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# The New Nitric Oxide Donor, FOR 911B, Induces Relaxation in Isolated Rat Aorta Involving the NO/sGC/cGMP Pathway and $K^+$ Channels

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**Abstract: Background:** Nitric oxide (NO) is a gaseous molecule considered to be a protagonist in the dilation of blood vessels, and its property and/or bioavailability are reduced in pathophysiological conditions such as cardiovascular diseases. Therefore, its exogenous administration becomes attractive, and new classes of compounds able to induce NO release have emerged to minimize the adverse effects found by existing NO donor drugs. **Objective**: Our aim was to investigate the vasorelaxant effect and mechanism of action induced by the ruthenium complex, which contains nitric oxide in its structure, $[Ru(phen)_2(TU)NO](PF_6)_3$ (FOR 911B), in isolated rat aorta. **Methods**: The animals were euthanized, and the aorta artery was identified, removed, and immediately placed in modified Krebs–Henseleit solution. To verify tissue viability, a contraction was obtained with phenylephrine (Phe) (0.1 μM), and to assess endothelial integrity, acetylcholine (ACh) (1 μM) was added. **Results**: In the present study, we demonstrated, for the first time, that FOR 911B promotes vasorelaxation in a concentration-dependent manner in isolated rat aortic artery rings. After the removal of the vascular endothelium, the potency and efficacy of the relaxation were not altered. With pre-incubation with hydroxocobalamin, the relaxing response was abolished, and with the use of ODQ, the main NO receptor blocker, the vasorelaxant effect was attenuated with a shift of the curve to the right. To investigate the participation of $K^+$ channels, the solution concentration was changed to KCl (20 and 60 mM), and it was pre-incubated with the non-selective $K^+$ channels blocker (TEA). Under these conditions, relaxation was altered, demonstrating that $K^+$ channels are activated by FOR 911B. By selectively blocking the different subtypes of $K^+$ channels with specific blockers, we demonstrated that the subtypes $K_V$, $K_{IR}$, $SK_{Ca}$, and $BK_{Ca}$ are involved in the vasodilator effect induced by FOR 911B. **Conclusions**: The results obtained demonstrated that FOR 911B promotes vascular relaxation in aortic artery rings in a concentration-dependent manner and independent of the vascular endothelium through the participation of the NO/sGC/cGMP pathway, as well as with the involvement of different $K^+$ channels.

**Keywords:** nitric oxide donors; metallodrugs; vasodilation



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## 1. Introduction

Nitric oxide (NO) is a highly reactive gaseous molecule with a short half-life that easily diffuses through cell membranes [1]. It is responsible for several physiological processes and plays a key role in the cardiovascular system, being characterized as a protagonist molecule in vasodilation [2–4].

NO is synthesized by the enzyme Nitric Oxide Synthase (NOS), which has three isoforms: neuronal NOS, inducible NOS, and endothelial NOS. Both isoforms use the amino acid L-arginine as a substrate, in addition to some cofactors such as oxygen ($O_2$), reduced

nicotinamide adenine dinucleotide phosphate (NADPH), and 6R-5,6,7,8-tetrahydrobiopterin ($BH_4$) [5–9].

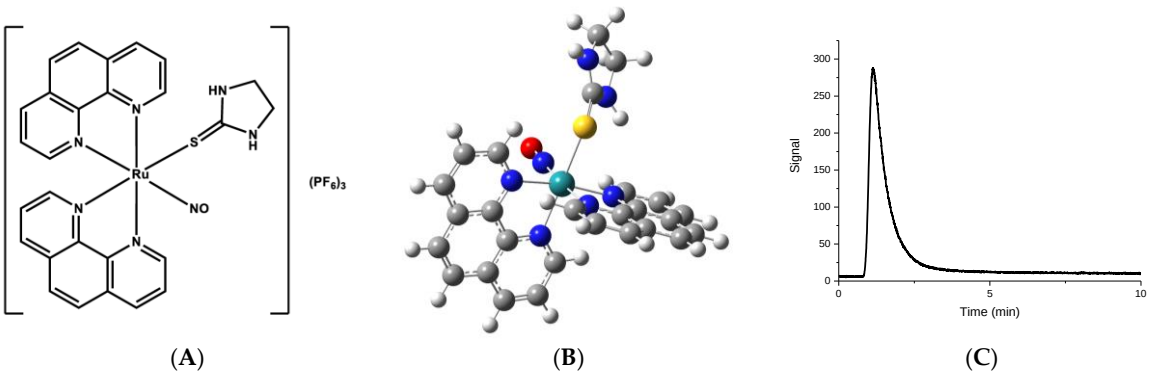
In the vascular endothelium, the NO produced easily diffuses through the membrane to the vascular smooth muscle cell (VSMC), activating soluble guanylyl cyclase (sGC), which is the primary mediator of its NO bioactivity, producing the second messenger cGMP, which activates protein kinase-G (PKG). PKG, in turn, exerts several effects that contribute to the reduction of free calcium concentration and consequently promote vascular relaxation [10,11].

Therefore, exogenous administration becomes attractive, and new strategies to increase NO production and signaling are being explored [12–14] to minimize undesirable effects such as short duration (half-life), high reactivity, low tissue selectivity, and development of tolerance with frequent dosing of existing donors like the classic sodium nitroprusside (SNP) and glyceryl trinitrate (GTN), which mimic the role of endogenous NO in biological systems [15–17].

