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Pharmacological characterization of the cardiovascular effect of Nibethione: *ex vivo*, *in vivo* and *in silico* studies

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Keywords
antihypertensive effect; calcium channel; mechanism of action; molecular docking; Nibethione; vasorelaxant activity

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Abstract

Objective This work describes the vasorelaxant and antihypertensive effects and the mechanism of action on vascular smooth muscle cells of Nibethione, a synthetic thiazolidinedione derivative. Additionally, evidence of its cytotoxicity is assessed.

Methods Nibethione (NB) was synthesized, and its vasorelaxant effect and mechanism of action were assessed through *ex vivo* experiments. Molecular docking studies were used to predict the mode of interaction with L-type Ca^{2+} channel, and *in vivo* antihypertensive activity was assayed on spontaneously hypertensive rats (SHR). The cytotoxicity potential was evaluated in porcine aortic endothelial cells (PAECs) from primary explants.

Key findings Nibethione vasorelaxant effect was efficient on KCl (80 mM) and NE-contraction. This effect was deleteriously modified in the presence of potassium channel block drugs, while the maximal contraction induced with NE was significantly decreased by NB; the $CaCl_2$-induced contraction was abolished entirely. *In vivo* experiments showed that NB decreased diastolic blood pressure in 20.3 % after its administration on SHR. The molecular docking showed that NB blocks L-type Ca^{2+} channel, and *in vitro* tests showed that NB did not produce cytotoxic activity on PAECs (IC_{50} >1000 μM).

Conclusions Nibethione showed *in vivo* antihypertensive and *ex vivo* vasorelaxant effects with implication of voltage-dependent L-type Ca^{2+} channel blocking, and this may contribute to the research of novel antihypertensive drugs.

Introduction

Cardiovascular diseases (CVD) comprise cerebrovascular disease, coronary heart disease and hypertension. Almost 17.9 million people die due to CVD every year worldwide. This number accounts for 31% of global deaths in low- and middle-income countries. Hypertension is the most prevalent CVD, and it is associated with high morbidity and mortality.[1] Current hypertension therapies reduce morbidity and mortality in the affected population. However, long-term use of this medication is associated with severe adverse events that could induce drug intolerance, poor therapy compliance and high economic burden due to complications.[2,3] In this context, drug discovery and pharmacological research of new antihypertensive agents remain an ongoing challenge in medical sciences. 2-Thioxo-1,3-thiazolidin-4-one and 2,4-thiazolidinedione derivatives are five-membered heterocyclic molecules that have emerged as central scaffold for design and synthesis of new drug with therapeutic potential chronic non-degenerative diseases.[4] Besides, they are both a privileged class of molecules due to possess a large number of biological effects, such as anticonvulsant, antibacterial, antiviral, antidiabetic and cardiovascular benefits among others.[5–9] Bhandari *et al.* evaluated thiazolidine derivatives in electrocardiographic, antiarrhythmic, vasorelaxant and

antihypertensive activity as well as for *in vitro* nitric oxide (NO) releasing ability and found that some of these heterocycles are very potent.[10] Thereby, the study of 2-thioxo-1,3-thiazolidin-4-one and 2,4-thiazolidinedione derivatives occupies a prominent place in the science of chemistry mainly because of their biological importance. Thus, the aim of current work was synthesizing Nibethione, a 2-thioxo-1,3-thiazolidin-4-one derivative and pharmacologically characterize its potential as an antihypertensive agent by *ex vivo*, *in vivo* and *in silico* assays.

Material and Methods

Chemicals and supplies

Most chemical reagents (dimethylsulfoxide), raw material (4-nitrobenzaldehyde, 98%) and pharmacological agents (2-aminopyridine, carbamylcholine chloride, calcium chloride, captopril, glibenclamide, nifedipine, norepinephrine L-bitartrate hydrate, pentobarbital, tetraethylammonium chloride and potassium chloride) were purchased from a Sigma-Aldrich's local provider (St. Louis, MO, USA). Reagents for Krebs solution were acquired from Fermont (Monterrey, NL, Mexico).

Cell culture reagents and supplies were purchased from different providers; Dulbecco's modified Eagle's medium (DMEM) and penicillin-streptomycin solution (100×, Ref. PSL01) were purchased from Caisson Laboratories (Smithfield, UT, USA); Gibco's fetal bovine serum (FBS) was purchased from Invitrogen (Waltham, MA, USA; Cat. No. 16000044); sodium bicarbonate was purchased from Sigma-Aldrich; Cell Titer-Blue cell viability assay was acquired from Promega (Madison, WI, USA; Cat. No. G8081); Trypsin-EDTA solution (0.25% Trypsin, 0.53 mM EDTA; ATCC-30-2101) was purchased from ATTC (Manassas, VA, USA).

Chemistry

Procedures and devices

All chemical reactions were monitored by thin-layer chromatography (1.5 × 3.0 cm) and were visualized by UV light at 254 nm. The uncorrected melting point was determined by the melting point apparatus EZ-Melt MPA12 (Stanford Research Systems). NMR experiments were performed in a 400 MHz-Varian Inova 400 device (Varian Inc., Palo Alto, CA, USA). Chemical shifts (δ_H and δ_C) and coupling constants (*J*) values are given in ppm and Hz, respectively. TMS ($\delta_H = 0$, $\delta_C = 0$) in $CDCl_3$ was used as standard reference for NMR experiments. The following abbreviations represent signal multiplicities: s (singlet), d (doublet), t (triplet), q (quartet) and m (multiplet). Mass spectroscopy was performed in a Jeol JMS-700 MStation (JEOL, Ltd., Peabody, MA, USA).

Synthesis of (Z)-5-(4-nitrobenzylidene)-2-thioxothiazolidin-4-one (Nibethione)

Nibethione was prepared in a one-step reaction, beginning from nitrobenzaldehyde (0.5 g, 3.30 mmol), which was condensed with 2-thioxo-1,3-thiazolidin-4-one (1.5 Equiv), following Knoevenagel reaction conditions: benzoic acid, piperide, toluene as solvent under reflux[8] (Figure 1).

Pharmacological evaluation

Animals

Adult Wistar rats were obtained from Unidad de Producción, Cuidado y Experimentación Animal from Universidad Juárez Autónoma de Tabasco (Villahermosa, Tabasco, México). Spontaneously hypertensive rats (SHR) and Wistar–Kyoto (WKY) rats were obtained from Instituto de Fisiología Celular at Universidad Nacional Autónoma de México (Mexico City, Mexico). All animals were male with bodyweight ranged 250–300 g.

