **ERJ-500,** ASA, and MOL, respectively in hemolytic activities, which further confirm that our aspirin derivative seems to be a safe compound.

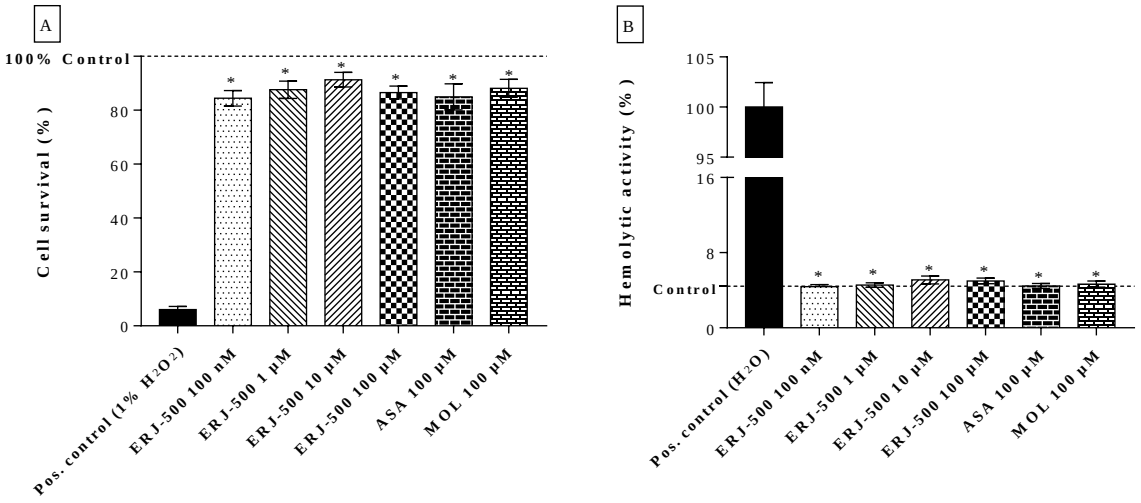


Figure 1. Safety evaluation of ERJ-500 A. Cytotoxicity test. The bar chart represents cell survival rates in percentage compared to the control group, which served by the solvent only (phosphate buffered saline-PBS). ERJ-500 100 nM - 100 µM; ASA 100 µM; MOL 100 µM; and 1% H_2O_2. Results are expressed as mean ± SEM. n=20-67 cells in each group. *p <0.05 in comparison with the positive control group (Pos. control).

B. Hemolysis test. The bars represent hemolytic activity in percentage referring to the control group, which contained the solvent (PBS) only. ERJ-500 100 nM – 100 µM, ASA 100 µM, MOL 100 µM. Results are expressed as mean ± SEM. n=8-11 in each group. *p < 0.05 in comparison with the positive control (H_2O) group (Pos. control).

Vasoactive effects of ERJ-500

To study the vasoactive effects of the **ERJ-500** in the myocardium, the drug was dissolved in the perfusion buffer at a concentration rate of 1 µM to 100 µM, and isolated hearts were perfused. During Langendorff perfusion, the **ERJ-500** did not produce any incidence of ventricular tachycardia or ventricular fibrillation. In addition, heart rate was not significantly changed in comparison with the drug-free control group (Figure 2.A.). Coronary flow was significantly increased by about 50% in the group treated with 100 µM **ERJ-500** (Figure 2.B.). During Langendorff perfusion, the coronary flow is influenced by the heart rate, the perfusion pressure, and the coronary dimension. Since the perfusion pressure used in the present study is constant and the heart rate is not significantly altered, the increased coronary flow could be a result of the coronary relaxation. Although in the present study, the concentration of NO was not directly measured, and it would be the subject of another study, our results support the hypothesis that NO may originate from the ASA-MOL compound

(**ERJ-500**), since salicylic acid shows no vasodilator activity in the myocardium [38, 39]. Cardioprotective effects of ASA and salicylic acid related derivatives can be attributed to affect the platelet activation related to cyclooxygenase enzyme activities (COX1 and COX2) and heat stress protein expression in the diseased myocardium [40, 41].