Understanding the potential of this molecule in the treatment of cardiovascular disorders, new chemical classes of NO donors have been synthesized and characterized. In this context, ruthenium complexes stand out for having a high affinity for NO and forming nitrosyl complexes [18], for being like the iron ion and thus easily binding to biological molecules, as is the case with transferrin, which serves as a vehicle for its elimination preventing its toxic effects [19], by presenting a stable active form under physiological conditions and for releasing NO in a specific biological target [20–22].

Recently, new nitrosyl-ruthenium compounds, named FOR, have been produced and tested in different biological systems such as cis-$[Ru(bpy)_2(2\text{-MIM})(NO)](PF_6)_3$ (FOR811A) was studied in a murine model of allergic asthma, and it prevented the bronchoconstriction during asthma [23]. Another ruthenium complex from the same family, cis-$[Ru(NO_2)(bpy)_2(5NIM)]PF_6$, showed a potential pharmacological application as an antioxidant and anti-inflammatory (inhibition of pro-inflammatory cytokines) in in vitro studies [24]. In the cardiovascular system, the cis-$[Ru(bpy)_2(ImN)(NO)]^{3+}$ (FOR0811) induced a decrease in blood pressure, demonstrating a long-lasting effect without reflex tachycardia in L-$N^G$-Nitro arginine methyl ester (L-NAME) hypertensive rats [25]. The authors also detected a vasodilator effect in aortic rings mediated by the sGC–cGMP pathway after the addition of FOR0811 [13,25].

Based on the promising effects elicited by the *FOR* complexes cited above, especially in the cardiovascular system, in addition to the new chemical synthesis of a new complex called FOR 911B ($[Ru(phen)_2(TU)NO]$ $(PF_6)^{3+}$), we hypothesized whether FOR 911B, a ruthenium complex studied for the first time here, could induce vasodilation through NO release (since it contains nitric oxide in its structure) and NO/sGC/cGMP pathway activation in the isolated aorta of rats. Therefore, the aim of this research was to investigate the vasorelaxant effect and the mechanism of action induced by the inedited ruthenium complex $[Ru(phen)_2(TU)NO]$ $(PF_6)^{3+}$ (FOR 911B) (Figure 1).




(**A**) (**B**) (**C**)

**Figure 1.** FOR 911B. (**A**) Planar structure of FOR911B, (**B**) 3D-structure depiction of the coordination complex, and (**C**) NO detection assay using a chemiluminescent NO detector.

## 2. Materials and Methods

### *2.1. Animals*

Male Wistar rats weighing between 250 and 350 g were used and kept in the animal facility of the Academic Center of Vitória (CAV/UFPE) under controlled temperature (22 ± 1 °C) and light conditions (12/12 h light/dark cycle) and had access to water and food ad libitum. All procedures were performed in accordance with ethical principles submitted to and approved by the Ethics Committee for the Use of Animals of the Federal University of Pernambuco (CEUA/UFPE # 0066/2019).

### *2.2. Obtaining the Ruthenium Complex*

The ruthenium compound complexed with the NO molecule, $[Ru(phen)_2(TU)NO](PF_6)_3$ represented by FOR 911B, was synthesized in the Bioinorganic Laboratory of the Department of Organic and Inorganic Chemistry of the Federal University of Ceará, according to the technique described by Gouveia-Júnior (2023), replacing 2,2′-bipyrudine (bpy) for phenantroline (phen) [26]. We also carried out a NO detection assay using a chemiluminescent NO detector (Sievers Nitric Oxide Analyzer NOATM 280i, GE Analytical Instruments, Boulder, CO, USA), and the result is shown in the graphic below. Briefly, a 0.1 uM FOR0911B solution was mixed with a 2.0 μM L-glutathione solution in 0.1 M DTPA buffer solution at pH 7.4 and injected into the detector. A strong signal was observed, indicating the rapid formation of nitric oxide. This qualitative result evidences that FOR0911B works as a NO donor in the presence of mild organic reducing agents, such as L-glutathione.

### *2.3. Vascular Reactivity Studies*

#### 2.3.1. Tissue Preparation

The animals were euthanized, and the aorta artery was identified, removed, and immediately placed in modified Krebs–Henseleit solution (composition (in mmol/L): NaCl 130.0; KCl 4.7; $KH_2PO_4$ 1.2; $CaCl_2$ 1.6; $MgSO_4$ 1.2; $NaHCO_3$ 14.9; glucose 5.5) for dissection and sectioning of the vessels into rings (1–2 mm in length). When necessary, the endothelium was removed by mechanical friction between the internal walls of the vessel and a metal rod. Each ring was immersed in tanks (10 mL) at 37 °C, aerated with a mixture of 95% $O_2$ and 5% $CO_2$ (carbogen), and attached to a force transducer, subjected to a basal tension of approximately 1.5 g for a stabilization period of 60 min. Changes in isometric tension were captured by the AQCAD acquisition system (version 2.3.3.0; AVS, São Bernardo do Campo, Brazil).

To verify tissue viability, a contraction was obtained with phenylephrine (Phe) (0.1 μM), and to assess endothelial integrity, acetylcholine (ACh) (1 μM) was added. Rings with relaxation greater than 80% due to phenylephrine-induced contraction were considered to have functional endothelium, and rings with relaxation less than 20% were considered to have no endothelium. Then, to elucidate the vasorelaxant effect, increasing concentrations of FOR 911B (0.0001—10 μM) were added in a cumulative manner to obtain a concentration–response curve in the presence or absence of vascular endothelium in arteries pre-contracted with Phe (0.1 μM).

