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Cardiovascular Safety of VIAN-c4551, an Antiangiogenic Peptide Derived From Vasoinhibin

Miguel A. García-González[1,2] | Alejandro D. Miguel-Martínez[3] | Abimael González-Hernández[1] | Magdalena Zamora[1] | Elva Adán-Castro[1] | Xarubet Ruíz-Herrera[1] | María A. Carbajo-Mata[1] | Jakob Triebel[4] | Thomas Bertsch[4] | José G. Lopez-Lopez[5] | Gonzalo Martínez de la Escalera[1] | Juan Pablo Robles[1,6] | Carmen Clapp[1] | Carlos M. Villalón[3]

[1]Instituto de Neurobiología, Universidad Nacional Autónoma de México (UNAM), Querétaro, Qro, Mexico | [2]Instituto de Fisiología, Benemérita Universidad Autónoma de Puebla (BUAP), Puebla, Pue, Mexico | [3]Departamento de Farmacobiología, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, Unidad Sede Sur, Mexico City, Mexico | [4]Laboratory Medicine and Transfusion Medicine, Institute for Clinical Chemistry, Nuremberg General Hospital, Paracelsus Medical University, Nuremberg, Germany | [5]Departamento de Farmacia, Benemérita Universidad Autónoma de Puebla (BUAP), Puebla, Pue, Mexico | [6]VIAN Therapeutics, San Francisco, California, USA

Correspondence: Carmen Clapp (clapp@unam.mx) | Carlos M. Villalón (cvillalon@cinvestav.mx)

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ABSTRACT

Vasoinhibin is a potent antiangiogenic protein that blocks the activation of endothelial nitric oxide synthase (eNOS) in response to vascular endothelial growth factor, bradykinin, and acetylcholine (ACh). In this regard, VIAN-c4551, a new synthetic vasoinhibin analog, has therapeutic potential for targeting angiogenesis in oncology and ophthalmology. Given that cardiovascular actions are common complications of antiangiogenic drugs and eNOS inhibitors, this multidisciplinary study investigated the cardiovascular safety of VIAN-c4551. Administered acutely, VIAN-c4551 inhibited ACh-induced eNOS phosphorylation/activation in cultured endothelial cells and in lung tissue treated in vivo, as well as the ACh-induced relaxation of rat aortic segments. However, daily intravenous (i.v.) injections of VIAN-c4551 (1 or 3 mg kg^{-1}) for 5 days failed to significantly modify the vasodepressor responses to ACh and the baseline values of blood pressure in anesthetized rats (intact, vagotomized, or pithed). Furthermore, daily i.v. injections of 1 mg kg^{-1} VIAN-c4551 (for 5 days) did not alter: (i) blood pressure or heart rate values in awake rats; (ii) cardiac autonomic and histological outcomes in anesthetized animals; or (iii) inflammatory (tumor necrosis factor-α [TNF-α] and interleukin-6 [IL-6]) and apoptotic (caspase-3) markers. Although VIAN-c4551 inhibited ACh-induced eNOS activation in vitro and in vivo and vasorelaxation in *ex vivo* assays, in vivo experiments consistently showed no significant cardiovascular effects produced by this synthetic peptide. Thus, VIAN-c4551 appears to be cardiovascularly safe for targeting angiogenesis-related diseases.

Miguel A. García-González and Alejandro D. Miguel-Martínez contributed equally to this study. Carmen Clapp and Carlos M. Villalón share a senior author position.

1 | Introduction

The formation of new blood vessels (angiogenesis) underlies the progression of high-impact diseases including cancer and diabetic retinopathy (Carmeliet 2003; Fallah et al. 2019). Although antiangiogenic drugs that block vascular endothelial growth factor (VEGF) signaling pathways have reached broad clinical use, drawbacks such as resistance and off-target effects, including hypertension, cannot be overlooked (De jesus-Gonzalez et al. 2012). In this regard, endogenous angiogenesis-inhibitory proteins (e.g., vasoinhibin) could be safer and more effective therapeutic alternatives (Nyberg et al. 2005; Rao et al. 2015).

Briefly, vasoinhibin is an endogenous protein involved in the regulation (inhibition) of angiogenesis, vasopermeability, and vasodilation (Clapp et al. 2006; Clapp et al. 2015), in part by blocking the production of nitric oxide (NO) derived from the activation of endothelial nitric oxide synthase (eNOS) by vascular endothelial grow factor (VEGF), bradykinin and acetylcholine (ACh) (Clapp et al. 2015). Altered vasoinhibin levels underlie the progression of several pathologies, including diabetic retinopathy (Triebel et al. 2022), retinopathy of prematurity (Zepeda-Romero et al. 2017), peripartum cardiomyopathy (Hilfiker-Kleiner et al. 2007), pre-eclampsia (González et al. 2007; Zamora et al. 2024), rheumatoid arthritis (Ortiz et al. 2020), and cancer (Nguyen et al. 2007). However, the therapeutic use of vasoinhibin is limited by difficulties in recombinant production (Moreno-Carranza et al. 2019). These obstacles were overcome by the development of VIAN-c4551, a cyclic heptapeptide vasoinhibin analog with full activity and potency that is easily produced, stable, and active after oral administration and topical eye delivery (Robles et al. 2022; Adán-Castro et al. 2025).

Hypertension is a common side effect of anti-VEGF agents used in oncology (An et al. 2010; Robinson et al. 2010) and ophthalmology (Rasier et al. 2009; Hanna et al. 2020). The proposed mechanisms include reduced production of vascular NO, elevation of systemic vascular resistance, and subsequent hypertension (Facemire et al. 2009). However, decreased levels of NO metabolites may not be associated with reduced flow-mediated vasodilation (Mayer et al. 2011), and NO inhibitors produce hypertension through mechanisms involving not only reduced endothelium-dependent vasodilation (Baylis et al. 1992; Manning et al. 1993), but also increased sympathetic vasoconstriction (Sander et al. 1995; Sander et al. 1997; Sander and Victor 1999).

Given that vasoinhibin blocks vascular NO production stimulated by VEGF, bradykinin and ACh (Gonzalez et al. 2004; García et al. 2008) and that VIAN-c4551 is a promising therapeutic drug, in this multidisciplinary study we have: (i) investigated the effects of VIAN-c4551 on ACh-induced eNOS activation in vitro and in vivo and ACh-induced aortic relaxation ex vivo; and (ii) examined the potential consequences of these actions on diastolic, mean and systolic blood pressure as well as cardiac outcomes in anesthetized (intact, vagotomized and pithed) and awake rats. Despite its in vitro and in vivo inhibitory effects on ACh-induced NO phosphorylation/activation and NO-mediated aortic relaxation, our results show that VIAN-c4551 does not alter blood pressure and cardiac function in vivo.

2 | Materials and Methods

2.1 | Animals

Seventy-five male Wistar rats (300–400 g) were housed under standard laboratory conditions (22 ± 2°C; 12 h/12 h light/dark cycle; free access to food and water). The experiments were approved by the Ethics Committees of both the Institute of Neurobiology of the National University of Mexico (UNAM) and the Center for Research and Advanced Studies (Cinvestav) of the National Polytechnic Institute (IPN) (Approval Numbers 033 and 0139-15, respectively), according to the US National Research Council's Guide for the Care and Use of Laboratory Animals (Eighth Edition, National Academy Press, Washington, DC, USA). These animals were divided into six groups ($n = 9$, 10, 20, 12, 10, 14; see below).

2.2 | eNOS Phosphorylation in Endothelial Cells In Vitro and Lung Tissue Treated In Vivo

In vitro: human umbilical vein endothelial cells (HUVECs) were obtained as previously reported by Baudin et al. (2007) and cultured in F12K medium supplemented with 20% fetal bovine serum, 100 $\mu g\,mL^{-1}$ heparin (Sigma-Aldrich, St. Louis, MO, USA), and 25 $\mu g\,mL^{-1}$ endothelial cell growth supplement (ECGS; Corning, NY, USA), as previously reported (Robles et al. 2022). HUVECs were incubated with or without 100 nM VIAN-c4551 for 1 h, followed by addition of 10 μM ACh (Sigma-Aldrich) for 10 min. In vivo: A group of nine rats was placed in an acrylic restrainer and divided into 3 subgroups that received an intravenous (i.v.) bolus injections of either vehicle (physiological saline, 1 $mL\,kg^{-1}$) or VIAN-c4551 (3 $mg\,kg^{-1}$) 10 min before the i.v. bolus injection of 100 $\mu g\,kg^{-1}$ ACh and 5 min later rats were euthanized by CO_2 inhalation and lungs harvested for immediate protein extraction. HUVEC and lungs were homogenized in RIPA lysis buffer supplemented with a 1:100 halt protease-phosphatase inhibitor cocktail and 5 mM EDTA (both from Thermo Scientific, Waltham, MA, USA). Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blotting, as previously reported (Robles et al. 2022), probed first with antibodies against phospho-eNOS (9571,1:250) and then re-probed with antitotal eNOS antibodies (9572, 1:500) (both from Cell Signaling, Danvers, MA, USA).