To further confirm vasoactive effects of **ERJ-500** and to study any possible beneficial effects of the compound on the mechanical activity of the hearts, we tested the molecule on the isolated working heart perfusion system as well, at a concentration, which seemed the most advantageous previously. In the working heart perfusion, when other mechanisms also involved to compensate measurable vasoactive effects, coronary flow was still significantly elevated in treated hearts with 100 µM **ERJ-500** (Figure 2.D.). Stroke volume was also significantly increased, thus, **ERJ-500** can be an additive effect to improve myocardial contraction force (Figure 2.E.). As previously measured in Langendorff heart preparation, heart rate did not change notably in working heart preparation also (Figure 2.C.). Rest of the measured, non-significant myocardial parameters can be found in the supplementary material.

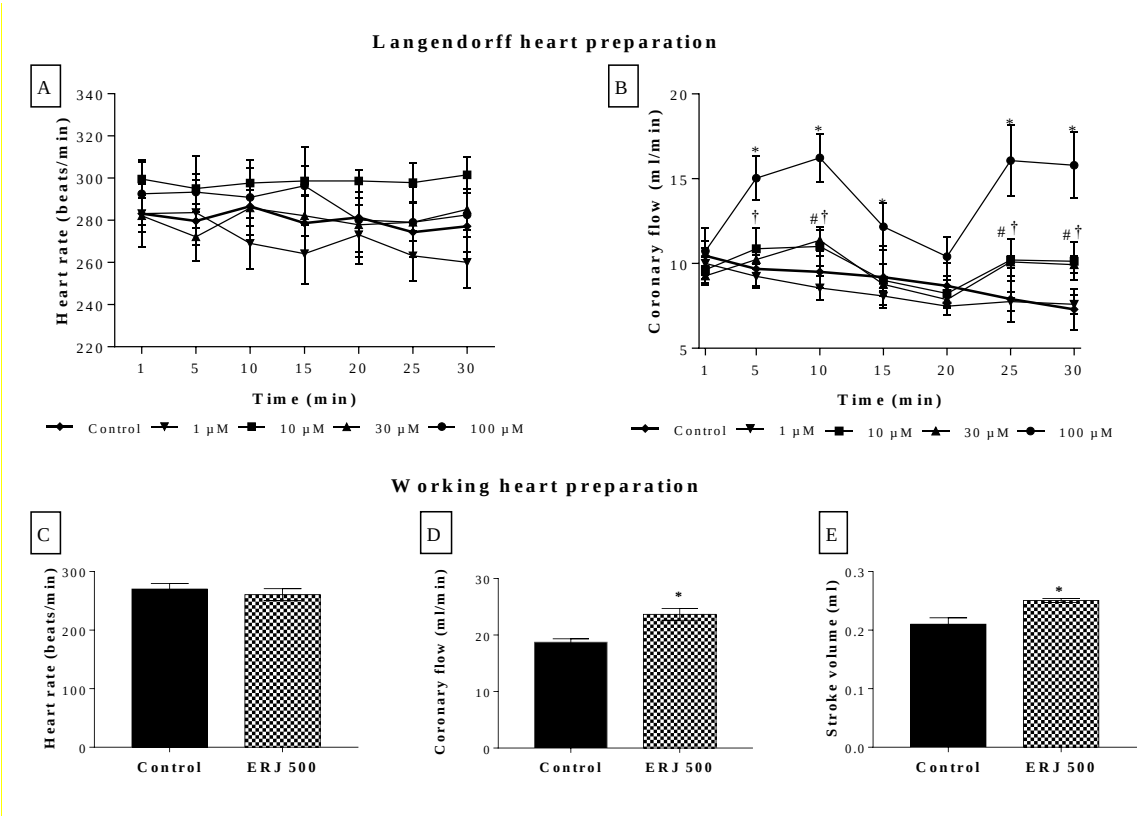


Figure 2. Effects of ERJ-500 on cardiac functions of isolated Langendorff and working heart.

A., Alteration of heart rate and **B.**, coronary flow in the presence of the ERJ-500 at different concentrations (1-100 µM) when the heart is mounted on the "Langendorff" apparatus. *p < 0.05 in comparison with the control values at the same time points. n=5 in each group.

C., Alteration of heart rate, **D.**, coronary flow, and **E.**, stroke volume in the presence (ERJ-500) or the absence (Control) of 100 µM ERJ-500, when the heart is mounted on the isolated working heart apparatus. No significant differences were observed among groups. *p < 0.05 in comparison with the control values. n=11 in the control group, n=6 in the treated group.