#### 2.3.2. Investigation of the Involvement of the NO/GCs Pathway in the Vasorelaxant Effect of FOR 911B

To investigate the participation of the NO-GCs pathway in the vasorelaxant response promoted by the ruthenium complex, some pharmacological tools were used, such as Hydroxocobalamin (HDX) (30 μM) [27], a NO radical scavenger; Nω-nitro-L-arginine methyl ester (L-NAME) (100 μM) [28] and $N^G$-Methyl-L-arginine acetate salt (L-NMMA) (100 μM) [29], non-selective NOS inhibitors; and 1H-[1,2,4]oxadiazolo [4,3-a]quinoxalin-1-one (ODQ) (1 μM [30], a potent inhibitor that prevents the activation of sGC by NO.

2.3.3. Evaluation of the Participation of Potassium Channels in the Vasodilatory Effect Promoted by FOR 911B

To evaluate the participation of $K^+$ channels in the vasorelaxant response induced by FOR 911B, modified saline solutions with KCl (20 and 60 mM) were used. The rings were also pre-incubated with tetraethylammonium (TEA) at a concentration of 3 mM, which is a non-selective blocker of potassium channels. To try to understand which subtype of $K^+$ channel would be participating in the vasorelaxant effect of the NO donor, different specific blockers of these channels were used, such as TEA (1 mM), iberiotoxin (20 nM), apamin (100 nM), 4-aminopyridine (0.3 mM), glibenclamide (10 μM), and barium chloride (100 μM).

### *2.4. Statistical Analysis*

Relaxation responses to cumulative concentrations of FOR 911B were calculated as a percentage of inhibition of the Phe-induced maximal contraction. The relaxing effect (R) of the substances was calculated for each concentration as a function of the maximum contraction provided by the agonist, according to the following expression: $R = (T_A - T_S/T_A) \times 100$, where $T_A$ and $T_S$ are, respectively, the tensions resulting from the action of the agonist (Phe) and a given substance (FOR 911B in this case). We use the 'log(agonist) vs. response—Variable slope' to analyze the vascular reactivity data. The graphs were then created based on the average values of the magnitude of the vasodilator effect, calculated for each concentration of the substance (after logarithmic transformation). Such data were used to construct concentration–effect curves using nonlinear regression analysis. To address this, the model that uses a sigmoid function of the type was taken as a basis: $y = a + ((b - a)/\ 1 + 10^{(\log CE50 - x)*S})$, where y corresponds to the response measure (relaxing effect), x to the decimal logarithm of the concentration, a to the minimum response, and b to the maximum response. The constant is called the slope factor and determines the angle of the curve.

The results were expressed as mean ± standard error of the mean (S.E.M). A Student's *t*-test was performed. Differences were considered significant when $p < 0.05$. For the concentration–response curves, the values of ME (maximum effect promoted in the percentage of relaxation) and $pD_2$ (negative logarithm of $EC_{50}$—is the molar concentration of an agonist that produces 50% of the maximal possible effect of that agonist) were obtained through nonlinear regression. The statistical program used was GraphPad Prism® version 8.0.

## 3. Results

### *3.1. Effect of FOR 911B on Isolated Rat Aortic Rings Pre-Contracted with Phe*

The NO donor, FOR 911B, promoted vascular relaxation in a concentration-dependent manner in isolated aortic artery rings with endothelium (ME = 111.55 ± 6.77%; $pD_2$ = 6.35 ± 0.36). In rings without functional endothelium, there was no change in the efficacy and potency of the vasorelaxant response promoted by the compound, as demonstrated by the values (ME = 116.25 ± 5.33%; $pD_2$ = 6.29 ± 0.79) (Figure 2A). This result suggests that the vasodilator response of the organic nitrate is independent of the relaxing factors derived from the vascular endothelium. Thus, in all subsequent experiments, we used the aortic rings without endothelium to investigate the mechanism of action involved in the vasorelaxant effect promoted by FOR 911B.

### *3.2. Effect of FOR 911B in Isolated Rat Aortic Rings on Contraction Induced by an Electrochemical Contracting Agent*

In rings without endothelium pre-contracted with KCl (60 mM), there was attenuation of the efficacy of the vasorelaxant effect promoted by FOR 911B when compared to the results found in rings pre-contracted with Phe (ME = 36.03 ± 5.68% vs. 116.25 ± 5.33%, respectively, $p < 0.05$) (Figure 2B). This finding demonstrates that $K^+$ channels participate in the vasorelaxant effect promoted by NO donors.

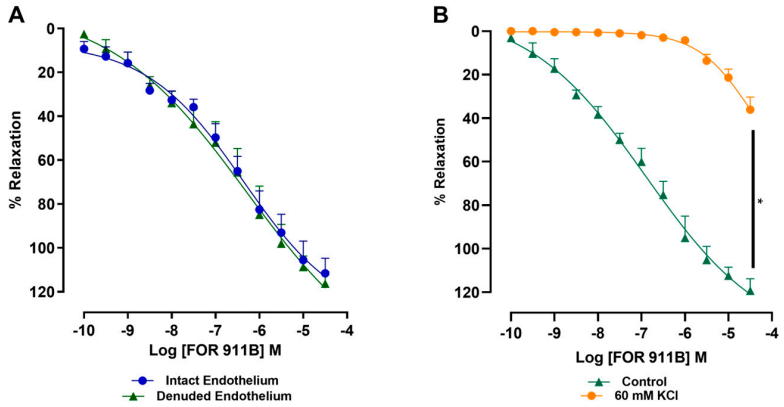



**Figure 2.** Concentration–response curve for the vasorelaxant effect induced by FOR911B in isolated rat aortic rings, with and without endothelium, pre-contracted with Phe (0.1 μM) (**A**) and without endothelium, pre-contracted with Phe and KCl (60 mM) (**B**). Values are expressed as mean ± S.E.M. (n = 6). * $p < 0.05$ vs. Control, Student's *t*-test.