Animals were housed under standard laboratory conditions (humidity, temperature and light/dark cycle) and were *ad libitum*-fed with rodent diet and tap water. All procedures were conducted following Mexican regulations for animal experimentation and care from SAGARPA (NOM-062-ZOO-1999, México).[11] This protocol was revised and approved by Internal Committee for the Care and Use of Laboratory Animals of Universidad Juárez Autónoma de

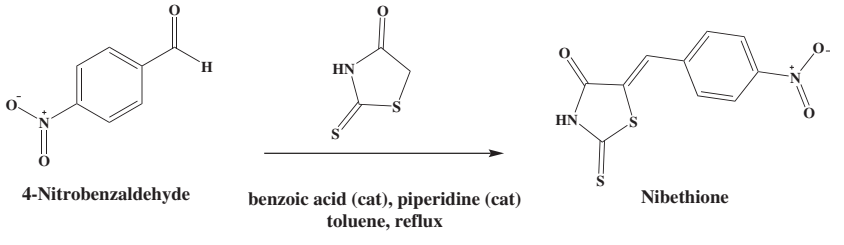

Figure 1 Scheme of synthesis of (Z)-5-(4-nitrobenzylidene)-2-thioxo-1,3-thiazolidin-4-one (Nibethione).

Tabasco with assigned code CICUAL-001. Also, experimental procedures met the US Guide for Care and Use of Laboratory Animals (National Academy Sciences, No. 85-23, revised 1996).[12] *Ex vivo* experiments were performed with six animals per group and euthanized by cervical dislocation technique after deep anaesthesia with pentobarbital (65 mg/kg, i.p). *In vivo* experiments were performed with five animals per group.

Ex vivo experiments

General procedure

Following euthanasia, thoracic aorta was dissected and cleaned of adjacent and connective material, then cut into rings of 3 mm in length. In addition, the endothelial layer of some aortic rings was gently removed by manual rubbing process. Then, aortic rings were fixed to the bottom of the chambers using stainless-steel hooks at a tension of 3 g. Tissues were allowed to stabilize for 10 min in incubation chambers at 37°C containing Krebs–Henseleit Solution constantly bubbled with an O_2: CO_2 (95:5%) mixture. Tension variations were measured using force transducers (Astro Med, West Warwick, RI, USA) connected to an MP-150 analyzer (BIOPAC 4.1 Instruments) as described previously by Estrada-Soto.[13]

The vasorelaxant activity of Nibethione on contraction induced by norepinephrine

By using a standard protocol, the vasorelaxant activity was measured.[14] After a period of stabilization, the aortic rings were submitted to sensitization period, using norepinephrine (NE; 0.1 μM); tissue stimulation was carried out during a period of 15 min; then, they were washed with Krebs–Henseleit Solution, allowing stabilizing for 15 min; this whole process was carried out in triplicate. The presence of a functional endothelium was assessed by testing the ability of acetylcholine/carbachol to reduce the phenylephrine-induced contraction by a value ≥50%. After sensitization, tissues were stabilized for 20 min, then were contracted with norepinephrine (0.1 μM) and Nibethione (1.14–376 μM), vehicle (DMSO; 1% final concentration) and positive controls (carbachol for endothelium-intact aortic rings: E+ or nifedipine for endothelium-denuded aortic rings: E−, (0.11–10 μM)) were added in cumulative concentrations (Concentration–Response Curves (CRC)). The vasorelaxant effect of test samples was determined by measuring its capacity to reduce the maximal vascular contraction induced by norepinephrine and comparing tissue tension before and after sample addition.

Determination of the vasorelaxant mechanism of action of Nibethione

To elucidate the vasorelaxant mechanism of action of Nibethione, the following experiments were performed after tissue sensitization:

(a)*Adrenergic pathway involvement*. For establishing an antagonism of the adrenergic receptor or disruption of the norepinephrine (NE) pathway, the following procedures were performed on endothelium-denuded aortic rings. A cumulative NE-induced contraction CRC (0.1–10 μM) was established as a positive control (control-CRC). In another experiment, aortic rings were pre-incubated with Nibethione (30, 20, 14 or 9 μM) for 15 min. Then, the CRC to NE-induced contraction was performed to compare the NE-induced contraction in the absence and presence of Nibethione.

(b)K^+ *channel involvement*. To identify the role of K^+ channels in the Nibethione-induced vasorelaxation effect, endothelium-denuded aortic rings were pre-incubated with tetraethylammonium chloride (a large-conductance K_{Ca} channel blocker (10 μM)), glibenclamide (an ATP-sensitive potassium channel blocker (10 μM)) or 2-aminopyridine (a voltage-gated potassium channel blocker (100 μM)) during 15 min prior to the contraction with norepinephrine (1 μM). The CRC of Nibethione (1.14–376 μM) was obtained as previously described. The highest vasorelaxant effect of Nibethione was compared in the absence and presence of channel blockers.

(c)Ca^{2+} *channel involvement*. For identifying the role of Ca^{2+} channels in the Nibethione-induced vasorelaxant effect, endothelium-denuded aortic rings were contracted by depolarization with a high potassium solution (KCl (80 mM)), and the CRC of Nibethione (1.14–376 μM) was built as described previously. In this test, nifedipine (a calcium channel blocker, (0.11 nM–10 μM)) was used as the positive control.

(d)Ca^{2+} *influx involvement*. To identify whether the inhibition of extracellular Ca^{2+} influx was responsible for the Nibethione-induced vasorelaxation. Endothelium-denuded aortic rings were washed with Ca^{2+}-free Krebs–Henseleit Solution containing high potassium concentration, after sensitization and were stabilized for 15 min. Then, a CRC for the $CaCl_2$-induced contraction (0.113 μM–10 mM) was built in the absence of Nibethione, and this experiment was considered as control. After the tissue was washed with Ca^{2+}-free Krebs–Henseleit Solution, 20 min later Nibethione (EC_{50} = 20 μM) or nifedipine (positive control, (10 μM)) was incubated for 15 min, and CRC of $CaCl_2$ was built again. The maximal contractile effect induced by $CaCl_2$ was compared in the absence and presence of test samples.

In silico evaluation of L-type Ca^{2+} Channel

Molecular modelling of the Human L-type Ca^{2+} Channel Model (LCCM)

The amino acid sequence of human voltage-dependent L-type Ca^{2+} Channel (VLCC) was acquired from National Center for Biotechnology Information,[15] with accession number Q13936 and aligned as described in the Results and Discussion sections. PSI-BLAST server was used against the Protein Data Bank (PDB) database for the comparative analysis and similar sequence search.