2.3 | *Ex vivo* Vascular Reactivity

A group of 10 rats was subdivided into 2 subgroups ($n = 5$ each) that received, for 5 days, daily i.v. bolus injections of either vehicle (physiological saline, 1 $mL\,kg^{-1}$) or VIAN-c4551 (1 mg kg^{-1}). Then, they were deeply sedated by an intraperitoneal (i.p.) injection of a mixture of ketamine and xylazine (60 mg kg^{-1} and 4 mg kg^{-1}, respectively) and subsequently euthanized by opening the thorax. At this point, the thoracic aortic segment was removed and immediately placed for cleaning in Krebs-Henseleit solution (118 mM NaCl, 4.75 mM KCl, 25 mM $NaHCO_3$, 1.2 mM; $MgSO_4$, 2.0 mM $CaCl_2$, 1.2 mM KH_2PO_4, and 11 mM glucose, pH 7.4) at 37°C; then aortic rings were obtained

to evaluate vascular reactivity ex vivo, as described by Gonzalez et al. (2004). Briefly, the aorta was cut into 3 mm-wide ring segments, each suspended from an isometric transducer in oxygenated tissue baths containing Krebs–Henseleit solution. Isometric tension of 2 g was applied, and the aortic segments were allowed to equilibrate for 10 min. The contractile response was standardized to the effect induced by 80×10^{-3} M KCl (incubated for 45 min), whereas the relaxation was standardized to the effect generated by 10^{-6} M phenylephrine (Phen, incubated for 10 min).

The aortic rings were contracted with 10^{-6} M Phen and, when the development of Phen-induced tension reached a plateau, they were incubated in the presence or absence of VIAN-c4551 (10^{-10} M) for 20 min. Then, different concentrations of ACh (10^{-9}–3×10^{-5} M) were added to induce endothelial cell-dependent relaxations. In other experiments, the effect of 10^{-10} M VIAN-c4551 on contractile vascular responses was tested using dose–response (D-R) curves against Phen (10^{-9}–3×10^{-5} M) or the thromboxane A_2 agonist U-46619 (10^{-9}–3×10^{-5} M). Real-time data were recorded using a force transducer (GRASS FT-03) plus amplifier (GRASS 7-DAJK) system and analyzed using LabChart software (version 7.0; ADInstruments, Colorado Springs, CO, USA).

2.4 | Blood Pressure in Anesthetized Rats (i.e., Intact, Vagotomized, or Pithed)

For this experimental protocol, 20 rats (subdivided into 4 subgroups; $n = 5$ each) were anesthetized with sodium pentobarbital (60 mg kg^{-1}, i.p.) and subjected to tracheal cannulation. They were artificially ventilated with room air using an Ugo Basile pump (Ugo Basile Srl, Comerio, VA, Italy) at a rate of 56 strokes min^{-1} and a stroke volume of 20 mL kg^{-1} as previously established (Kleinman and Radford 1964). A Grass pressure transducer (P23 XL) coupled to a Grass model 7D polygraph (Grass Instrument Co., Quincy, MA, USA) was connected to the left carotid artery to record systolic blood pressure (SBP), mean blood pressure (MBP) and diastolic blood pressure (DBP). Animals were kept warm (37.5 ± 0.5°C) throughout the experiments and 30 min were allowed to elapse for hemodynamic stabilization.

Subgroup 1 ($n = 5$) consisted of intact rats, namely, animals with intact vagus nerves and an intact central nervous system (CNS); this subgroup was used to investigate the acute effect of consecutive i.v. bolus injections of VIAN-c4551 (1 and 3 mg kg^{-1}) on the blood pressure responses elicited by dose–response curves to ACh (0.01, 0.03, 0.1, 0.3 and 1 μg kg^{-1}, i.v.). For this purpose, catheters were placed into the right and left femoral veins, one for administering VIAN-c4551 or saline as vehicle (1 mL kg^{-1}) and the other for administering increasing doses of ACh (0.01 to 1 μg kg^{-1}). Three dose–response (D-R) curves to ACh were obtained for each animal, which were initiated with an i.v. bolus injection of ACh into the femoral vein every 5–10 min, as we waited until blood pressure had returned to baseline values. The first D-R curve to ACh was followed by a 10 min wash with saline (1 mL kg^{-1}), a bolus injection of VIAN-c4551 (1 mg kg^{-1}) or saline (1 mL kg^{-1}) into the right femoral vein, with an interval of 10 min; a second D-R curve to ACh, a 10 min wash, a bolus of VIAN-c4551 (3 mg kg^{-1}), and, after 10 min, a third D-R curve to ACh.

In addition, the effect of VIAN-c4551 on resting blood pressure was tested in anesthetized rats that were intact (i.e., with intact vagus nerves and an intact CNS; $n = 5$), bilaterally vagotomized ($n = 5$) (González-Hernández et al. 2018), or pithed (including bilateral vagotomy; $n = 5$) by inserting a stainless-steel rod through the orbit and *foramen magnum* and down the vertebral *foramen*, as reported by Rivera-Mancilla et al. (2018). The rats were ventilated and catheterized, and their SBP, MBP, and DBP were recorded, as described above. After a 10 min hemodynamic stabilization period, blood pressure was assessed before (0 min) and 10 min after consecutive i.v. bolus injections of VIAN-c4551 (1 and 3 mg kg^{-1}).

2.5 | Blood Pressure in Awake Rats

In a group of 12 awake rats, SBP, MBP and DBP were measured using a noninvasive tail-cuff system (IITC 179 Blood Pressure Analyzer; Life Sciences, Woodland Hills, CA, USA). Animals were trained and acclimatized for 2 weeks to the restraint procedure of the tail-cuff-based system in a quiet and isolated environment. Briefly, rats were positioned in a custom-made rat restraint apparatus for 5 min (first week) and 10 min (second week), with the cuff and sensor at the base of the tail. Blood pressure was then measured using an inflating bulb (deflation time of 5 s), and traces were recorded on thermal paper. Measurements were taken 2 h after the last daily injection of saline (1 mL kg^{-1}; $n = 6$) or VIAN-c4551 (1 mg kg^{-1}; $n = 6$) into the tail vein for 5 consecutive days. All measurements were taken between 13:00 and 15:00 h to minimize the impact of diurnal variation.

2.6 | Electrocardiogram, Heart Rate, Ventricular Remodeling and Histological Changes

Electrocardiographic recordings were obtained in a group of 10 rats previously habituated for 2 weeks in a dark and quiet environment under movement restriction, with 3 electrodes on the shaved thorax positioned according to derivative II of Einthoven's frontal plane (Howarth et al. 2004; Howarth et al. 2008). These animals were subdivided into two subgroups that consisted of: (i) awake rats ($n = 5$); and (ii) rats under i.p. anesthesia with a mixture of ketamine and xylazine (60 and 4 mg kg^{-1}, respectively; $n = 5$).

All animals were injected daily with 1 mg kg^{-1} VIAN-c4551 or 1 mL kg^{-1} saline into the tail vein for 5 days, and electrocardiogram recordings were obtained 24 h before the first administration of VIAN-c4551 and 24 h after the last administration of VIAN-c4551. The electrodes were connected to an amplifier (BIO Amp CF, ADInstruments, Colorado Springs, CO, USA) and Powerlab 4/35 (ADInstruments), and the signals were collected and stored using LabChart 7 software (ADInstruments). The total recording time was 15 min.

The electrocardiogram software analysis measured the heart rate (HR), QRS, RR, QT, and corrected QTc (QT/√RR). Five minutes electrocardiograms free of artifacts were selected using Kubios HRV 2.1. software, and heart rate variability (HRV) was analyzed in the time domain (SDNN and RMSSD), frequency domain (LF/HF), and nonlinear methods (SD1 and SD2).

At the end of the experiments, the hearts were dissected and perfused with saline until traces of blood were removed. The left and right ventricles were then isolated, fixed (4% formaldehyde), and dehydrated for kerosene embedding (Leica Biosystems, Leica Microsystems Inc., Deer Park, IL, USA). Five micrometers thick ventricular sections were subjected to hematoxylin-eosin staining for blood vessel evaluation and Mason's trichrome staining for collagen analyses, as described previously (Sun et al. 2024; Zhao et al. 2016). The number of blood vessels and the area positive for collagen per unit area were counted using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.7 | Cardiac Ventricle Expression of Inflammatory and Apoptotic Markers

An additional group of 14 rats, divided into two subgroups, were injected daily with $1\,mg\,kg^{-1}$ VIAN-c4551 ($n = 7$) or $1\,mL\,kg^{-1}$ saline ($n = 7$) into the tail vein for 5 days. The isolated left and right ventricles were processed for RT-qPCR. RNA was isolated using TRIzol (Invitrogen, Thermo Fisher Scientific) and retrotranscribed with the high-capacity cDNA reverse transcription kit (applied Biosystems, Thermo Fisher Scientific).

Polymerase chain reaction (PCR) products were obtained and quantified using Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific) in a final reaction containing 20 ng of cDNA and 0.5 μM of each of the following primer pairs for rat genes: tumor necrosis factor-α (TNF-α) fwd (GGGCTTG TCACTCGAGTTTT), TNF-α rev (TGCCTCAGCCTCTTCTCA TT), interleukin-6 (IL-6) fwd (TCCAACTCATCTTGAAAGCA), IL-6 rev (TTCATATTGCCAGTTCTTCG), caspase-3 fwd (ATAG TAACCGGGTGCGGTAG), caspase-3 rev (GAAAGCCGAAACT CTTCATCA), GAPDH fwd (GTCCACTGGCGTCTTCACCA) and GAPDH rev (GTGGCAGTGATGGCATGGAC). Amplification consisted of 40 cycles of 10 s at 95°C, 30 s at the annealing temperature of each primer pair, and 30 s at 72°C. The mRNA expression levels were calculated by the $2^{-\Delta\Delta C_T}$ method.