### *3.3. Effect of FOR 911B in Isolated Rat Aortic Rings on the NO/GCs Pathway*

After the addition of L-NAME in denuded rings, we observed a shift to the right of the concentration–effect curve for FOR 911B in rat aorta with decreased efficacy and potency (ME = 96.84 ± 9.79%; $pD_2$ = 5.35 ± 0.09 vs. ME = 116.25 ± 5.33%; $pD_2$ = 6.29 ± 0.79, respectively, $p < 0.05$) (Figure 3A).

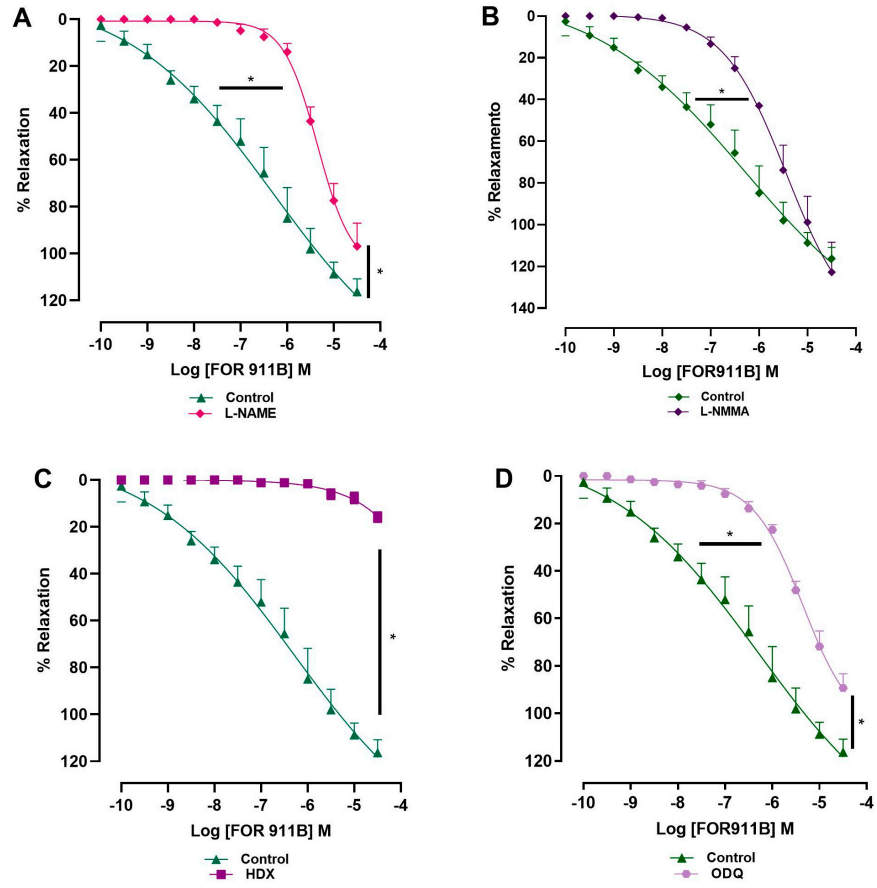



**Figure 3.** Concentration–response curve for the vasorelaxant effect induced by FOR911B in isolated endothelium-denuded rat aortic rings pre-contracted with Phe (0.1 μM) in the presence of L-NAME (100 μM) (**A**), L- NMMA (100 μM) (**B**), HDX (30 μM (**C**), and ODQ (1 μM) (**D**). Values are expressed as mean ± S.E.M. (n = 6). * $p < 0.05$ vs. Control, Student's *t*-test.

A similar result could be observed with the use of L-NMMA, with a decrease in the potency of the vasodilatory effect induced by the compound ($pD_2$ = 5.43 ± 0.24 vs. $pD_2$ = 6.29 ± 0.79, respectively, $p < 0.05$), but without a change in ME (Figure 3B).

In the presence of HDX, the vasodilatory effect promoted by the metallodrug was abolished (ME = 15.83 ± 1.89) when compared to the control (ME = 116.25 ± 5.33%). In this context, because the vasorelaxant effect induced by FOR 911B was suppressed, it was not possible to calculate the $pD_2$ value (Figure 3C).

The analysis of the vasorelaxant action of FOR 911B in rings pre-incubated with ODQ was also performed, which promoted a reduction in the maximum effect and potency of the donor under study when compared to the control group, as observed in Figure 3D (ME = 89.30 ± 5.96%; $pD_2$ = 5.35 ± 0.13 vs. ME = 116.25 ± 5.33%; $pD_2$ = 6.29 ± 0.79, respectively, $p < 0.05$). This suggests that the compound under study induces its vasodilatory effect through the release of the NO radical and that the sGC/cGMP pathway participates in this effect.