Anti-ischemic effect of ERJ-500

To further analyze the effect of **ERJ-500** on the rat myocardium, infarct size was evaluated using the triphenyl-tetrazolium-chloride-staining method (TTC). Following 30 min of ischemia and 90 min reperfusion, infarct zones of TTC-stained hearts were expressed in a percentage of the whole myocardium. Figure 3. shows that hearts perfused with ERJ-500 containing buffer resulted in a significantly decreased infarct size.

This result indicates that ERJ-500 has a cardioprotective effect, which could be a consequence of the vasorelaxant property, however, other mechanisms may also contribute to this effect.

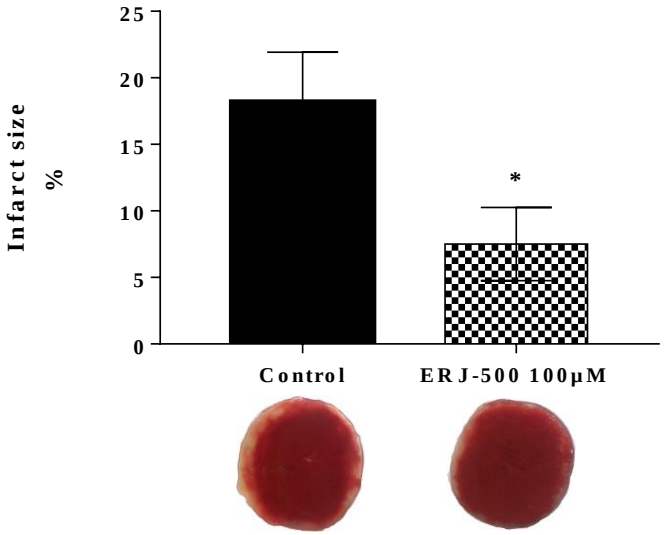


Figure 3. Effects of ERJ-500 on infarct site. Changes in infarct size after 30 min ischemia followed by 90 min reperfusion, when the hearts are mounted on the isolated working heart apparatus TTC staining method was used. *$p < 0.05$ in comparison with the control value. n=5 in each group.

3. CONCLUSION

In the present study, an attempt was made to synthesize a new NO-releasing ASA derivative and ascertain whether the release of NO from the MOL conjugate could be associated with enhanced myocardial circulation, and consequently, giving a chance to the survival of cardiac cells and tissues by preserving the oxygen supply via the dilation of coronary vessels.

Based on our observations, the new molecule ERJ-500 appears to be nontoxic and stable under oxidative conditions. Furthermore, our pharmacological studies indicate vasoactive and anti-ischemic properties for the molecule. However, further in vivo studies are needed to investigate the effect on whole organism.

4. EXPERIMENTAL

4.1. Chemistry

MOL derivative **7** was prepared according to literature procedures [37]. All reagents were purchased from commercial suppliers and used without further purification. TLC was performed on Kieselgel 60 F_{254} (Merck, Darmstadt, Germany) with detection by UV-light (254 nm) and immersing into sulfuric acidic ammonium-molibdenate solution followed by heating. Flash column chromatography was performed on Silica gel 60 (Merck 0.040-0.063 mm). Organic solutions were dried over Na_2SO_4 or $MgSO_4$ and concentrated in vacuum. The ^{1}H NMR (400 MHz) and ^{13}C NMR (101 MHz) spectra were recorded with a Bruker DRX-400 spectrometer at 25 ºC. Chemical shifts are referenced to Me_4Si (0.00 ppm for ^{1}H) and to the residual solvent signals ($CDCl_3$: 77.1 for ^{13}C). MALDI-TOF MS analyses of the compounds were carried out in the positive reflectron mode using a BIFLEX III mass spectrometer (Bruker, Karlsruhe, Germany) equipped with delayed-ion extraction. 2,5-Dihydroxybenzoic acid (DHB) was used as matrix and $F_3CCOONa$ as cationising agent in DMF.