The query sequence had a high sequence identity with two sequences of the structures with the PD-ID: 5GJV (69%), 3JBR (69%). The crystallographic structure of 5GJV (Resolution of 3.6 Å) showed 83% of query cover, while 3JBR (Resolution of 4.2 Å) only 77%. For this reason, the sequence 5GJV was used as a template and it was aligned with the query sequence, and Figure 2a shows a sequence alignment between template crystal structure and query sequence. Then, the heterotetrameric pore assembly was built in MODELLER v9.18[16–19] according to the structure of the mammalian voltage-gated calcium channel Cav1.1 (PDBID: 5GJV) as a structural template. One hundred 3D-dimensional models were constructed. The best model was selected based on DOPE score, and the geometry was optimized to avoid some steric clashes. For this purpose, the MMFF94 force field was applied. Although the heterotetrameric pore modelled were compared with the template of the crystallographic structure (5GJV) were obtained analogous residues into the two binding pockets. All representations of molecular docking results were carried out using PyMOL (Molecular Graphics System, v1.2, Schrödringer).

Molecular docking simulations

Docking of Br-dihydropyridine (UK-59811) and Nibethione was performed with AutoDock 4.2 software package.[20] Polar hydrogens were added for LCC model by using the hydrogen module in AutoDock Tools (ADT). Later, Gasteiger charges were given for all ligands and receptors. The semi-rigid molecular docking performed an automated dock of the ligand with user-specified dihedral flexibility within a rigid binding site of a protein; it was carried out using the Lamarckian Genetic algorithm, applying the standard parameters. The two grids were centred at the geometric centre of the cavity of the pore with dimensions of 50 × 50 × 50 points (site 1), while the second had dimensions of 40 × 40 × 40 points covering site 2, both binding pockets with points separated by 0.375 Å. The program performed 100 runs. After molecular docking, all solutions were clustered into groups with RMSD of 2.0 Å. The reproducibility of interaction mode between LCC and Br-dihydropyridine was used for internal validation of the homology model due to its conformation, and two binding sites in the molecular structure of CavAb (PDB-ID: 5KLG) were previously reported.[21] The results of molecular docking for Br-dihydropyridine in site 1 and site 2 are shown in Figure 2b and 2c, respectively. MOE 2018.01[22] and Pymol 1.7.4. were employed for adequate visualization.

Cytotoxicity activity

Cell culture

Porcine Aortic Endothelial Cells (PAECs) were obtained from primary culture. Cells were cultured in T-25 polystyrene flasks with DMEM/High glucose, phenol red-free, 2 mM L-glutamine supplemented with 10% inactivated FBS, antibiotics (100 U/ml of penicillin and 100 μg/ml of streptomycin) and grown at 37 °C in a humidified atmosphere of 5% CO_2 in air. Cultured cells (80–90% confluence monolayer) were trypsinized and seeded in 96-well plate at a density of 5×10^3 cells/well. PAECs were used at passages 4–6 for all experiments.

Resazurin reduction assay for cytotoxicity assessment

In vitro cytotoxicity of Nibethione was tested against PAECs through the measure of cell viability in proliferation using resazurin reduction assay.[23] Cells were seeded and incubated for 24 h at 37°C with 5 % CO_2. Then cells were treated with increasing concentrations of test sample (125, 250, 500, 750 and 1000 μM). After 24 h of exposition, 20 μl of CellTiter-Blue reagent (1 : 5 v/v) was added and absorbance measured at 570 and 600 nm in 96-well plates. Multifunctional microplate reader Cytation 3 (Biotek Instruments, Inc., Winooski, VT, USA), Gen 5 Microplate Reader and Imager Software (Biotek Instruments, Inc.,Winooski, VT, USA) were used for this step. The percentage of cells showing cytotoxicity relative to the control group (Ctrl) was then determined. A higher percentage of reduction of Resazurin reflects a more significant cell proliferation. The concentration leading to 50 % inhibition of viability (IC_{50}) was calculated by regression analysis (per cent survival versus log concentration). The criteria used to categorize cytotoxicity of Nibethione against PAECs were based on U.S. National Cancer Institute (NCI) and were as follows: $IC_{50} \leq 20$ μg/ml = highly cytotoxic, IC_{50} ranged between 21 and 200 μg/ml = moderately cytotoxic, IC_{50} ranged between 201 and 500 μg/ml = weakly cytotoxic and $IC_{50} > 501$ μg/ml = no cytotoxicity.[24]