### *3.4. Effect of FOR 911B on Isolated Rat Aortic Rings on Potassium Channels*

In denuded aortic rings, depolarization induced by KCl at a concentration of 20 mM was able to promote significant reduction in the efficacy and potency (ME = 98.69 ± 7.35%; $pD_2$ = 5.46 ± 0.06 vs. ME = 116.25 ± 5.33%; $pD_2$ = 6.29 ± 0.79, respectively, $p < 0.05$) of the effect promoted by the ruthenium complex under study when compared to its control (Figure 4A).

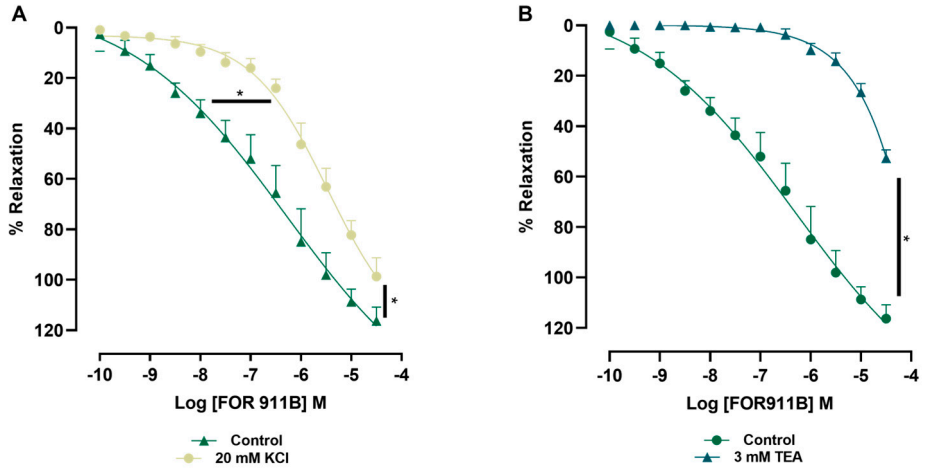



**Figure 4.** Concentration–response curve for the vasorelaxant effect induced by FOR911B in isolated endothelium-denuded rat aortic rings pre-contracted with Phe in the presence of 20 mM KCl (**A**) and 3 mM TEA (**B**). Values are expressed as mean ± S.E.M. (n = 6). * $p < 0.05$ vs. Control, Student's *t*-test.

In the presence of TEA (3 mM), which non-selectively blocks $K^+$ channels, the concentra tion–response curve induced by the NO donor was shifted to the right with a 50% reduction in ME (52.65 ± 3.23%, $p < 0.05$) when compared to rings without functional endothelium pre-contracted with Phe. Due to this decrease, it was not possible to calculate $pD_2$ (Figure 4B). These results corroborate the previous findings of these studies (Figure 2B), which indicate the participation of potassium channels in the effect promoted by the ruthenium complex.

### *3.5. Vasorelaxant Effect Promoted by FOR 911B Against Different Subtypes of $K^+$ Channels*

As there was a change in the vasorelaxant response promoted by the NO donor in the presence of TEA (Figure 4B), we selectively blocked the different subtypes of $K^+$ channels in aortic artery rings. Evaluating the participation of $K_{ATP}$ with the incubation of glibenclamide, it was observed that there was no difference in the relaxation promoted by the compound when compared to the rings without the blocker (ME = 117.32 ± 7.03%;

$pD_2$ = 6.41 ± 0.20) (Figure 5A). In contrast, in the presence of 4-AP, a selective $K_V$ blocker, there was a shift in the curve to the right, with a decrease in the efficacy (ME = 95.37 ± 4.73, $p < 0.05$) and potency ($pD_2$ = 5.89 ± 0.07, $p < 0.05$) of the vasorelaxant effect promoted by FOR 911B compared to the control (Figure 5B). A similar result with a decrease in the efficacy and potency of FOR 911B was found when there was prior treatment with apamin, which blocks $SK_{Ca}$, as demonstrated by the values (ME = 92.60 ± 2.42; $pD_2$ = 5.52 ± 0.06, $p < 0.05$) (Figure 5C).