2.8 | Statistical Analysis

Data analysis and graphs were generated using GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA, USA). All results were presented as mean ± S.E.M. Statistical changes in the relative intensity of p-eNOS are expressed with a value of $p < 0.001$. In the D-R curves, statistical differences ($p < 0.05$) were obtained by comparing the effects of the treatment groups vs those produced by vehicle and are represented with empty symbols. The changes in blood pressure produced in the treatment groups (Figure 2) were compared vs those produced by the corresponding vehicle. The daily (Day 1–5) blood pressure results (Figure 3) were compared versus blood pressure value obtained before VIAN-c4551 administration (Day 0). Results (Tables and Figures) were analyzed with a two-way ANOVA test coupled to a Šídák's post hoc test, except for Figures 1B and 4B analyzed with a one-way ANOVA and Tukey's multiple comparison test and Table 4 evaluated with unpaired *t* test.

3 | Results

3.1 | Effects of VIAN-c4551 on ACh-Induced eNOS Phosphorylation at Ser^{1177}

ACh is a potent vasodilator whose activity is mediated by eNOS activation and NO synthesis in endothelial cells (Palmer et al. 1987). Since eNOS activation occurs through phosphorylation of Ser^{1177} (Boo and Jo 2003), we evaluated whether VIAN-c4551 modified ACh-induced eNOS phosphorylation in HUVEC using western blot analysis (Figure 1A). ACh enhanced eNOS phosphorylation at Ser^{1177} compared to no treatment, whereas preincubation with VIAN-c4551 for 1 h before ACh addition reduced ACh-induced eNOS phosphorylation. Phosphorylation levels were similar between the untreated group and VIAN-c4551 alone. Differences in phosphorylated eNOS levels were quantified after normalization of total eNOS levels on the blot (Figure 1B).

3.2 | Effects of VIAN-c4551 on ACh-Induced Relaxation of Rat Aortic Segments

Given that vasoinhibin inhibits eNOS derived NO-mediated vasodilation in response to ACh (Gonzalez et al. 2004), we tested whether VIAN-c4551 conserved this property by evaluating its effect on ACh-induced relaxation of rat aortic segments precontracted with the α_1-adrenoceptor agonist Phen.

Our results show that VIAN-c4551 significantly ($p < 0.05$) reduced the vasorelaxation induced by high concentrations of ACh ($> 10^{-7}$ M) (Figure 2A). In this respect: (i) the maximal relaxation (E_{max}) to ACh was lower ($p < 0.05$) in VIAN-c4551-treated segments than in untreated segments ($70.1 \pm 4.3\%$ vs. $86.2 \pm 3.3\%$; $p < 0.05$); and (ii) the sensitivity (EC_{50}) for ACh was reduced ($p < 0.05$) compared to untreated segments ($0.3 \pm 0.1\,\mu M$ vs. $0.8 \pm 0.2\,\mu M$; $p < 0.05$) (Figure 2A, Table 1). These findings support the ability of VIAN-c4551 to inhibit endothelial NO-mediated vasodilation, which may result in vasoconstriction.

In view that in vivo treatment with vasoactive substances can alter the relaxant and contractile responses of isolated arteries in vitro (Momoi et al. 2003), we further investigated whether daily treatment with VIAN-c4551 for 5 days modified the *ex vivo* action of VIAN-c4551 on ACh-induced relaxation of aortic segments. As shown in Figure 2A, the in vivo treatment with VIAN-c4551 prevented the inhibitory action of VIANc-4551 observed in vitro. Accordingly, ACh E_{max} (85.0 ± 7.5 vs. 86.2 ± 3.3) and EC_{50} (0.5 ± 0.1 vs. 0.8 ± 0.2) values were like those of untreated rings (Figure 2A, Table 1).

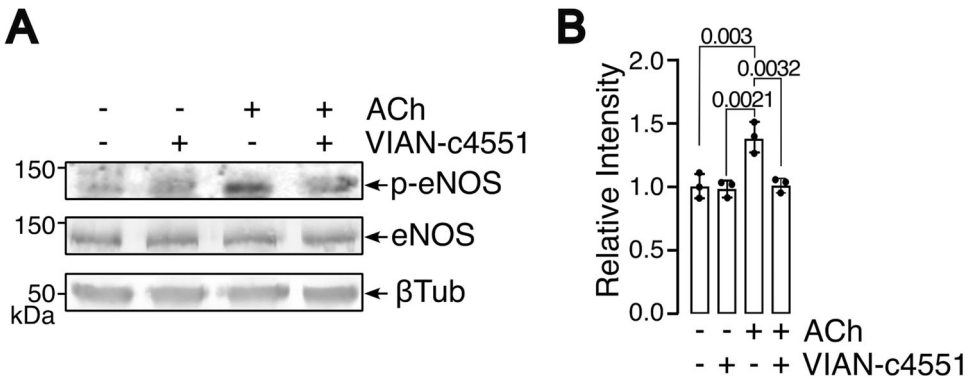

FIGURE 1 | VIAN-c4551 reduces ACh-induced eNOS phosphorylation in endothelial cells. (A) Representative western blot analysis of the phosphorylation of eNOS at Ser^{1177} in human umbilical vein endothelial cells preincubated with 100 nM VIAN-c4551 for 1 h and then treated with 10 μM ACh for 10 min. Total eNOS and β-tubulin were used as loading controls. (B) Quantification of eNOS phosphorylation by densitometry after normalization for total eNOS and β-tubulin. Bars are means ± S.E.M. from three independent experiments. Individual values are shown and *p* values indicated (One-way ANOVA, Tukey). ACh, acetylcholine; p-eNOS, phosphorylated endothelial nitric oxide synthase; eNOS, endothelial nitric oxide synthase; βTub, β-tubulin.

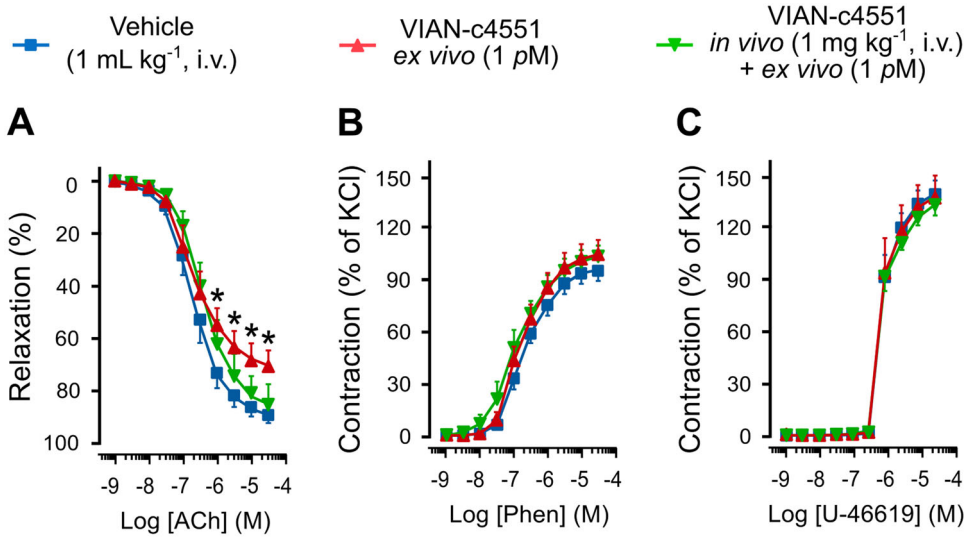

FIGURE 2 | VIAN-c4551 reduces ACh-induced relaxation of rat aortic rings. (A) ACh-induced vascular relaxation of ex vivo aortic segments obtained from rats injected daily with saline (vehicle; 1 mL kg^{-1}) or VIAN-c4551 (1 mg kg^{-1}) for 5 days. Isolated aortic segments were treated with or without 100 pM VIAN-c4551 for 25 min and submaximally contracted with phenylephrine (Phen), followed by treatment with increasing concentrations of ACh. Vasorelaxation was expressed as the percentage of contraction induced by Phen. Vasoconstriction of isolated aortic segments from rats treated with saline or VIAN-c4551 for 5 days and incubated *ex vivo* with or without VIAN-c4551 (100 pM) and different doses of Phen (B) or U46619 (C). Values are presented as the mean ± S.E.M ($n = 5$). *$p < 0.05$ versus vehicle (two-way ANOVA, Šídák).

It is noteworthy that downregulation of endothelial cell-derived NO can enhance the vasoconstrictor effect of norepinephrine (Feitelson et al. 2003), but not that of thromboxane A_2 (Momoi et al. 2003). These findings led us to search for a possible mechanism mediating the in vivo influence of VIAN-c4551. Hence, we next tested whether VIAN-c4551 can alter the contractile concentration-response curves to the α_1-adrenoceptor agonist Phen (10^{-9}–3×10^{-5} M) or to the thromboxane A_2 agonist U-46619 (10^{-9}– 3×10^{-5} M) in rat aortic rings; these rings were obtained from rats previously treated or not with five daily injections of VIAN-c4551. Our results show that the concentration-response curves to Phen (Figure 2B, Table 1) and U-46619 (Figure 2C, Table 1) were not modified by incubation with VIAN-c4551 with or without in vivo treatment.