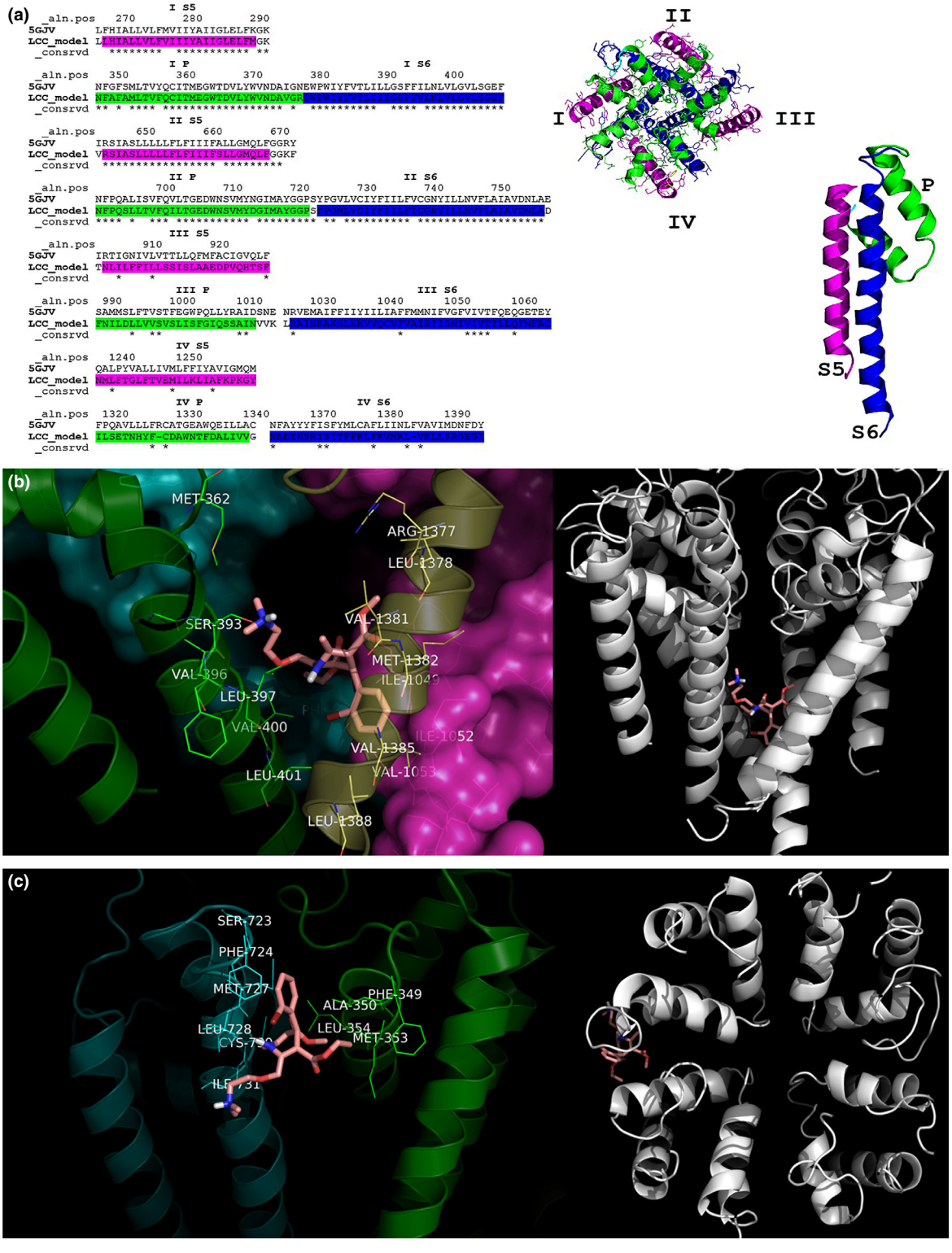

Figure 2 (a) 5GJV sequence alignment between template crystal structure and query sequence (b) proposed binding of the Br-dihydropyridine into the site 1 and (c) into the site 2 for the LCC receptor. [Colour figure can be viewed at wileyonlinelibrary.com]

The antihypertensive effect of Nibethione

The antihypertensive effect of Nibethione was assessed in spontaneously hypertensive rats (SHR) and Wistar–Kyoto (WKY) rats were used as the control group.[25] Animals were allotted into three groups (five animals each): Control rats (group 1), treated rats (group 2) and positive control (group 3). Group 1 was administrated with a single intragastric dose of purified water (5 ml/kg), group 2 was administrated with Nibethione (187.76 μmol/Kg), and group 3 was administrated with Captopril (138.06 μmol/Kg). Blood pressure (systolic and diastolic) and heart rate values were recorded before (T0) and after the administration test sample at 1, 3, 5 and 7 h (T1–T7) by a tail-cuff method using a LE 5001 automatic blood pressure computer (PanLab, S.L.U; Harvard Apparatus, Barcelona, Spain). Percentage of decrease in heart rate (HR), systolic blood pressure (SBP) and diastolic blood pressure (DBP) were calculated.

Data analysis

The plotted values are mean of the percentage of six experiments for isolated rat aortic ring assay (n = 6), three independents experiments for *in vitro* cytotoxicity assay (n = 3) considerating the mean of three replicates each experiment, and five experiments for *in vivo* experiments (n = 5) ± standard deviations. Concentration–response curves were plotted and fitted whit Origin Lab version 8.0 software (Microcal). Pharmacodynamics parameters such as maximum effect (E_{max}) and half-effective concentration (EC_{50}) were obtained fitting the Hill equation to dose–response curves with Origin Lab software.

Half-inhibitory concentration (IC_{50}) for cytotoxicity assay was calculated using regression analysis (survival % vs log concentration). Criteria used to categorize cytotoxicity were based on the U.S. National Cancer Institute criteria as follow: $IC_{50} \leq 20$ μg/ml as highly cytotoxic, IC_{50} 21–200 μg/ml as mildly cytotoxic, IC_{50} 201–500 μg/ml as weakly cytotoxic and IC_{50} >501 μg/ml as non-cytotoxic.[24]

Statistical significance was calculated by one-way analysis of variance (ANOVA) and post hoc Tukey's tests. All values with $p < 0.05$ were considered statistically significant (*).[26,27]

Results

Chemistry

The synthesis of a (*Z*)-5-(4-nitrobenzylidene)-2-thioxothiazolidin-4-one (Nibethione) was prepared in a one-step reaction. The nitrobenzaldehyde (0.5 g, 3.30 mmol) was condensed with 2-thioxo-1,3-thiazolidinone (1.5 equiv.), benzoic acid (0.12 g, 0.99 mmol, 30% mol) and piperidine (30% mol) in the presence of 10 ml of toluene. The reaction was heated under reflux with continuous elimination of water formed during the reaction using a Dean-Stark apparatus for 3–4 h. The reaction mixture was cooled, and the yellow solid was filtered off and dried to afford Nibethione compound. The crude solid was purified by conventional procedures.[28] The chemical structure of Nibethione was confirmed using spectroscopic and spectrometric data (NMR and mass spectra): yellow solid, melting point: 265–266°C, yield: 88 %; ^{1}H-NMR (400 MHz, DMSO-d6) δ: 7.76 (2H, d, H-2, H-6), 7.93 (1H, s, H-7), 8.26 (2H, d, H-3, H-5), 12.08 (1H, s, NH) ppm; ^{13}C-NMR (100 MHz, DMSO-d6) δ: 124.2 (C-3, C-5), 127.9 (C-1), 129.0 (C-7), 130.9 (C-2, C-6), 139.3 (C-5′), 147.3 (C-4), 162.3 (C = S), 167.3 (C = O) ppm; MS/FAB$^+$: m/z 267 $(M + H)^+$.