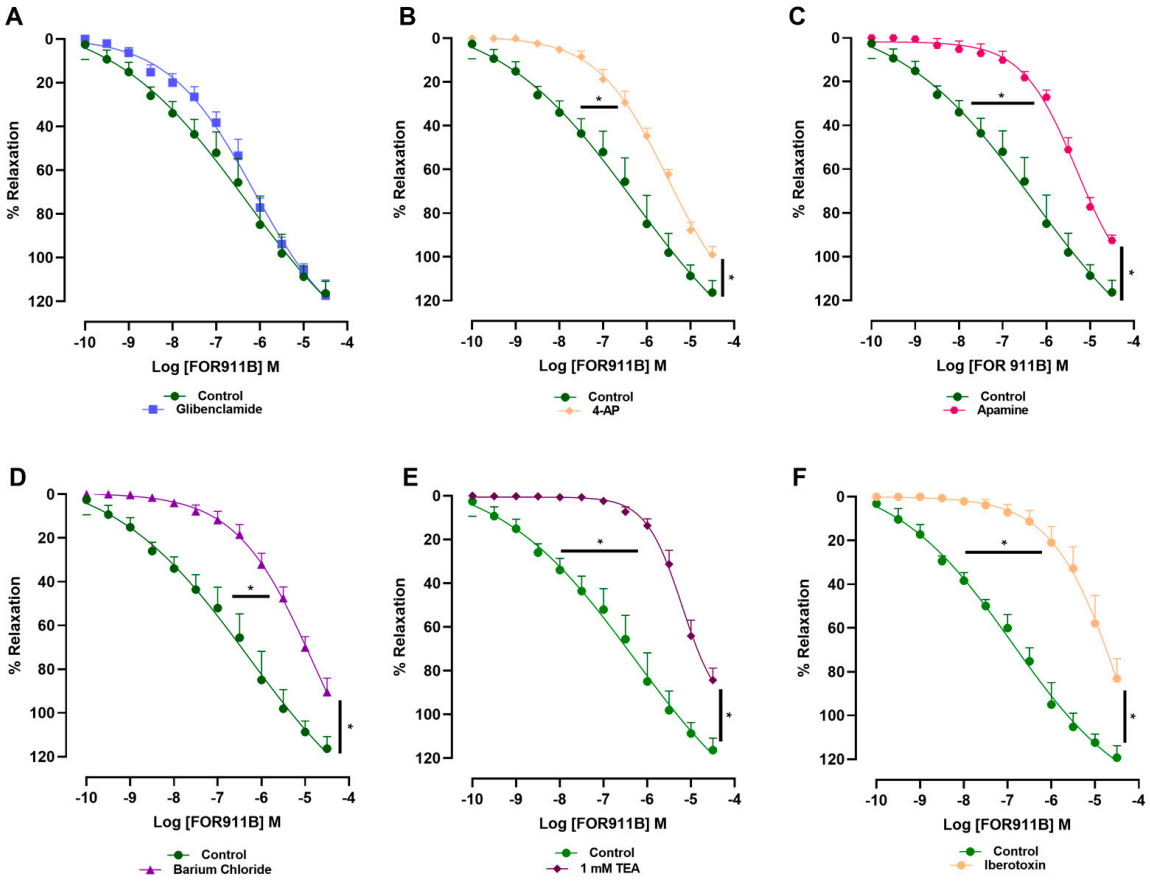



**Figure 5.** Concentration–response curve for the vasorelaxant effect induced by FOR911B in isolated endothelium-denuded rat aortic rings pre-contracted with Phe in the presence of Glibenclamide (10 μM) (**A**), 4-AP (0.3 mM) (**B**), Apamine (100 nM) (**C**), Barium chloride (100 μM) (**D**), TEA (1 mM) (**E**), and Iberotoxin (20 nM) (**F**). Values are expressed as mean ± S.E.M. (n = 6). * $p < 0.05$ vs. Control, Student's *t*-test.

With the use of Barium Chloride, a $K_{IR}$ blocker, the vasorelaxation promoted by the metallodrug was attenuated when compared to the control (ME = 90.41 ± 6.27%; $pD_2$ = 5.56 ± 0.08, $p < 0.05$) (Figure 5D). In the presence of TEA at a concentration of 1 mM, which acts as a selective blocker for $BK_{Ca}$, and Iberiotoxin, which promotes the same selective blockade, the vasorelaxant response produced by the cumulative addition of FOR 911B was reduced (ME = 84.26 ± 5.41; $pD_2$ = 5.11 ± 0.07, ME = 83.12 ± 9.13; $pD_2$ = 5.21 ± 0.14, respectively) when compared to the effect of this ruthenium complex on rings pre-contracted with Phe (ME = 116.25 ± 5.33%; $pD_2$ = 6.29 ± 0.79) ($p < 0.05$) (Figure 5E,F). These results demonstrate the participation of different subtypes of potassium channels in the relaxation induced by FOR911B. The main findings of this study are outlined in Table 1.

**Table 1.** Summary of results.

| Protocol | ME | $pD_2$ |
|---|---|---|
| Intact Endothelium | 111.55 ± 6.77% | 6.35 ± 0.36 |
| Denuded Endothelium | 116.25 ± 5.33% | 6.29 ± 0.79 |
| KCl (60 mM) | 36.03 ± 5.68% * | - |
| L-NAME | 96.84 ± 9.79% * | 5.35 ± 0.09 * |
| L-NMMA | 122.68 ± 14.21% | 5.43 ± 0.24 * |
| HDX | 15.83 ± 1.89 * | - |
| ODQ | 89.30 ± 5.96% * | 5.35 ± 0.13 * |
| KCl (20 mM) | 98.69 ± 7.35% * | 5.46 ± 0.06 * |
| TEA (3 mM) | 52.65 ± 3.23% * | - |
| Glibenclamide | 117.32 ± 7.03% | 6.41 ± 0.20 |
| 4-AP | 95.37 ± 4.73 * | 5.89 ± 0.07 * |
| Apamine | 92.60 ± 2.42 * | 5.52 ± 0.06 * |
| Barium Chloride | 90.41 ± 6.27% * | 5.56 ± 0.08 * |
| TEA (1 mM) | 84.26 ± 5.41 * | 5.11 ± 0.07 * |
| Iberotoxin | 83.12 ± 9.13 * | 5.21 ± 0.14 * |

Values are expressed as mean ± S.E.M. (n = 6). * $p < 0.05$ vs. Control, Student's *t*-test.

## 4. Discussion

The present study evaluated a ruthenium complex that has a NO molecule in its structure called FOR 911B. The main results demonstrated that the compound promoted vascular relaxation in aortic artery rings in a concentration-dependent and endothelium-independent manner. The possible mechanism of action seems to be involved with the release of radical NO, activation of sGC, and activation of $K^+$ channels.