In summary, VIAN-c4551 inhibits ACh-induced aortic vasorelaxation and does not alter the contractile vascular responses to Phen or thromboxane A_2. With these findings in mind, we decided to explore the possibility that the inhibition by VIAN-c4551 of ACh-induced aortic vasodilation results in changes in cardiovascular function (i.e., blood pressure, HR, and electrocardiographic parameters). Accordingly, as a first experimental approach, blood pressure was investigated in anesthetized rats by testing the acute action of pharmacological doses of VIAN-c4551 on baseline blood pressure values as well as on the vasodepressor responses elicited by increasing doses of ACh.

TABLE 1 | Parameters of relaxation and contraction curves for acetylcholine (ACh), phenylephrine (Phen), and U46619 of rat aortic rings obtained from rats treated in vivo and ex vivo with saline (vehicle) and/or VIAN-c4551.

Agonist	Parameter	Vehicle	VIAN-c4551 ex vivo	VIAN-c4551 in vivo + ex vivo
ACh	E_{max} (%)	86.2 ± 3.3	70.1 ± 4.3*	85.0 ± 7.5
	EC_{50} (μM)	0.8 ± 0.2	0.3 ± 0.1*	0.5 ± 0.1
Phen	E_{max} (%)	92.9 ± 5.6	101.2 ± 7.9	99.8 ± 6.1
	EC_{50} (μM)	0.21 ± 0.03	0.18 ± 0.03	0.14 ± 0.05
U46619	E_{max} (%)	131.0 ± 7.1	130.1 ± 11.0	124.3 ± 6.5
	EC_{50} (μM)	0.9 ± 0.1	1.0 ± 0.2	0.8 ± 0.1

Note: Rats were injected i.v. with vehicle (1 mL kg^{-1}) for 5 days and their aortic segments were incubated ex vivo with vehicle or 100 pM VIAN-c4551 (100 pM) for 25 min. The other rats were injected i.v. with VIAN-c4551 (1 mg kg^{-1}) for 5 days, and their aortic segments were incubated ex vivo with 100 pM VIAN-c4551 for 25 min. Aortic segments were then submaximally contracted with phenylephrine (Phen) and incubated with increasing concentrations of ACh or directly with increasing concentrations of Phen or the thromboxane A_2 agonist, U46619. E_{max}: Maximal effect; EC_{50}: half-maximal effective concentration. Values are the mean ± S.E.M., $n = 5$, $^*p < 0.05$, vs. vehicle (two-way ANOVA, Šídák).

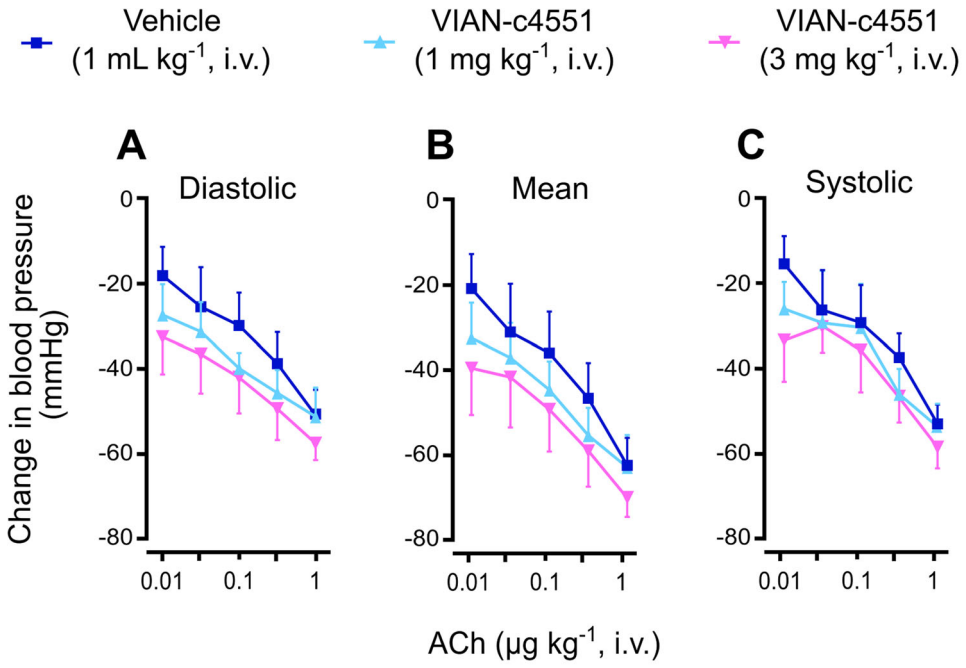

FIGURE 3 | VIAN-c4551 did not modify the vasodepressor responses to ACh in anesthetized intact rats. Decreases in diastolic (A), mean (B), and systolic (C) blood pressure values were induced by increasing intravenous bolus injections of ACh in anesthetized intact rats pretreated intravenously with vehicle (saline; 1 mL kg^{-1}) or VIAN-c4551 (1 or 3 mg kg^{-1}). Values are presented as mean ± S.E.M ($n = 5$). Note that no significant changes ($p > 0.05$) were produced after 1 or 3 mg kg^{-1} of VIAN-c4551 or the corresponding volume of vehicle.

3.3 | Effects of VIAN-c4551 on the Vasodepressor Responses to ACh in Anesthetized Intact Rats

As previously defined (Feher 2012; Pagoulatou et al. 2021; Lefferts et al. 2022): (i) diastolic blood pressure (DBP) is considered an approximate index of systemic vascular resistance/tone; (ii) systolic blood pressure (SBP) is an indicator of left ventricle heart contractility; and (iii) mean blood pressure (MBP = DBP + [SBP-DBP]/3) is the average blood pressure throughout a complete cardiac cycle, directly proportional to cardiac output and systemic vascular resistance.

Baseline values of DBP, MBP and SBP in anesthetized intact rats were 72 ± 9, 92 ± 8, and 130 ± 8 mmHg, respectively. The consecutive i.v. bolus injections of ACh resulted in the expected dose-dependent decreases in DBP, MBP and SBP (Figure 3A–C, respectively), which were significant ($p < 0.05$) when compared with their corresponding baseline values. Moreover, VIAN-c4551 failed to significantly modify ($p > 0.05$) the vasodepressor responses to ACh after i.v. administration of the two consecutive doses tested (1 and 3 mg kg^{-1}). This is a remarkable result because, contrary to what was expected from its inhibitory effect on ACh-induced eNOS phosphorylation/activation in vitro and aortic vasorelaxation *ex vivo* (Figure 2A, Table 1), VIAN-c4551 failed to attenuate the vasodepressor responses to ACh in vivo. The lack of in vivo effects is not related to the dose used. One milligram kg^{-1} i.v. VIAN-c4551 was the lowest dose displaying a maximal antiangiogenic effect in a mouse melanoma tumor model (Robles et al. 2022) and effective to inhibit melanoma-induced lung vascular permeability (Perez et al. 2025). Moreover, daily i.v. injections of 1 and 10 mg/kg VIAN-c4551 for 14 days in healthy mice displayed a safe

toxicity profile on body weight, biochemical and hematological parameters, and anatomical and histological evaluation of major organs (lungs, liver, spleen, kidneys) (Perez et al. 2025). Thereby, the lack of vasopressor responses likely reflects an in vivo VIAN-c4551-resistant systemic eNOS activation and/or vasodilatation in response to ACh.

3.4 | Effects of VIAN-c4551 on *In Vivo* ACh-Induced eNOS Phosphorylation at Ser^{1177}

To investigate whether VIAN-c4551 conserved the property to inhibit ACh-induced eNOS activation in vivo, we chose lung tissue as a suitable site, as eNOS regulates pulmonary vascular tone and is linked to pulmonary hypertension (Zhao et al. 2009). We evaluated whether VIAN-c4551 prevented the acute ACh-induced eNOS phosphorylation in lungs using western blot analysis (Figure 4A). ACh enhanced eNOS phosphorylation at Ser^{1177} compared to no treatment, whereas a single i.v. bolus injection of VIAN-c4551 10 min before i.v. ACh reduced ACh-induced eNOS phosphorylation. Differences in phosphorylated eNOS levels were quantified after normalization of total eNOS levels on the blot (Figure 4B). These findings indicate that the in vivo failure of VIAN-c4551 to alter the vasodepressor responses to ACh is not due to a lack of in vivo inhibition of eNOS activation.

3.5 | Effects of VIAN-c4551 on Baseline Blood Pressure Values in Anesthetized Rats (i.e., Intact, Bilaterally Vagotomized or Pithed)

The possible hemodynamic influence of VIAN-c4551 was additionally tested on the resting blood pressure values of anesthetized rats under three different experimental conditions, namely: (i) with intact vagus nerves and an intact CNS (i.e., intact rats); (ii) bilaterally vagotomized (i.e., devoid of autonomic [vagal] regulation); and (iii) pithed and bilaterally vagotomized (i.e., devoid of central influences on the cardiovascular system as the CNS pathways were eliminated at spinal and brain levels) [for further experimental details, see González-Hernández et al. 2018; Rivera-Mancilla et al. 2018]. Baseline DBP, MBP and SBP values were determined before and 10 min after two consecutive i.v. bolus injections of VIAN-c4551 (1 and 3 mg kg^{-1}; see Table 2).

As previously reported by González-Hernández et al. (2018), baseline blood pressure values ranged from 30 to 60 mmHg in pithed rats and, as expected (Tarizzo and Dahlöf 1989), they were steadily increased by and i.v. continuous infusion of the to the α_1-adrenoceptor agonist methoxamine (not shown). Importantly, VIAN-c4551 did not significantly affect ($p > 0.05$) the baseline blood pressure values in the anesthetized animals subjected to any of the three experimental conditions (i.e., intact, vagotomized, or pithed) (Table 2). These findings indicate that VIAN-c4551 is devoid of significant cardiovascular effects in vivo, at least under our experimental conditions, and in the short term.