Vasorelaxant effect

The vasorelaxant effect of Nibethione (1.14–376 μM) was evaluated on the NE-induced contraction on aorta rings isolated from rats. Figure 3 shows the vasorelaxant effect induced by Nibethione, which was efficient (Maximum Effect: E_{max} = 101.54 %) on intact-endothelium aortic rings (E+) compared with carbachol (E_{max} = 74.20 %), a muscarinic agonist (the positive control). As we observed, Nibethione induced an endothelium-independent vasorelaxation (E−) effect that completely abolished contraction induced by norepinephrine (E_{max} = 103.46 %). Nifedipine a calcium channel blocker was used as E-positive control (E_{max} = 99.8 %). Table 1 shows the pharmacodynamics parameters obtained from the concentration–response

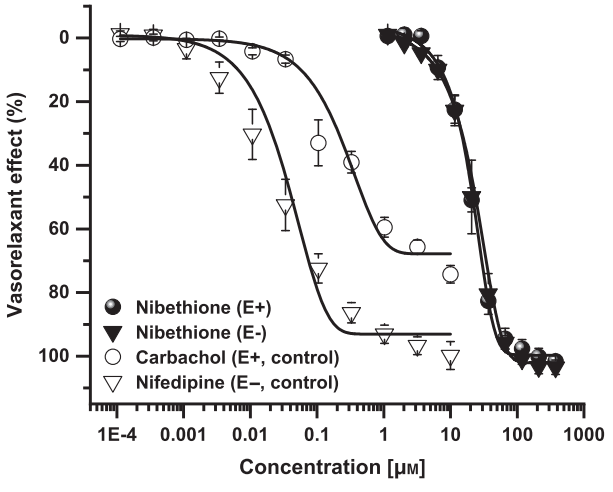

Figure 3 Concentration–response curves of vasorelaxant effect of Nibethione on aortic rings precontracted with norepinephrine (0.1 μM). All results are expressed as the mean ± SEM of six experiments. No statistically significant difference between Nibethione groups E+ and E− (ANOVA: E_{max}: F = 0.02, P = 0.8790; CE_{50}: F: 0.08, P = 0.7901).

Table 1 Pharmacological parameters of the vasorelaxant effect of Nibethione and positive controls used

	Nibethione			Control	
Vasorelaxant effect	E_{max} (%)	EC_{50} (μM)		E_{max} (%)	EC_{50} (μM)
Aorta rings with endothelium (E+)	101.54 ± 1.78	19.07 ± 2.67	Carbachol	74.2 ± 2.75	0.47 ± 0.05
Aorta rings without endothelium (E−)	103.46 ± 2.28	20.6 ± 2.23	Nifedipine	99.8 ± 4.38	0.03 ± 0.02

E_{max}, maximum effect (Efficacy); EC_{50}, half-effective concentration (Potency), no statistically significant difference between Nibethione groups E+ and E− (E_{max}; ANOVA: $F = 0.02$, $P = 0.8790$; CE_{50}; ANOVA: F: 0.08, $P = 0.7901$).

curve obtained from Nibethione and positive controls. CE_{50} values of Nibethione groups E+ and E− did not show statistically significant difference by one-way ANOVA: E_{max}: $F = 0.02$, $P = 0.8790$; CE_{50}: F: 0.08, $P = 0.7901$.

Determination of the vasorelaxant mechanism of action of Nibethione

Since Nibethione showed an endothelium-independent vasorelaxant following mechanistic, experiments were focused on studying the vascular smooth muscle-related molecular pathways involved, such as the adrenergic pathway, as well as K+ or Ca^{2+} channel involvement.[29,30] The Figure 4a showed how Nibethione abolished NE-induced contraction in a concentration-dependent fashion (NB at (10 μM): $E_{max} = 7.0 \pm 0.39$ g; (14 μM): $E_{max} = 4.0 \pm 0.5$ g (ANOVA: F = 21.07, p = 0.0013); (20 μM): $E_{max} = 3.5 \pm 0.3$ g (F = 50.86, p = 0.0001); and (30 μM): $E_{max} = 3.0 \pm 0.3$ g (F = 51.66, p = 0.0001)) compared with the control (NE-contraction curve without Nibethione; $E_{max} = 7.15 \pm 0.33$ g). On the other hand, Figure 4b showed that the concentration–response curve of Nibethione was shifted right in aortic rings pre-incubated with each K^+ channel blocker proved (glibenclamide CE_{50}:110 μM (ANOVA: $F = 11.25$, $P = 0.0153$), TEA CE_{50}: 109 μM ($F = 9.99$, $P = 0.0195$) and 2-aminopyridine CE_{50}: 128 μM ($F = 14.60$, $P = 0.0087$)) in relation to the control (Nibethione in the absence of blockers, CE_{50}: 20.6 μM). This evidence suggested that the mechanism of action of Nibethione is through an ion flux modulation or ionic channel regulation. To confirm either ionic channel or ion flux involvement, the vasorelaxant effect of test sample was challenged on aorta rings contracted with depolarizing high-concentration KCl solution (80 mM), which produces Ca^{2+} entry through voltage-operated Ca^{2+} channels.

Figure 4c showed the efficient vasorelaxant effect of Nibethione (E_{max} = 87%) on depolarization by KCl solution (80 mm). Besides Ca^{2+} influx involvement was evaluated when a $CaCl_2$ solution was added to the bath in a Ca^{2+}-free incubation medium. Figure 4d shows $CaCl_2$-induced contraction curve in the presence of Nibethione (EC_{50} = 20 μM). In this case, the vascular contraction was completely abolished (ANOVA: $F = 124.7$, $P = 0.0001$), similarly as nifedipine control. Once we identified an active involvement of calcium channel on vasorelaxant mechanism induced by Nibethione, we investigated the molecular interaction of this compound on L-type calcium channel, a large-conductance voltage-operated calcium channel widely expressed on vascular smooth muscle cells.

In silico evaluation of L-type Ca^{2+} Channel (LCC)

Molecular interaction

The building of voltage-operated L-type Ca^{2+} channel model allowed us to hypothesize some overall characteristics for a molecular binding mechanism of dihydropyridine and Nibethione (used as control). We could identify common residues involved in binding sites. To analyze protein–ligand interaction and binding aspects, we performed molecular docking studies on this template.

Two binging pockets were identified, and the molecular docking was performed into these sites, amino acid residues: (I-S6) S393, V396, L397, N398, V340, L401; (II-S6) L745, F748; (III-S6) T1048, I1049, I1052, V1053; (IV-S6) L1378, V1381, M1382, L1384, V1385, L1387 and L1388 comprised a hydrophobic pocket (site 1) (Figure 5a) whereas a second site was comprised of following residues: (II-S6) S723, F724, M727, L728, C730, I731, I734; (I-S6) F349, A350, M353 and L354 where the Nibethione and Br-dihydropyridine also they bind (Figure 5b).