In recent years, NO has become a molecule of great chemical interest as it mediates several physiological processes [3,4]. Changes in the production and/or release of NO are caused by endothelial dysfunction, such as in CVD, leading to impairment of its function through the NO/sGC/cGMP pathway. In this scenario, with the search for compounds capable of donating NO in a stable and modulated manner, ruthenium-based metallic compounds are being continuously studied as a new class of nitric oxide donors [30–36].

Initially, we verified the vasorelaxant effect promoted by FOR 911B after pre-contraction with phenylephrine, which is a selective agonist and induces contraction through the activation of α1-adrenergic receptors [27,37]. FOR 911B induced relaxation in isolated rat aortic artery rings in a concentration-dependent manner. Also, understanding the importance of the vascular endothelium in synthesizing and releasing EDRFs that regulate vascular tone [38], we investigated the involvement of endothelium in the vasorelaxant response promoted by the ruthenium complex. The response induced by FOR 911B was not altered after the removal of the endothelium, suggesting that EDRFs are not involved in the vasodilator effect induced by the new NO donor.

When evaluating Cis-$[Ru(bpy)_2ImN(NO)](PF_6)_3$ (FOR 0811), Costa et al. (2020) found a similar result, where the removal of the endothelium did not promote any change in the concentration–response curve to FOR 0811 [23]. On the other hand, TERPY, another NO-donating metallodrug, produced a reduction in the vasorelaxant potency under the same experimental conditions, suggesting a relevant role of the endothelial layer in the mechanism of action of that compound. The authors suggested that this probably occurs due to the neutralization of $O^{2-}$ and thromboxane A2, contractile factors from endothelial cells [39]. This shows that ruthenium complexes can have opposite effects in relation to the presence or absence of the vascular endothelium.

To evaluate the participation of NOS isoforms in the effect promoted, we performed pre-incubation with L-NAME, a non-selective inhibitor of the different NOS isoforms [40]. Interestingly, with mechanical removal of the endothelial layer in the presence of the blocker, we observed a decrease in the efficacy and potency of the vasorelaxant effect promoted by the compound, a result that had not previously been reported with ruthenium complexes under the same experimental conditions. The same phenomenon was found

using another NOS inhibitor (L-NMMA), corroborating the reduction of the vasorelaxant effect in the presence of L-NAME. The literature demonstrates that NO derived from nNOS can participate in the local regulation of vascular tone independently of the central nervous system, and in smooth muscle cells, inhibition of this isoform increases responses to several vasoconstrictors, suppresses cGMP production, and exacerbates neointimal formation [41]. However, the mechanism by which the NOS inhibition induces a decrease in the vasodilation in the aortic ring without endothelium still needs to be properly clarified.

NO presents different redox states, and the radical type is synthesized by different NOS isoforms and can also be provided by NO donors [30]. To confirm the release of the radical form of NO, the most found NO subtype for inducing the relaxing effect, we incubated the rings with HDX, a radical NO scavenger [42], which contains a cobalt-centered coryne core that can interact with NO to form a new complex, leading to a reduction in its availability [43].

With pre-incubation with HDX, the effect induced by FOR 911B was abolished. Therefore, it is suggested that the studied metallodrug possibly induces vasorelaxation by releasing radical NO. A similar result was found by Costa et al. (2020), who used HDX in their preparations, as well as L-Cysteine, a NO- scavenger. Treatment with HDX completely inhibited the vasodilation induced by FOR0811 [23]. This was also seen by Braz (2022), wherein the evaluation of the participation of the NO radical with the use of this blocker, the maximum effect produced by its compound was reduced; these results reinforce the idea that one of the ways in which the ruthenium compound FOR611A acts is through the NO donation pathway [43]. These findings agree with what was reported by Dierks and Burstyn in 1996, who demonstrated that only NO· is capable of directly activating GCs and promoting vascular relaxation [44]. The GCs enzyme is considered the primary receptor of NO, which perfectly activates this enzyme by nitrosylation of iron in its heme portion, which increases the synthesis of cGMP from GTP, which acts as a second messenger, in turn activating PKG [45]. To elucidate the participation of the NO/GCs/cGMP pathway in the relaxation induced by FOR 911B, we used ODQ as a pharmacological tool, a selective inhibitor of this enzyme, which promotes changes in the oxidation state of the heme portion of GCs and consequently prevents the formation of cGMP [46,47]. In the presence of ODQ, the results demonstrate that this pathway is important in donor-induced vasodilation since the potency and efficacy of the compound decreased with the use of this blocker, thus suggesting that this ruthenium complex possibly induces vascular relaxation through this pathway. The action of NO donors in activating GCs has been previously reported under similar experimental conditions and corroborates the results found here [22,23,30,35].

We also investigated whether the effect promoted by FOR911B involved the participation of $K^+$ channels [48]. Potassium channels directly contribute to the regulation of cell membrane potential, being an important factor in maintaining vascular tone [49]. For this, the concentration of this ion was altered, and we used KCl at a concentration of 60 mM, an agent capable of promoting electromechanical contraction. The relaxation induced by FOR 911B was attenuated under this experimental condition. The induced contraction occurs independently of the activation of receptors, where the increase in the concentration of potassium in the extracellular medium promotes inhibition of its efflux, there is a depolarization of the membrane and opening of the channels for $Ca^{2+}$, and consequent contraction in the smooth muscle [50–52]. This attenuation suggests that the NO donor possibly promotes its vasorelaxant effect through direct activation of the $K^+$ channels in the membrane.