Compound 5

Compound **4** (4.37 g, 10 mmol) was dissolved in dry dichloromethane (50 ml) and Et_3N (2 ml) was added to the stirred solution. Compound **3 (**1.99 g, 10 mmol) dissolved in dry dichloromethane (10 ml) was added dropwise at 0 °C to the reaction mixture and it was stirred for 5 h at room temperature. The reaction mixture was quenched with satd. aq. $NaHCO_3$ (30 ml), stirred for further 15 min, then it was diluted with dichloromethane (100 ml) and extracted with 10% $NaHSO_4$ (30 ml) and water (30 ml), dried over Na_2SO_4, filtered and evaporated at 35 ºC in vacuum. The crude product was purified by flash column chromatography (*n*-hexane:acetone 7:3) to give **5** as a pale yellow syrup (4.0 g, 67%). R_f 0.34 (*n*-hexane:acetone 7:3);^{1}H NMR (400 MHz, $CDCl_3$): δ 8.03 (dd, J = 7.8 Hz, J = 1.8 Hz, 1H, arom), 7.53 (td, J = 7.8 Hz, J = 1.8 Hz, 1H, arom), 7.47–7.45 (m, 6H, arom), 7.30–7.19 (m, 10H, arom), 7.08 (dd, J = 8.1 Hz, J = 0.8 Hz, 1H), 4.41–4.39 (m, 2H, TEG-CH_2), 3.78–3.76 (m, 2H, TEG-CH_2), 3.70–3.65 (m, 10H, 5 x TEG-CH_2), 3.23 (t, J = 5.2 Hz, 2H, TEG-CH_2), 2.34 (s, 3H, CH_3 Ac); ^{13}C NMR (101 MHz, $CDCl_3$): δ 169.9 (1C, C_q Ac), 164.5 (1C *C*OO), 150.8 (1C, C_q arom), 144.2 (3C, C_q arom), 134.0, 132.0, 128.8, 127.9, 127.0, 126.1, 123.9 (19C, arom), 123.3 (1C, Cq arom), 86.6 (1C, C_q Tr), 70.9, 70.8, 70.7, 69.2, 64.4, 63.4 (8C, 8 x TEG-CH_2), 21.10 (1C, CH_3 Ac); MS (MALDI-TOF): m/z calcd for $C_{36}H_{38}NaO_8$: 621.25 $[M+Na]^+$; found: 621.32.

Compound 6

Compound **5 (**1.2 g, 2.0 mmol) was added to the mixture of hexafluoroisopropanol (7.5 ml), $BF_3 \cdot Et_2O$ (50 µl, 0.2 equiv.) and Et_3SiH (1.2 ml, 3.8 equiv.). After complete conversion of the starting compound (cc. 15 min) the reaction was quenched with satd. aq. $NaHCO_3$ solution (2 ml). The mixture was concentrated in vacuum and the residue was purified by flash column chromatography (*n*-hexane:acetone 1:1) to give compound **6** as a colourless syrup (460 mg, 65%). R_f 0.25 (*n*-hexane:acetone 1:1); ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.05 (dd, J = 7.8 Hz, J = 1.6 Hz, 1H, arom), 7.56 (td, J = 7.9 Hz, J = 1.6 Hz, 1H, arom), 7.32 (td, J = 7.6 Hz, J = 1.2 Hz, 1H, arom), 7.11 (dd, J = 8.1 Hz, J = 1.2 Hz, 1H, arom), 4.45–4.43 (m, 2H, TEG-*C*H_2), 3.81–3.78 (m, 2H, TEG-*C*H_2), 3.74–3.65 (m, 10H, 5 x TEG-*C*H_2), 3.60–3.58 (m, 2H, TEG-*C*H_2), 2.62 (s, 1H, TEG-O*H*), 2.36 (s, 3H, *C*H_3 Ac); ^{13}C NMR (101 MHz, $CDCl_3$): δ 169.9 (1C, C_q *C*OO), 164.5 (1C, C_q Ac), 150.8 (1C, C_q arom), 134.1, 132.0, 126.1, 123.9 (4 C, arom), 123.2 (1C, C_q arom), 72.5, 70.8, 70.7, 70.6, 70.4, 69.2, 64.3, 61.8 (8C, 8 x TEG-*C*H_2), 21.1 (1C, *C*H_3 Ac); MS (MALDI-TOF): m/z calcd for $C_{17}H_{24}NaO_8$: 379.36 $[M+Na]^+$; found: 379.21.