Cytotoxicity activity

Cell viability of PAECs was not modified for different concentrations of sample test: 125 μM (130.30 ± 9.08%), 250 μM (131.89 ± 11.89%), 500 μM (105 ± 6.28%), 750 μM (105 ± 7.15%) or 1000 μM (112 ± 9.08%) after 24 h. Calculated IC_{50} was greater than 1000 μM suggesting no cytotoxicity under the US National Cancer Institute criteria.[31]

The antihypertensive effect of Nibethione

The *in vivo* acute antihypertensive activity was evaluated in spontaneously hypertensive rats (SHR) by the non-invasive tail-cuff method. Figure 6 shows the acute effect of Nibethione on arterial pressure. Intragastric dosing of

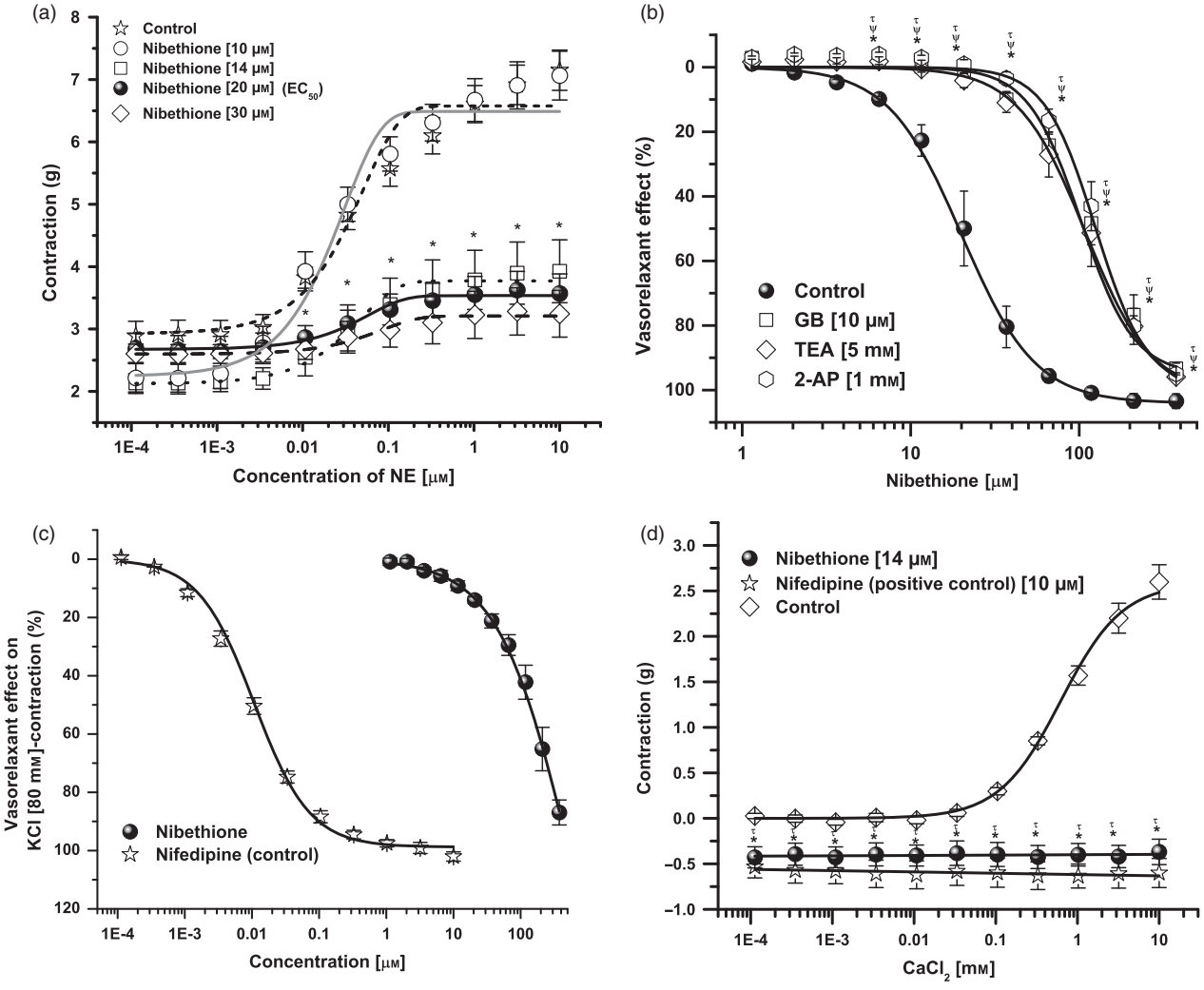

Figure 4 (a) Inhibitory effect of Nibethione on NE-contraction in aortic rings, ANOVA: (10 μM; $F = 0.02$, $P = 0.8890$), (14 μM; $F = 21.07$, $P = 0.0013$); (20 μM; $F = 50.86$, $P = 0.0001$); and (30 μM; $F = 51.66$, $P = 0.0001$), (b) effect of potassium channel blockers (Glibenclamide: GB (ANOVA: $F = 11.25$, $P = 0.0153$), tetraethyl ammonium: TEA ($F = 9.99$, $P = 0.0195$) and 2-aminopyridine: 2-AP ($F = 14.60$, $P = 0.0087$) on Nibethione-induced vasorelaxant effect in aortic rings precontracted with norepinephrine (0.1 μM), (c) vasorelaxant effect of Nibethione on the KCl (80 mM)-induced contraction and (d) inhibitory effect of Nibethione (20 μM) on the $CaCl_2$ contraction (ANOVA: $F = 124.7$, $P = 0.0001$). All experiments were realized in endothelium-denuded aortic rings, and the results are presented as the mean ± SEM of six experiments; $^*P < 0.05$ represents a significant difference compared with control.

187 μmol/kg led to a moderate decrease in diastolic blood pressure (DBP) at one hour (20.3%) (ANOVA: $F = 16.07$, $P = 0.0102$) and three hours (14.19%) ($F = 7.96$, $P = 0.037$) postadministration (Figure 6a). The systolic blood pressure (SBP) and heart rate (HR) were not significantly modified respect to control rats (WKY) (Figure 6b, $F = 1.74$, $P = 0.2447$ and 6(c) $F = 0.01$, $P = 0.9083$).