3.6 | Effects of Daily VIAN-c4551 for 5 Days on Blood Pressure in Awake Rats

The above findings in anesthetized rats with acute administration of VIAN-c45551 were complemented by evaluating in awake rats the resting blood pressure values 2 h after daily i.v. administration (1 mg kg^{-1}) of VIAN-c4551 for five consecutive days. Interestingly, VIAN-c4551 did not significantly modify ($p > 0.05$) DBP, MBP or SBP values throughout the 5 days of administration (Figure 5). Similar results were obtained with an equivalent volume of vehicle (saline; 1 mL kg^{-1}) (not shown).

3.7 | Effects of VIAN-c4551 on Heart Rate or Electrocardiographic Parameters in Awake and Anesthetized Rats

To analyze its potential effects on HR and electrocardiographic parameters, VIAN-c4551 (1 mg kg^{-1}, i.v.) was daily injected for 5 days in previously habituated awake rats and anesthetized rats. The electrocardiogram (ECG) was recorded before the first

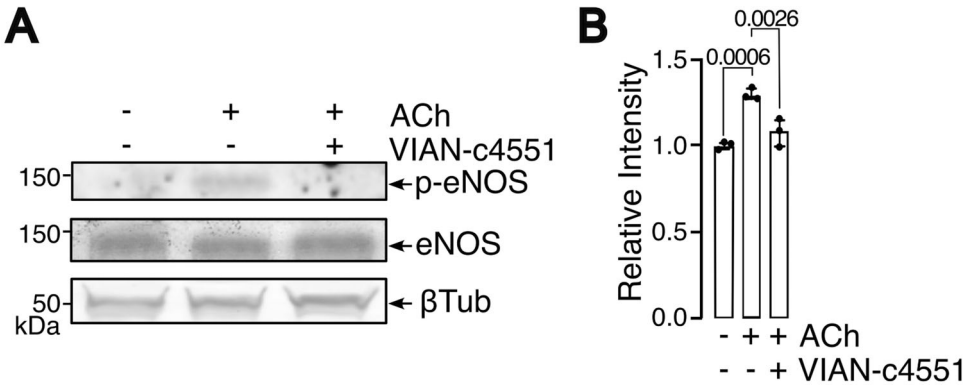

FIGURE 4 | VIAN-c4551 reduces the in vivo ACh-induced eNOS phosphorylation in lungs. (A) Representative western blot analysis of the phosphorylation of eNOS at Ser^{1177} in lung tissue collected from rats injected intravenously with 3 mg kg^{-1} VIAN-c4551 followed 10 min later by the intravenous injection of 100 μg kg^{-1} ACh (~10 μM). Lungs were harvested 5 min after ACh administration. Total eNOS and β-tubulin were used as loading controls. (B) Bars are means ± S.E.M. from three independent experiments. Individual values are shown and *p* values indicated (one-way ANOVA, Tukey). ACh, acetylcholine; eNOS, endothelial nitric oxide synthase; p-eNOS, phosphorylated nitric oxide synthase; βTub, β-tubulin.

TABLE 2 | Values of diastolic (DBP), mean (MBP), and systolic (SBP) blood pressure before and 10 min after two i.v. doses of VIAN-c4551 in anesthetized (intact, vagotomized or pithed) rats.

	VIAN-c4551			
	1 mg kg^{-1}		3 mg kg^{-1}	
INTACT	**Before (0 min)**	**After (10 min)**	**Before (0 min)**	**After (10 min)**
DBP (mmHg)	92 ± 8	91 ± 10	95 ± 5	95 ± 7
MBP (mmHg)	98 ± 8	98 ± 9	101 ± 5	103 ± 6
SBP (mmHg)	111 ± 8	113 ± 8	114 ± 5	120 ± 6
VAGOTOMIZED				
DBP (mmHg)	87 ± 12	83 ± 11	88 ± 7	83 ± 7
MBP (mmHg)	93 ± 12	88 ± 11	94 ± 7	88 ± 6
SBP (mmHg)	106 ± 12	99 ± 11	105 ± 7	99 ± 6
PITHED				
DBP (mmHg)	40 ± 7	38 ± 3	38 ± 5	39 ± 2
MBP (mmHg)	45 ± 7	43 ± 2	43 ± 4	44 ± 3
SBP (mmHg)	56 ± 7	52 ± 2	54 ± 3	54 ± 3

Note: Values are presented as mean ± S.E.M. ($n = 5$). Note that no significant changes ($p > 0.05$) were produced after 1 or 3 mg kg^{-1} of VIAN-c4551 (two-way ANOVA, Šídák).

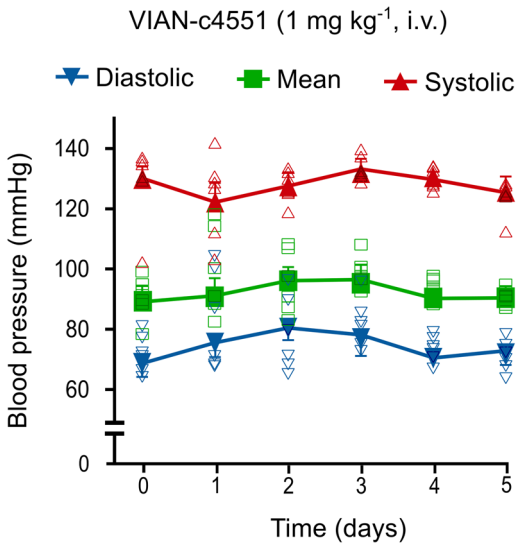

FIGURE 5 | Daily administration of VIAN-c4551 for 5 days did not alter blood pressure in awake rats. Systolic, mean, and diastolic blood pressure values in awake rats 2 h after daily intravenous administration (1 mg kg^{-1}) of VIAN-c4551 for five consecutive days. Values are mean ± S.E.M. ($n = 6$). Individual values are shown. No significant changes ($p > 0.05$) were produced after VIAN c4551. Similar results were obtained with an equivalent volume of vehicle (saline; 1 mL kg^{-1}) (not shown).

administration of VIAN-c4551 and 24 h after the last administration of VIAN-c4551.

In addition to HR, ECG parameters included the QRS complex representing the depolarization of ventricles, the corrected QT interval (QTc) that measures ventricular repolarization, and HRV indicators, namely: the standard deviation of the normal-to-normal interval (SDNN), the root mean square of successive differences (RMSSD), the low- and high-frequency components of HR variability (LF/HF), measurement of short-term HRV (SD1), and measurement of short- and long-term HRV (SD2). Table 3 shows that there were no significant differences in HR or any of the above ECG components after the VIAN-c4551 treatment in awake and anesthetized rats.

3.8 | Effects of VIAN-c4551 on Cardiac Ventricular Remodeling, Cardiac Histology, or Expression of Inflammatory (TNF-α and IL-6) and Apoptotic (Caspase-3) Markers

Consistent with the above findings, as shown in Table 4, in isolated ventricles obtained from rats treated daily for 5 days with VIAN-c4551 (1 mg kg^{-1}) or vehicle (1 mL kg^{-1}), these compounds failed to: (i) alter the Fulton index (a gross indicator of ventricular remodeling; Covington et al. 2023); (ii) cause cardiac histological changes in the size of cardiomyocytes, capillary density, and collagen deposition; or (iii) modify the expression levels of the inflammatory (TNF-α and IL-6) and apoptotic (caspase-3) markers. In addition, we observed that VIAN-c4551 did not change body weight or blood glucose levels in awake rats (Table 4).

4 | Discussion

4.1 | General

In addition to the implications discussed below, several lines of evidence highlight the clinical antiangiogenic potential of the vasoinhibin analogue VIAN-c4551. Indeed, this novel compound inhibits the angiogenic effects of VEGF and blocks pathological angiogenesis in experimental models of cancer and diabetic retinopathy (Robles et al. 2022). In this context, it must be highlighted that medications such as VEGF inhibitors are first-line therapies in oncology and retinopathy

TABLE 3 | Heart rate, electrocardiogram parameters and HRV domains of awake and anesthetized rats before and after the i.v. administration VIAN-c4551 (1 mg kg^{-1}).