Compound ERJ-500

The starting materials were dried over P_2O_5 overnight. Compound **7** (2.01 g, 6 mmol) was suspended in dry acetonitrile (100 ml) and compound **6** (2.49 g, 7 mmol) dissolved in dry acetonitrile (10 ml) was added. The reaction mixture was stirred at reflux temperature for 2 h, then it was evaporated. The crude product was purified by flash column chromatography (*n*-hexane: acetone 6:4 →1:1) to give **ERJ-500** as a colorless syrup (758 mg, 41%).

R_f 0.16 (CH_2Cl_2:acetone 8:2); ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.03 (dd, J = 7.9 Hz, J = 1.7 Hz, 1H, arom), 7.7 s1H, C*H* sydnone, 7.56 (ddd, J = 8.1, 7.4, 1.8 Hz, 1H, arom), 7.31 (td, J = 7.7 Hz, 1.1 Hz, 1H, arom), 7.10 (dd, J = 8.1 Hz, J = 1.1 Hz, 1H, arom), 4.43– 4.41 (m, 2H, *C*H_2 morpholine), 4.26–4.24 (m, 2H, *C*H_2 morpholine), 3.94–3.92 (m, 4H, 2 x TEG-*C*H_2), 3.80 – 3.78 (m, 2H, *C*H_2 morpholine), 3.74–3.72 (m, 2H, *C*H_2 morpholine), 3.68-3.63 (m, 8H, 4 x TEG-*C*H_2), 3.51–3.49 (m, 4H, 2 x TEG-*C*H_2), 2.35 (s, 3H, *C*H_3 Ac); ^{13}C NMR (101 MHz, $CDCl_3$): δ 174.2 (1C, C_q carbamate), 169.7 (1C, C_q COO), 164.4 (1C, C_q Ac), 161.2 (1C, C_q sydnone), 150.6 (1C, C_q arom), 133.8, 131.8, 125.9, 123.7 (4C, arom), 123.2 (1C, C_q arom), 70.6, 70.5, 69.3, 69.0, 65.4, 64.6, 64.3, 54.6 (13C, 1 x sydnone-*C*, 4 x morpholine-*C*H_2, 8 x TEG-*C*H_2), 20.9 (1C, *C*H_3 Ac). MS (MALDI-TOF): m/z calcd for $C_{24}H_{32}N_4NaO_{11}$: 575.20 $[M+Na]^+$; found: 575.31.

4.2. Oxidation by synthetic porphyrin and the chemical Fenton system

Two reactions were carried out to test the stability of **ERJ-500** molecule under oxidative conditions, based on the method as reported by Csepanyi et al. [42] recently, with minor modifications as follows: 50 µl of **ERJ-500** dissolved in acetonitrile was used for synthetic porphyrin oxidation in 10 mM concentration. 400 µl of **ERJ-500** in 2.5 mM concentration for the Fenton reaction. Samples were drawn at 1 h in the Fenton reactions prior to injecting them instantly to the HPLC and further investigation. Reaction mixtures for blank contained acetonitrile only without **ERJ-500**. The control mixtures contained no peroxide.

4.3. Biological characterization

Determination of cytotoxicity by MTT assay

Assessment of the cytotoxicity of the **ERJ-500,** ASA, and MOL on cellular survival was accomplished using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay based on the method described by Csepanyi et al. [42]. Briefly, H9c2 cells were treated with 100 nM, 1 µM, 10 µM, 100 µM of **ERJ-500**, 100 µM of MOL, 100 µM of ASA and 1% H_2O_2 (positive control) containing medium for 24 h on 96 well plates. Then, MTT solution was added to the medium and incubated for 3.5 h at 37 °C. After eliminating the solution from the cells, isopropanol was added and incubated for 0.5 h at 37 °C to dissolve the formazan aggregates. Absorbance was measured at 570 nm and 690 nm.

Animals

Female Sprague Dawley (SD) rats with an average weight of 248 ± 6 g were used in the present study. Animals were nutrified with standard rodent chow pellets (R/M-Z+H, ssniff Spezialdiäten GmbH, Soest, Germany) *ad libitum* with free access to water and kept at an ambient temperature of 25 ± 2 °C, with a relative humidity of 55 ± 5%, and a 12-hour light-dark cycle. All animals were treated according to the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research, and the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH Publication no. 86-23, revised in 1996). Breeding and handling of animals were approved by the Institutional Animal Care and Use Committee of the University of Debrecen, Debrecen, Hungary.