To confirm this participation of the $K^+$ channels, we also used a Krebs solution modified to 20 mM. Unlike the previous experimental condition (with KCl 60 mM), at this concentration, there is a partial attenuation of the efflux of this ion and of the relaxation promoted mediated by the opening of these channels [49]. The effects induced by FOR 911B were reduced, with a shift of the curve to the right, strengthening the findings of this study that the K+ channels participate in this vasorelaxant response.

Considering the previous findings, we used TEA at a concentration of 3 mM, a non-selective blocker of $K^+$ channels [37]. In the presence of this blocker, there was a significant reduction in the maximum relaxation promoted by the donor, a result like that found with 60 mM KCl. It has been reported that other NO donors, such as FOR 011A, RuBPY, cis-[Ru(H-dcbpy)$_2$(Cl)(NO)] (DCBPY), and trans-[RuCl([15]aneN$_4$)NO]$^{2+}$ also promote vascular relaxation by activating potassium channels [53–57].

We then performed pre-incubation with different blockers specific to the $K^+$ channel subtypes, aiming to elucidate which one would be participating in this response promoted by FOR 911B. The literature shows that there are four subtypes found in VSMC, namely ATP-sensitive channels ($K_{ATP}$), inward rectifier channels ($K_{IR}$), voltage-sensitive channels ($K_V$), and $K_{Ca}$, which can be subdivided according to their conductance: small or low ($SK_{Ca}$) and large or wide ($BK_{Ca}$) [58,59].

By selectively blocking $K^+$ channels, the results demonstrated that there was no change in the concentration–response curve when GLIB was added, ruling out the possible participation of $K_{ATP}$. GLIB has been used as a $K_{ATP}$ blocker in several studies, including ours and a recent study using other nitric oxide donors [60,61], which utilized the same GLIB concentration as ours. In addition to the well-known effect on insulin secretion [62], GLIB has been shown to be safe and with few side effects, inducing a protective effect in vascular endothelial cells, yet GLIB-induced intracellular $Ca^{2+}$ increases in these cells [63]. Moreover, the vascular specificity of GLIB as a $K_{ATP}$ blocker is supported by the fact that it does not interfere with the hypotensive effects of a large number of compounds [64].

On the other hand, with the blockade of $K_V$, $K_{IR}$, $SK_{Ca}$, and $BK_{Ca}$, we observed significant changes in the potency and efficacy of the vasodilatory effect promoted by FOR911B. Besides the blockage of $K_V$, controversially, 4-AP has been shown to evoke an increase in Kv7.4 current and hyperpolarization that were independent of 4-AP-mediated changes in intracellular pH; however, this effect occurred in the pre-contracted mesenteric artery, already in rest tone, and 4-AP (1 mM) had no effect [65]. In the current research, we used a lower concentration of 4-AP (0.3 mM) in a basal tone, and we observed a shift to the right and decreases in ME and pD2 after a cumulative concentration of FOR 911B in the isolated aorta.

Here, the participation of $K_{IR}$ was shown by the use of $BaCl_2$. It is demonstrated that, besides the $K_{IR}$ blockage, $BaCl_2$ is able to block the KATP channel in resistance vessels [66]. However, several previous findings by us and others demonstrated that $BaCl_2$ in that concentration (100 µM) is effective in assuming the involvement of the $K_{IR}$ channels [60,61]. In addition, Brunt et al., 2013 reported that $BaCl_2$ at 100 µM was the concentration that induced the most effective inhibition of Kir-mediated vasodilation [67].

As we demonstrated, a shift to the right was shown in the vasorelaxation curve with the use of TEA (1 mM) and IbTX [68], which strongly indicates the participation of $BK_{Ca}$. TEA was found to selectively block the $BK_{Ca}$ channels in concentrations lower than 1 mM (Kd = 0.29 mM) [69], while at higher concentrations, up to $10^{-2}$ M, TEA could block other subtypes of potassium channels [69,70]. These data demonstrate that these subtypes are involved in the NO donor-induced response.

Lunardi et al., in 2009, under similar experimental conditions as ours, observed that with the use of a highly selective blocker of $K_{ATP}$ channels, glibenclamide, there was no blockade of the relaxation of rat aortic rings induced by RUNOCL, as found with FOR 911B. This RUNOCL complex activates two of the four main subtypes of $K^+$ channels: $BK_{Ca}$ and $SK_{Ca}$ [32]. With RuBPY, a widely studied ruthenium complex, selective blockers such as apamin and 4-AP did not alter the induced relaxation. With paxillin, a selective $BK_{Ca}$ blocker, there was a decrease in induced vasorelaxation. These results suggest that $BK_{Ca}$ may be involved in the relaxation induced by RuBPY [34]. Many vasodilators, including NO, activate $K_{Ca}$ directly or indirectly via the activation of kinases [71], with high-conductance $K_{Ca}$ ($BK_{Ca}$) being the main subtype and in greater numbers in VSMCs [72].

## 5. Conclusions

The results obtained demonstrated that the ruthenium complex $[Ru(phen)_2(TU)NO](PF_6)^{3+}$ (FOR 911B) promotes vascular relaxation in aortic artery rings in a concentration-dependent manner and independent of the vascular endothelium through the release of radical NO with the participation of the NO/sGC/cGMP pathway, as well as with the involvement of different $K^+$ channels (especially $K_V$, $K_{IR}$, $SK_{Ca}$, and $BK_{Ca}$). These effects indicate a therapeutic potential for the ruthenium complex FOR 911B as a NO donor, being promising for the treatment of cardiovascular dysfunctions in the future or its use as a pharmacological tool in other studies.

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