	Awake			Anesthetized		
	VIAN-c4551 (1 mg kg^{-1})			VIAN-c4551 (1 mg kg^{-1})		
Parameter	**Before**	**After**	***p* value**	**Before**	**After**	***p* value**
HR (beats min^{-1})	477 ± 9	491 ± 9	0.99	433 ± 27	384 ± 33	0.36
QRS (ms)	23.9 ± 0.7	23.4 ± 0.3	0.81	23.8 ± 0.3	24.2 ± 0.4	0.81
QTc (ms)	3.2 ± 0.1	3.5 ± 0.2	0.56	3.6 ± 0.3	4.8 ± 0.6	0.97
SDNN (ms)	6.2 ± 0.7	4.3 ± 0.8	0.44	2.0 ± 0.4	2.6 ± 0.6	0.82
RMSSD (ms)	3.7 ± 0.8	2.5 ± 0.2	0.59	1.6 ± 0.2	1.7 ± 0.3	0.97
LF/HF	0.8 ± 0.1	0.8 ± 0.2	0.99	0.6 ± 0.3	0.4 ± 0.1	0.78
SD1 (ms)	2.6 ± 0.6	1.7 ± 0.2	0.51	1.1 ± 0.1	1.2 ± 0.2	0.99
SD2 (ms)	8.3 ± 0.9	5.8 ± 1.1	0.47	2.6 ± 0.6	3.4 ± 0.8	0.76

Note: VIAN-c4551 (1 mg kg^{-1}, i.v.) was daily injected for 5 days in previously habituated awake and anesthetized rats, and the electrocardiogram (ECG) was recorded 24 h before the first administration of VIAN-c4551 and 24 h after the last administration of VIAN-c4551. Values are mean ± S.E.M., $n = 5$. Note that no significant changes ($p > 0.05$) were produced after 1 mg kg^{-1} of VIAN-c4551 (two-way ANOVA, Šídák).
Abbreviations: HR, heart rate (beats min^{-1}); LF/HF, ratio low-frequency oscillation (LF (Hz))/high frequency (HF (Hz)); QRS, complex QRS; QTc, interval QR corrected (with Bazzett's formula); RMSSD, variance or RR interval; SDNN, standard deviation of RR interval; SD1, short-term term dispersion; SD2, long-term dispersion.

TABLE 4 | Cardiac remodeling/histology parameters or inflammatory/apoptotic markers in isolated heart ventricles from rats treated daily for 5 days with vehicle (1 mL kg^{-1}, i.v.) or VIAN-c4551 (1 mg kg^{-1}, i.v.), as well as body weight and blood glucose levels in awake rats.

Parameter	**Vehicle (1 mL kg^{-1})**	**VIAN-c4551 (1 mg kg^{-1})**
Fulton index	0.30 ± 0.02	0.32 ± 0.01
Cardiomyocytes area (μm^2)	756.6 ± 48.5	745.8 ± 28.1
Collagen (%)	3.5 ± 0.5	3.1 ± 0.3
Vascular density (vessels/mm^2)	8.2 ± 1.3	7.4 ± 0.7
TNF-α (%)	100 ± 7.7	96.4 ± 13.9
IL-6 (%)	100 ± 23.4	132 ± 30
Caspase-3 (%)	100 ± 10.2	89.2 ± 8.7
Body weight (g)	262.1 ± 5.9	258.8 ± 6.4
Glucose (mg dL^{-1})	117.1 ± 5.5	103.3 ± 4.4

Note: Values are presented as the mean ± S.E.M., $n = 5$–7. The parameters evaluated in the heart ventricles were right vs. right and left vs. left (1 mL kg^{-1} vehicle vs. 1 mg kg^{-1} VIAN-c4551). Statistical analysis was performed using an unpaired *t* test. Note that no significant differences ($p > 0.05$) were observed between the groups treated with vehicle and VIAN-c4551.

(Carmeliet 2003; Fallah et al. 2019); notwithstanding, they are associated with cardiovascular side effects, especially hypertension (Jiang et al. 2020; Dong et al. 2021).

By applying a multidisciplinary experimental approach, in the present investigation we provide further evidence that reinforces the cardiovascular safety of VIAN-c4551. This approach considered, before and after treatment with VIAN-c4551: (i) biochemical biology assays (ACh-induced eNOS phosphorylation at Ser^{1177}); (ii) in vitro experiments (ACh-induced vasodilatation in aortic rings as well as cardiac remodeling and histological parameters in isolated heart ventricles); and (iii) in vivo studies analyzing blood pressure (i.e., DBP, MBP and SBP) and the corresponding changes produced by ACh in anesthetized rats under three different experimental conditions (i.e., intact, vagotomized or pithed), as well as HR and electrocardiographic parameters in anesthetized and awake animals.

The preclinical findings obtained from this multidisciplinary approach may represent a clear therapeutic advantage of VIAN-c4551 (i.e., lack of cardiovascular side effects) over VEGF inhibitors. In this sense, it is worthy of note that: (i) hypertension induced by VEGF inhibitors involves attenuation of eNOS/NO-mediated vasodilatation (Touyz et al. 2017; Dong et al. 2021); and (ii) vasoinhibin inhibits the stimulation of eNOS/NO elicited by VEGF and other endogenous vasodilators such as bradykinin and ACh (Gonzalez et al. 2004; García et al. 2008).

4.2 | Capability of VIAN-c4551 to Reduce Both ACh-Induced eNOS Phosphorylation at Ser^{1177} and ACh-Induced Aortic Vasorelaxation

Is known that: (i) ACh can activate PI3K/Akt-dependent phosphorylation of eNOS at Ser^{1177} (human eNOS) (Trott et al. 2013), leading to eNOS-dependent vasodilation; and (ii) transgenic mice expressing only the phosphomimetic form of eNOS (Ser^{1117}) respond better to vasorelaxant agents and display increased blood reperfusion during ischemia (Atochin et al. 2007). In relation to these findings, our results show that VIAN-c4551 could reduce both ACh-induced phosphorylation of eNOS (Ser^{1177}) in human endothelial cells (Figure 1) and lung tissue in vivo (Figure 4), and ACh-induced relaxation of rat aortic rings (Table 1, Figure 2A). These results are consistent with the previously observed inhibition of ACh-induced vasodilation produced by vasoinhibin (Gonzalez et al. 2004) and could suggest a possible prohypertensive action in vivo.

However, this inhibitory effect of VIAN-c4551 observed after acute treatment was not replicated when the aortic rings were obtained from rats treated during 5 days with VIAN-c4551 (Table 1). Interestingly acute VIAN-c4551 prevented the increase of ACh-induced eNOS phosphorylation in lung tissue (Figure 4). These findings imply that compensatory mechanisms regulating vasodilator/vasodepressor responses occur. Indeed, eNOS-derived NO helps control the coronary vascular tone under acute eNOS inhibition, but not under the chronic inhibition of the enzyme (Gödecke et al. 1998). Apart from NO, coronary tone is influenced by several vasoactive factors, such as prostacyclins, adenosine, endothelin and angiotensin II, which can compensate for the absence of NO signaling (Bassenge 1995; Huang et al. 1995; Cheng et al. 2023).

Furthermore, cardiac baroreflex mechanisms may also play a contributing role. For instance, while some acute pharmacological treatments may increase or decrease blood pressure, cardiac baroreflex mechanisms can adjust sympathetic nerve outflow, leading to blood pressure compensation (Salah et al. 2025).

The effect of VIAN-c4551 was clearly specific for ACh-induced vasodilatation, in view that this vasoinhibin analog failed to significantly modify the contractile responses to Phen (Table 1, Figure 2B) or U-46619 (Table 1, Figure 2C) in the aortic segments of rats treated with or without VIAN-c4551. In keeping with this suggestion, other studies have demonstrated that treatment of rats with vasoactive substances can alter the relaxant and contractile responses of isolated arteries in vitro (Momoi et al. 2003) by mechanisms such as the enhancement of norepinephrine-induced vasoconstriction (Feitelson et al. 2003).

Altogether, our in vitro and ex vivo assays show that acute VIAN-c4551 specifically reduced eNOS phosphorylation and NO-mediated aortic vasorelaxation in response to ACh without affecting the vasoconstrictor responses to Phen or U-46619. Notwithstanding, it is important to note, as previously described (di Gioia et al. 2023; Michel et al. 2022), that the aorta is a blood vessel of conductance (not resistance) that allows blood flow due to its anatomical and viscoelastic properties. This fact is of relevance to help explain why the attenuation of ACh-induced aortic vasodilatation caused by VIAN-c4551 did not translate into a reduction of ACh-induced decreases in blood pressure (i.e., ACh-induced systemic vasodilatation; Figure 3) or an increase in resting blood pressure when given acutely (Table 2) or during 5 days (Figure 4) (see below).

4.3 | VIAN-c4551 Failed to Modify the Vasodepressor Responses to ACh in Anesthetized Intact Rats

The fact that acute i.v. administration of two supramaximal doses of VIAN-c4551 (i.e., 1 and 3 mg kg^{-1}) was devoid of significant effects on the ACh-induced decreases in DBP, MBP and SBP (i.e., ACh-induced systemic vasodilatation; Figure 3) clearly contrasts with the capability of VIAN-c4551 to attenuate ACh-induced aortic vasodilatation (Table 1, Figure 2A). This apparent discrepancy, however, deserves further consideration, particularly about the contribution of conductance and resistance arteries to the total peripheral resistance and, consequently, to blood pressure. Within this context, it is noteworthy that: (i) the mesenteric arterial bed, which receives 25% of cardiac output and branches into first-, second-, third- and fourth-degree side branches, contributes significantly to total peripheral resistance and, consequently, to arterial blood pressure (Christensen and Mulvany 1993); (ii) hypertensive states (e.g., spontaneously hypertensive rats) mainly involve pathophysiological changes in vascular reactivity along the whole branching of the mesenteric arterial bed (Tatchum-Talom et al. 2011); and (iii) the aorta and its larger branches are conductance (not resistance) arteries (di Gioia et al. 2023; Michel et al. 2022).

On this basis, since MBP is directly proportional to cardiac output and systemic vascular resistance (Feher 2012; Pagoulatou et al. 2021; Lefferts et al. 2022), and the attenuation by VIAN-c4551 of ACh-induced aortic vasodilatation did not translate into a reduction of ACh-induced decreases in blood pressure, it is most likely that VIAN-c4551 is not acting at the level of the mesenteric arterial bed. Consistent with this view, as shown in Figure 3, neither did VIAN-c4551 attenuate ACh-induced decreases in DBP (an approximate index of systemic vascular resistance/tone) and SBP (an indicator of cardiac left ventricle contractility) (Feher 2012; Pagoulatou et al. 2021; Lefferts et al. 2022).