Discussion

Smooth muscle cells (SMCs) comprise functional layers in many contractile tissues such as blood vessels, airways, uterus, penis, gut and bladder.[30] Vascular SMCs play a pivotal role in the physiology of vessels contraction and relaxation. Thus, impaired contractibility may cause or contribute to cardiovascular diseases, such as hypertension, vascular stroke and heart failure.[32] Nibethione (Nitrobenzylydenethiazolidinethione) is a chemical structure that is present in a wide range of molecules, and it is easy to obtain, which is being prepared in a one-step reaction using the Knoevenagel condensation. Indeed, this core (2-thioxo-1,3-thiazolidin-4-one) is considered as a sulfur analog of thiazolidinedione, which is present into pioglitazone or rosiglitazone structures.[33] Regarding the mentioned

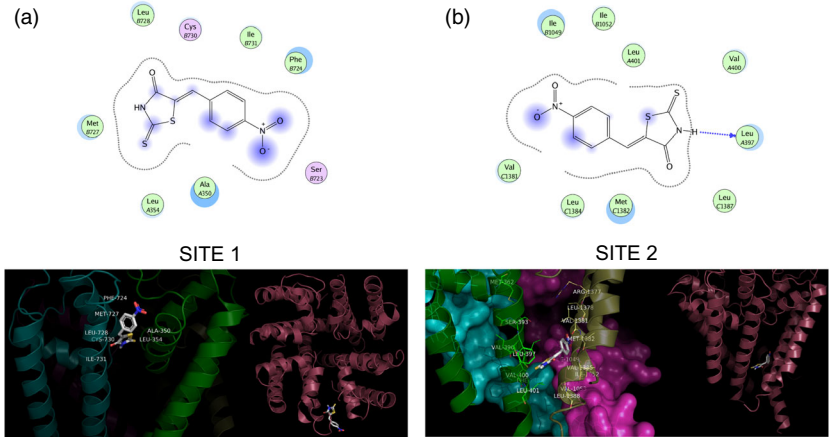

Figure 5 Bidimensional and Tridimensional image proposed to binding of the Nibethione (a) into the site 1 and (b) the site 2 for the L-type Ca^{2+} channel receptor. [Colour figure can be viewed at wileyonlinelibrary.com]

moiety, it has been reported that thiazolidinedione derivatives have emerged as potent antidiabetic agents, which act in several pharmacological targets for the treatment of diabetes and could be attributed antihypertensive properties.[34,35]

Results exposed in Figure 3 showed that Nibethione produces a significantly vasorelaxant effect in aortic rings. Based on this, its underlying vasorelaxant mechanism of action was determined through *ex vivo* functional experiments. The SMCs account in the wall of blood vessels (VSMC) represents a central point in the modulation of vasoconstriction processes and subsequently hypertension initiation. VSMC contraction begins when agonists as angiotensin II, norepinephrine or endothelin bind to receptors coupled to a heterotrimeric $G_{q/11}$ protein ($PG_{q/11}$), which actives the phospholipase C (PLC). This enzyme hydrolyses the membrane lipids as phosphatidylinositol 4,5-bisphosphate (PIP_2) which catalyses the formation of two secondary messengers: diacylglycerol (DAG) and inositol triphosphate (IP_3). The IP_3 binds to receptors in the sarcoplasmic reticulum (RS) resulting in the Ca^{2+} release into the cytosol, whereas DAG activates protein kinase C (PKC), which phosphorylates specific target proteins. PKC has contraction-promoting effects through phosphorylation of L-type Ca^{2+} channels.[36] This contraction is regulated by increasing intracellular calcium concentration and myosin and actin contractile proteins activation.[30,36,37] VSMC relaxation occurs either removal of contractile stimulus or directed action of a substance that stimulates inhibition of the contractile mechanism. Regardless, the relaxation process needs a decreased intracellular Ca^{2+} concentration and increased MLC phosphatase activity.[30] In addition, vascular endothelium contributes to relaxation through release endothelium-derivative relaxant factors (EDRF), such as nitric oxide (NO), endothelium-derivative hyperpolarizing factor (EDHF) and vasorelaxant prostacyclin (PGI_2), which stimulate the synthesis of cyclic nucleotides.[38] Vasorelaxant effect of Nibethione was independent of endothelium presence; for this reason, EDRF involvement was promptly discarded.[39]

Once endothelium involvement was discarded, we decide to explore a well-known VSM mechanism, such as α_1-adrenoceptors antagonism, potassium channel opening, and Ca^{2+} channel blockade or Ca^{2+}–CaM complex activity inhibition.[40–43] Interestingly, Nibethione at 14, 20 and 30 μM caused a dose-dependent depletion of NE-induced contraction (Figure 4a). The curve was non-parallel shifted with suppression of maximum effect in a concentration-dependent manner. So, this picture is characteristic of non-competitive antagonism of adrenergic receptor or disruption of the norepinephrine mechanism.[36] This evidence suggested that Nibethione could be involved in a mechanism as inactivation of contractile intracellular proteins, the blockade of calcium influx, or the opening of potassium channels.[30,36,37] It is well known that a variety of ion channels, pumps, transporters and exchangers are important in regulating the ionic balance and smooth muscle membrane potential. These proteins regulate vascular tone, especially modulating the vascular resistance, where systemic blood pressure is regulated.[44] Large-conductance calcium-activated potassium channels (BK_{Ca}) and ATP-sensitive potassium channels (K_{ATP}) significantly contribute to the regulation since these channels are associated with an open–close probability of voltage-gated Ca^{2+} channels.[45] Conversely, K^+ channels opening results in charge switching, membrane hyperpolarization, and consequently vasorelaxation.[45]

The vasorelaxant effect of Nibethione was significantly modified in presence of glibenclamide, 2-aminopyridine, and tetraethylammonium (Figure 4b), suggesting the