Determination of hemolytic activity

Hemolysis tests were performed as described by Roka et al. [43] with some minor modifications. Rat blood samples were collected to K_3EDTA containing vacuum tubes (BD, Plymouth, UK) and were treated with 100 nM, 1 µM, 10 µM, 100 µM **ERJ-500**, 100 µM of MOL and the same concentration of ASA in phosphate buffered saline (PBS). The percentage of hemolysis was expressed as the ratio of hemoglobin in the supernatant of the different chemical solutions related to the hemoglobin concentration after the complete hemolysis of erythrocytes in water.

Langendorff heart preparation and assessment of heart rate and coronary flow

Rats were anesthetized with an intraperitoneal pentobarbital sodium injection (60 mg/kg), with heparin as an anticoagulant (1000 U/kg). Following the induction of deep anesthesia, chest cavities were opened, hearts were excised and placed in ice-cold modified Krebs-Henseleit bicarbonate (KHB) buffer (containing 118 mM NaCl, 5.8 mM KCl, 1.8 mM $CaCl_2$, 25 mM $NaHCO_3$, 0.36 mM KH_2PO_4, 1.2 mM $MgSO_4$, and 5.0 mM glucose). After excision, aortas were cannulated and each heart was perfused with modified KHB buffer at a filling pressure of 100 cm of water, using the "non-working" Langendorff mode for 5 min in order to flush blood out from the myocardium. The setup was assembled with two buffer-chambers at the same constant pressure. The one contained the KHB buffer only, the other contained **ERJ-500** dissolved into the KHB buffer at different concentrations (1 µM, 10 µM, 30 µM, 100 µM). At the end of the washout period, baseline cardiac parameters were registered, including coronary flow (CF) and heart rate (HR), and the inflow was switched to serve the hearts from the chamber containing **ERJ-500** for 10 min. Next, 10 min of washout period, followed by 10 min of adding once more the **ERJ-500** containing buffer. A continuous pressure signal was recorded during the whole experiment with the help of a pressure transducer (ADInstruments, PowerLab, Castle Hill, Australia), which was calibrated before each experiment. HR was calculated from the continuously recorded pressure signal. CF was assessed by the time-collecting of the coronary effluent.

Isolated working heart preparation to assess cardiac parameters and infarct size

To measure cardiac function, isolated working heart preparations were carried out based on a previously described method by Czompa et al [44] on Sprague Dawley female rats (n=6) divided into two groups. After completing the isolated working heart preparation procedure followed by 10 minutes washout period, we registered the baseline working heart parameters

such as aortic flow [45], coronary flow (CF), aortic pressure (AOP), heart rate (HR) and derivated aortic pressure (AOdP/dT). Cardiac output (CO) was calculated by the sum of AF and CF and we got stroke volume (SV) by dividing the CO with HR. In the treated group, ERJ-500 was added to the KHB buffer by a dilution of a previously prepared stock solution, creating a 100 µM concentration of ERJ-500 in the heart inflow. The molecule-containing KHB buffer was presented after the washout and baseline registration period for 5 mins, followed by a 30 min ischemia followed by 90 min reperfusion. Results of AOP, AOdP/dT, CO and AF are included in the supplementary material.

To determine the degree of the infarcted area in the myocardium, triphenyl tetrazolium chloride (TTC) staining was performed according to a previously presented study by Czompa et al [46]. Briefly, following ischemia and reperfusion, 50 ml of 1 % TTC solution was perfused through the myocardium. Then, hearts were frozen, sectioned, digitalized and all heart sections were blotted dry and weighed. Risked and infarcted areas were quantified by an open-source planimetry software Fiji [47]. Percentage of the infarcted area compared to the whole risked area of the myocardium is represented on a bar chart.

Statistical analyses

All data are presented as the average magnitudes of each outcome in a group ± standard error of the mean [42]. Statistical analysis was performed using t-test or one- or two-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons test with GraphPad Prism software for Windows (GraphPad Software Inc., La Jolla, CA, USA). Probability values (p) less than 0.05 were considered statistically significant.

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