In view of these results, we further investigated the effects of VIAN-c4551 on resting blood pressure under different experimental conditions.

4.4 | VIAN-c4551 Does Not Significantly Change Resting Values of Blood Pressure (DBP, MBP, and SBP) in Anesthetized Rats (i.e., Intact, Vagotomized or Pithed) or Awake Rats

In view that VIAN-c4551 specifically reduced NO-mediated aortic vasorelaxation in response to ACh, it would seem reasonable to assume that this inhibitory effect of VIAN-c4551 could also be reproduced in vivo and, consequently, result in a possible prohypertensive action. To further explore this possibility, we analyzed, as a first approach in anesthetized rats (i.e., intact, vagotomized or pithed), the effects of acute supramaximal doses of VIAN-c4551 (1 and 3 mg kg^{-1}, i.v.) on resting values of DBP, MBP, and SBP.

Interestingly, the fact that resting values of DBP, MBP, and SBP remained without significant changes ($p > 0.05$) after the two doses of VIAN-c4551 in intact, vagotomized or pithed rats (Table 2): (i) is apparently inconsistent with its inhibitory effects on ACh-induced aortic vasorelaxation (Table 1, Figure 2A); and (ii) clearly indicates that this vasoinhibin analog lacks cardiovascular effects of peripheral and central origin (Table 2). Undoubtedly, the peripheral cardiovascular effects of VIAN-c4551 can be excluded in view that it was devoid of significant effects on the blood pressure of bilaterally vagotomized rats (i.e., devoid of autonomic cardiovascular regulation at the neuroeffector level) and pithed rats bilaterally vagotomized (i.e.,

devoid of a functional CNS and, consequently, an absence of cardiovascular compensatory baroreflex mechanisms).

Indeed, as already discussed by Avilés-Rosas et al. (2017), the pithed rat model with bilateral vagotomy: (i) allows us to uncover potential prohypertensive effects produced by novel drugs with potential therapeutic usefulness, since this model lacks cardiovascular compensatory baroreflex mechanisms; and (ii) is certainly not representative of the physiological condition as the autonomic and central neurogenic control of blood pressure was eliminated. Despite this condition, such potential effects could be clinically relevant under a cardiovascular pathophysiological state (Avilés-Rosas et al. 2017).

Moreover, the potential central cardiovascular effects of VIAN-c4551 (if any) do not seem to be pharmacologically relevant under our experimental conditions, since this water-soluble heptapeptide (molecular weight: 768 Da) produced no significant effects on the blood pressure (including DBP, MBP, and SBP) of intact rats (Table 2). Certainly, in the CNS, NOS plays an important role in brain homeostasis by modulating cerebral blood flow and release of neurotransmitters (Northington et al. 1997). Hence, in the hypothetical case that VIAN-c4551 could cross the blood-brain barrier at concentrations sufficient to inhibit NOS phosphorylation in the CNS, one could speculate that this effect in intact rats could have caused a certain change in cerebral blood flow and neurotransmitter release that produced a small change in blood pressure. However, in this hypothetical case in intact rats, the initial increase in blood pressure could have been so small that it was completely overshadowed by centrally originating cardiovascular compensatory baroreflex mechanisms; these compensatory baroreflex mechanisms would, in turn, increase vagal tone resulting in a decrease in both HR and cardiac output.

These findings in intact rats, together with the fact that VIAN-c4551 produced no significant effects in vagotomized or pithed rats (Table 2), strongly suggest that this heptapeptide is devoid of cardiovascular effects at the central and peripheral levels.

Clearly, the above results were obtained with acute i.v. doses of VIAN-c4551 in anesthetized rats. Nevertheless, the possible interference by experimental conditions such as anesthesia (see, e.g., Tettelbaum et al. 1971; Hahn et al. 2022) and acute treatment cannot be categorically excluded. Accordingly, we decided to further investigate, in awake rats, the effects of daily i.v. administration of VIAN-c4551 ($1\,mg\,kg^{-1}$) for 5 consecutive days. However, even under these conditions, our results showing that VIAN-c4551 failed to significantly modify DBP, MBP and SBP (Figure 4) support the cardiovascular safety of this heptapeptide.

4.5 | Exclusion of Confounding Factors That May Have Overshadowed the Potential Prohypertensive Action of VIAN-c4551 in Anesthetized or Awake Rats

The fact that VIAN-c4551 produced no significant changes in blood pressure in anesthetized or awake rats (see above) leads us to consider the possible role of confounding factors that may have interfered with the potential prohypertensive action of VIAN-c4551. These confounding factors may include, among others:

- Pharmacokinetic factors. Admittedly, we do not know to what extent our results obtained in anesthetized and awake rats may have been influenced by pharmacokinetic factors such as metabolism and excretion in the i.v. administration of VIAN-c4551 (1 and/or $3\,mg\,kg^{-1}$). However, these i.v. doses are considered pharmacologically supramaximal when taking into account that, to achieve antiangiogenic effects, a 2.5 to 208-fold lower concentration of VIAN- c4551 (EC_{50}: 150 pM) is required compared to other proteins (e.g., endostatin EC_{50}: 31,150 pM; and angiostatin EC_{50}: 388 pM) or peptides (e.g., anginex EC_{50}: 2560 pM; and cilengitide EC_{50}: 1097 pM) under clinical research (Robles et al. 2022; Rosca et al. 2011). These results indicate that VIAN-c4551 is a more potent antiangiogenic drug.
- Duration of treatment, dosing and general anesthesia. These also seem unlikely factors confounding the potential prohypertensive action of VIAN-c4551. For example, in another study using anesthetized rats, NOS inhibitors such as L-NAME elevated blood pressure immediately after i.v. administration (Vág et al. 2002), while a long-term administration of VIAN-c4551 (once daily for 5 days) failed to modify blood pressure in anesthetized and awake rats (present study).
- Absence or presence of a functional CNS, autonomic influences or consciousness. The cardiovascular effects of vasoactive compounds, including serotonin, depend on experimental conditions such as the absence or presence of a functional CNS (spinal or pithed animals), autonomic influences (vagotomy or sympathectomy) or consciousness (Villalón and Centurión 2007). Notwithstanding, under our experimental conditions, VIAN-c4551 was devoid of cardiovascular effects regardless of whether the rats were anesthetized (with intact vagus nerves and CNS), bilaterally vagotomized, pithed or awake (Table 2, Figure 4). Accordingly, these confounding factors do not seem to play an important role.

4.6 | Lack of Effect of VIAN-c4551 on Some Cardiac Function Variables, Cardiac Remodeling/Histology and Well-Being in Awake or Anesthetized Rats

Overall, and in keeping with the above findings, when analyzing some cardiac function variables in anesthetized and awake rats pretreated for 5 days with VIAN-c4551, we found no significant changes in HR (indicating no direct cardiac pacemaker activity), electrocardiographic parameters, cardiac ventricular remodeling, cardiac histology, and well-being (body weight and blood glucose levels) (Tables 3 and 4). In marked contrast to VIAN-c4551, vasoinhibin has been associated with changes in cardiac tissue and proteins, such as decreased cardiac endothelial cell viability and eNOS expression (Nakajima et al. 2017), as well as clinical conditions associated with vasoinhibin mechanisms (Hilfiker-Kleiner et al. 2007; Kryczka et al. 2024).

4.6.1 | Inactivity of VIAN-c4551 on Heart Rate and Electrocardiographic Parameters

Since the electrocardiogram allows the identification of cardiac changes such as those induced by vasoinhibin, we evaluated whether VIAN-c4551 changes cardiac electrical activity and cardiac autonomic regulation. The fact that daily VIAN-c4551 (1 mg kg^{-1}, i.v.) for 5 days produced no changes in HR, QRS interval or QTc period (Table 3) is crucial because vasoinhibin causes alterations in calcium dynamics that directly correlate with changes in ventricular depolarization and repolarization (Arredondo Zamarripa et al. 2017).

Moreover, QTc period lengthening frequently appears secondary to the administration of antiangiogenic therapies and is an indicator of ventricular arrhythmias (Dobbin et al. 2021); therefore, VIAN-c4551 can be considered innocuous with respect to cardiac electrical activity (Table 3), perhaps mediated by its inability to elicit changes in calcium regulation in cardiomyocytes.

It is worthy of note that prolactin, the endogenous precursor of vasoinhibin (Triebel et al. 2015), can exert multiple effects on nervous system modulation (Martínez-Alarcón et al. 2022). Our evaluation indicates that VIAN-c4551 does not cause changes in cardiac autonomic regulation, as we found no significant differences in any of the domains of analysis of HRV (Table 3). This indicates that VIAN-c4551 does not modify the firing frequency of the sympathetic and parasympathetic nervous system, probably because no increase in systemic blood pressure was produced and, consequently, baroreflex mechanisms did not trigger any sympatho-vagal changes (Pereira-Junior et al. 2010). This is consistent with the fact that no chronotropic or geometric changes were observed in the analysis of consecutive R-R intervals.

On the other hand, as previously reported (Tiwari et al. 2021), HRV has been shown to be influenced by, among others, environmental, pathological, physiological and pharmacological factors, such that: (i) an increase in HR variability is associated with a healthy condition; and (ii) a reduction in HR variability has been considered to have prognostic value for adverse cardiac events. Accordingly, the fact that VIAN-c4551 did not cause a reduction in HRV in any domain (Table 3), along with the fact that it does not modify cardiac electrical capacity, strengthens the idea that VIAN-c4551 is safe at the cardiac level.