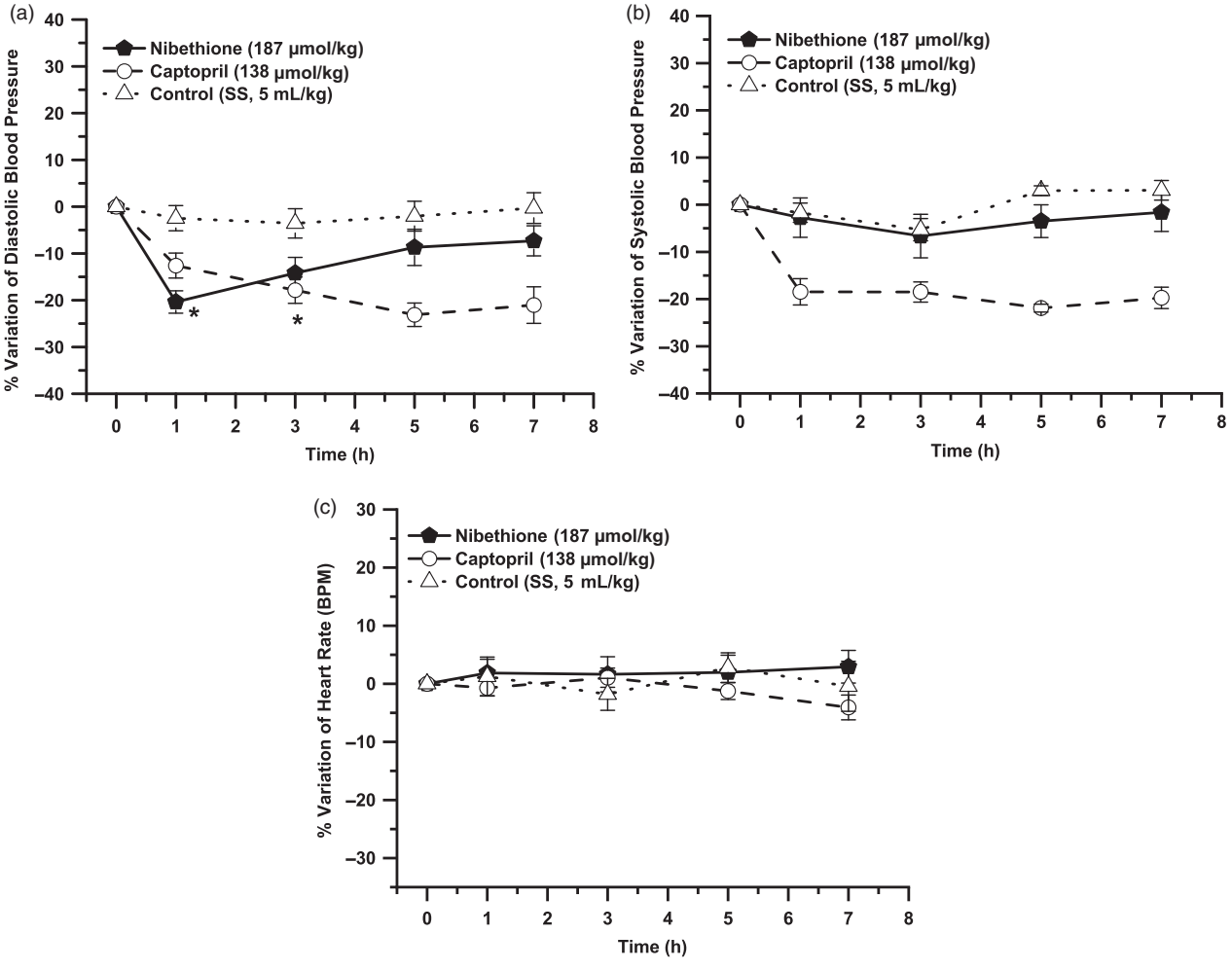

Figure 6 Maximal decrease in (a) diastolic blood pressure, (b) systolic blood pressure and (c) heart rate (BPM) elicited by a single intragastric administration of 187.76 µmol/kg of Nibethione in conscious SHR rats. Results are expressed as the means ± SEM of five rats per group. *Indicates significance respect to WKY (control) with $P < 0.05$ (ANOVA: $F = 16.07$, $P = 0.0102$ (T1), $F = 7.96$ $P = 0.037$ (T2)).

participation of K_{Ca}, K_{ATP} or Kv channels. K-channels opening leads to the closure of Ca^{2+} channels, and thus to the decrease of intracellular Ca^{2+} and VSMC relaxation.[46] To corroborate this hypothesis, we decided to explore the vasorelaxant effect related to Ca^{2+} channel blockade.

High-concentration KCl solution is a typical strategy for depolarizing cell membrane by an electrochemical force, which results in the opening of voltage-dependent L-type Ca^{2+} channels (VLCC).[46,47]

Nibethione showed an efficient vasorelaxation in KCl-induced contraction similar as nifedipine (a calcium channel blocker; Figure 4c); this behaviour suggests VLCC-blockade, thus leading to a decrease of intracellular Ca^{2+} repolarization, and VSMC relaxation.[47] Additionally, the $CaCl_2$-induced contraction curve was entirely inhibited by Nibethione, supporting a direct involvement of VLCC (Figure 4d). This possibility took relevance when we observed that Nibethione could bind active sites of dihydropyridine simultaneously. On the other hand, the antihypertensive experiment in SHR only showed an acute decrease in diastolic blood pressure (DBP) at 1 and 3 h after administration.

The DBP is the pressure in the arteries when the heart rests between beats, it refers to the function of filling the heart with blood between muscle contractions, and it describes that portion of the cardiac cycle opposite to contraction. Its elevation represents an increase of systemic vascular resistance (SVR), which refers to the resistance that the vascular system offers to blood flow. When blood vessels constrict (vasoconstriction), this leads to an increase in SVR, contrary to when blood vessels dilate (vasorelaxation), this leads to a decrease in SVR. If Nibethione produced vasorelaxant effect due to calcium channel blockage

in VSMC; this activity could decrease peripheral vascular resistance explaining DBP decrease.[48]

With the purpose of identifying the potential vascular toxicity of Nibethione, its cytotoxic activity was measured in porcine aortic endothelial cells (PAEC). Even though the effect of Nibethione was endothelium-independent, the presence of voltage-dependent calcium channels in endothelial cells has been described.[49] Results obtained from cytotoxicity assay in PAEC of Nibethione did not show changes in cell viability, and its IC_{50} was >1000 μM; this value suggests the absence of cytotoxicity. Nevertheless, it is necessary to perform acute and chronic studies of toxicity in rodents to discard its possible systemic toxicity.

In conclusion, this investigation suggests that Nibethione is a potential lead able to induce a vasorelaxant effect with participation of blockade of voltage-dependent L-type Ca^{2+} channels, and the findings presented here will have implications for the research of antihypertensive drugs.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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Author's contribution

Amanda Sánchez-Recillas performed major experiments and analysed data. Gabriel Navarrete-Vázquez carried out the design, synthesis and chemical characterization of Nibethione compound. Sergio Hidalgo-Figueroa designed *in silico* experiments, analysed, and interpreted data. Marcos Bonilla-Hernández participated in toxicity assays. Rolffy Ortiz-Andrade and Maximiliano Ibarra-Barajas participated in antihypertensive experiment. Víctor Yáñez-Pérez carried out the *ex vivo* experiments and statistical analysis. Juan Carlos Sánchez-Salgado contributed to the grammar review and final manuscript preparation. All authors contributed to drafted and revised the manuscript.

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