4.6.2 | Inactivity of VIAN-c4551 on Parameters of Cardiac Ventricular Remodeling/Histology or the Expression of Inflammatory and Apoptotic Markers

In the framework of cardiac pathophysiology, it is important to emphasize that cardiac ventricular remodeling is one of the main and most frequent consequences of increased vascular resistance and/or hemodynamic alterations. Cardiac remodeling is primarily characterized by fibrosis, hypertrophy, and/or vascular rarefaction, and it is frequently associated with heart failure (Gallo and Savoia 2024). In our results, we did not find evidence to suggest possible cardiac ventricular remodeling (Table 4), perhaps favored by the inactivity of VIAN-c4551 to produce significant changes in blood pressure (i.e., DBP, MBP and SBP; see Section 4.4 above).

It should be noted that our histological examination also aimed to identify potential direct effects of VIAN-c4551 on cardiomyocytes. In this respect, VIAN-c4551 has been described to play an important role in cardiac ventricular remodeling through neovascularizing activity modulated by the phosphatidylinositol 3-kinase (PI3K) pathway and cardiac endocrine activity (Hamid and Prabhu 2010). Likewise: (i) cardiac overexpression of VEGF can induce hypertrophy due to lipid alterations and mitochondrial damage (Lottonen-Raikaslehto et al. 2017); and (ii) in the presence of an anti-VEGF (bevacizumab), VEGF was shown to significantly modulate ventricular remodeling during post-infarction (Morishita et al. 2015). Together, these lines of evidence highlight the importance of studying cardiac remodeling mediated by vasoactive molecules in the cardiac vascular bed.

Besides having no effect on cardiac remodeling/histology, VIAN-c4551 failed to modify the expression of inflammatory (TNF-α and IL-6) and apoptotic (caspase-3) markers in the cardiac ventricles (Table 4). This inactivity of VIAN-c4551 on these markers (which could have revealed subclinical effects not visible by conventional cardiac histology), implies once again that its systemic administration is safe for the heart.

4.6.3 | Inactivity of VIAN-c4551 on Well-Being (Body Weight and Blood Glucose Levels)

Our findings show that VIAN-c4551 is ineffective to significantly change blood pressure, cardiac function and cardiac remodeling/histology, reinforcing its cardiovascular safety. Thus, we decided to further explore the potential effects of VIAN-c4551 on body weight and blood glucose levels and found no significant effects (Table 4). This represents additional convenience.

4.6.4 | Study Limitations

Given the potential clinical use of VIAN-c4551, an important limitation of the present study is that only a single daily dose (1 mg kg^{-1}, i.v.) and a short-term treatment (5 days) were explored. However, a toxicity analysis of VIAN-c4551 has recently demonstrated (Perez et al. 2025) that mice daily injected with vehicle (saline) or with two different doses of VIAN-c4551 (1 or 10 mg kg^{-1}, i.v.) for 2 weeks showed no detrimental effects in body weight, blood hematological parameters, serum biochemical parameters, and histopathology at the end of the experiments.

Another limitation is the absence of a positive control group treated with antiangiogenic agents known to induce hypertension. Such a comparison would have allowed us a more rigorous assessment of the sensitivity of our in vivo model. Nevertheless, reported data consistently show that VEGF inhibitors rapidly and markedly elevate blood pressure (Cooper et al. 2019; Dong et al. 2021). For example, a single i.v. dose of

aflibercept (a fusion protein targeting VEGF and placental growth factor), acutely elevates blood pressure and impairs vascular relaxation in mice (Dong et al. 2021). Similarly, vandetanib and pazopanib, two VEGFR2-targeting tyrosine kinase inhibitors (TKIs), produce sustained hypertension in normotensive rats (Cooper et al. 2019). In contrast, VIAN-c4551, even at supratherapeutic doses, produced no changes in blood pressure, heart rate, or cardiac histopathology, inflammatory and apoptosis parameters (present study). This suggests that, although a direct comparison was not included, the model itself is likely to be sensitive enough to detect hypertensive or vasotoxic effects of clinically relevant antiangiogenic agents.

4.7 | Final Considerations, Potential Clinical Perspectives, and Conclusions

Considering VIAN-c4551 within the context of drug development research, it is worthy of note its lack of an in vivo effect on blood pressure and cardiac function at supramaximal doses (up to 3 mg kg^{-1}, i.v.; present study) despite its ability to inhibit eNOS activation and NO-mediated aortic vasorelaxation to ACh in vitro and ex vivo. From a clinical perspective, given that this vasoinhibin analog has therapeutic potential for targeting angiogenesis in oncology and ophthalmology, it remains to be established whether its antiangiogenic doses achieve sufficient plasma concentrations to produce cardiovascular effects in humans; notwithstanding, this seems unlikely based on our preclinical results.

Interestingly, a recent study has linked vasoinhibin to the renin-angiotensin system (RAS), such that renin cleaves prolactin into vasoinhibin (Núñez et al. 2025), and the prolactin-vasoinhibin axis shares functional properties with the RAS, a major regulator of blood pressure, inflammation and angiogenesis (Kanugula et al. 2023). The prolactin-vasoinhibin axis is upregulated by water deprivation (Núñez et al. 2025) and, like angiotensin II (Kanugula et al. 2023), vasoinhibin has pro-inflammatory properties (Corbacho et al. 2000; Ortiz et al. 2020) and releases vasopressin from the hypothalamic-neurohypophyseal system (Mejía et al. 2003).

However, the various functions of vasoinhibin are segregated into distinct, nonadjacent, and independent motifs, namely: (i) the $H_{46}G_{47}R_{48}$ motif, contained in VIAN-c4551, is responsible for the inhibition of angiogenesis by vasoinhibin (Robles et al. 2022); and (ii) the $H_{30}N_{31}L_{32}S_{33}S_{34}E_{35}M_{36}$ motif is involved in the inflammatory, apoptotic, and fibrinolytic properties of vasoinhibin (Robles et al. 2024). In agreement with these findings: (i) VIAN c4551 conserves the potency to inhibit angiogenesis, vasopermeability and vasodilation (Robles 2022; present results), but lacks apoptotic and pro-inflammatory actions on endothelial cells as well as fibrinolytic activity (Robles et al. 2024); and (ii) both vasoinhibin and VIAN-c4551 act by blocking the phosphorylation/activation of eNOS leading to NO production (García et al. 2008; Robles et al. 2022; present study), an important signal for VEGF-induced proliferation, permeability, and dilation of blood vessels. The fact that inhibition of eNOS phosphorylation/activation by VIAN-c4551 is not enough to alter cardiovascular homeostasis in vivo (present findings), together with its broad action against several vasoactive factors (Clapp et al. 2015), highlights its future as a potent and safe drug against angiogenesis-dependent diseases.

Lastly, although the ability of VIAN-c4551 to release vasopressin has not yet been evaluated, the lack of pro-inflammatory (Robles et al. 2024) and prohypertensive (present results) properties suggests that RAS-like actions are absent in VIAN-c4551. Because pro-inflammatory and prohypertensive actions are undesirable side effects of antiangiogenic therapy, VIAN-c4551 stands as a selective and cardiovascularly safe agent for targeting pathological angiogenesis.

5 | Conclusion

Despite VIAN-c4551's inhibition of ACh-induced eNOS activation and aortic vasorelaxation in vitro and *ex vivo*, supramaximal doses of this vasoinhibin analog were devoid of cardiovascular effects in vivo. Therefore, this preclinical study reinforces the cardiovascular safety of VIAN-c4551 for targeting angiogenesis-related diseases.

Author Contributions

Miguel A. García-González: conception and design, acquisition, analysis, and interpretation of data, helped draft the manuscript. **Alejandro D. Miguel-Martínez:** conception and design, acquisition, analysis, and interpretation of data. **Abimael González-Hernández:** analysis and interpretation of data, helped draft the manuscript. **Magdalena Zamora:** acquisition and analysis of data. **Elva Adán-Castro:** acquisition and analysis of data. **Xarubet Ruíz-Herrera:** acquisition and analysis of data. **María A. Carbajo-Mata:** acquisition and analysis of data. **Jakob Triebel:** resources. **Thomas Bertsch:** resources. **José G. Lopez-Lopez:** resources. **Gonzalo Martínez de la Escalera:** review and editing. **Juan Pablo Robles:** conception and design, analysis and interpretation of data, helped draft the manuscript. **Carmen Clapp:** conception and design, analysis and interpretation of data, supervision, secured funding, wrote and edited the manuscript. **Carlos M. Villalón:** conception and design, analysis and interpretation of data, supervision, securedfunding [including payment of the article publication charge as per the transformative agreement between Wiley and Cinvestav (Mexico)], wrote and edited the manuscript. All authors critically reviewed and approved the submitted version of the present manuscript.

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Conflicts of Interest

J.P.R., M.Z., T.B., J.T., G.M.E., and C.C. are inventors of a submitted patent application (WO/2021/098996), which is owned by the Universidad Nacional Autónoma de México (UNAM) and J.T. J.P.R. is the CEO and founder of VIAN Therapeutics Inc. M.Z. and C.C. are consultants for VIAN Therapeutics. Inc.

Data Availability Statement

Generation and analysis of all original data are included in the figures and tables of this study.

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