Bioorganic & Medicinal Chemistry 22 (2014) 2783–2790

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Synthesis, antinociceptive activity and pharmacokinetic profiles of nicorandil and its isomers

Isabela C. César [a], Adriana M. Godin [a], Débora P. Araujo [b], Francinely C. Oliveira [b], Raquel R. Menezes [a], Julliana R. A. Santos [c], Mariana O. Almeida [a], Marcela M. G. B. Dutra [a], Daniel A. Santos [c], Renes R. Machado [a], Gerson A. Pianetti [a], Márcio M. Coelho [a], Ângelo de Fátima [b,*]

[a] Department of Pharmaceutical Products, School of Pharmacy, Universidade Federal de Minas Gerais, 31270-901 Belo Horizonte, MG, Brazil
[b] Department of Chemistry, Institute of Exact Sciences, Universidade Federal de Minas Gerais, 31270-901 Belo Horizonte, MG, Brazil
[c] Department of Microbiology, Institute of Biological Sciences, Universidade Federal de Minas Gerais, 31270-901 Belo Horizonte, MG, Brazil

ARTICLE INFO

Article history:
Received 6 January 2014
Revised 24 February 2014
Accepted 8 March 2014
Available online 19 March 2014

Keywords:
Nicorandil
Nicotinamide
Nitric oxide
NO-releasing drugs
Nitrite
Antinociceptive activity

ABSTRACT

Nicorandil (*N*-(2-hydroxyethyl)nicotinamide nitrate) is an antianginal drug, which activates guanylyl cyclase and opens the ATP-dependent K^+ channels, actions that have been suggested to mediate its vasodilator activity. We synthesized nicorandil and its two isomers, which vary in the positions of the side chain containing the nitric oxide (NO) donor, and also their corresponding denitrated metabolites. The activities of these compounds were evaluated in an experimental model of pain in mice. Pharmacokinetic parameters of nicorandil and its isomers, as well as the plasma concentrations of the corresponding denitrated metabolites and also nicotinamide and nitrite were determined. Nicorandil exhibited the highest antinociceptive activity, while the *ortho*-isomer was the least active. Nicorandil and *para*-nicorandil, which induced higher plasma concentrations of nitrite, exhibited higher antinociceptive activity, which suggests that the release of NO may mediate this activity.

1. Introduction

Nicorandil [*N*-(2-hydroxyethyl)nicotinamide nitrate (**1**; Fig. 1) was synthetized in 1976[1] and has been marketed since 1984 in Japan and other countries for the prevention and treatment of chronic angina pectoris.[2–4] It has been demonstrated that nicorandil releases nitric oxide (NO) and opens ATP-dependent K^+ channels, actions that have been suggested to mediate its vasodilator activity.[5] It is still unclear whether nicorandil induces its NO-independent effects directly or indirectly.[5] Regarding the opening of ATP-dependent K^+ channels induced by nicorandil, Simpson and Wellington (2004)[4] have demonstrated that *N*-(2-hydroxyethyl)-nicotinamide (**1A**; Fig. 1), the major metabolite, may mediate this effect. In addition, **1A** enhances the vascular effects of endogenous vasodilators, such as adenosine and adrenomedullin.[6,7] Another metabolite, nicotinamide, may be formed when the side chain of **1A** is cleaved.[3] Nicotinamide, an amide derivative of vitamin B3, similarly to nicorandil, induces vasodilation, but its potency is much lower than that of the parent compound.[8]

As NO and ATP-dependent K^+ channels are important molecules mediating many other biological actions, including inflammation[9,10] and nociception,[11] it would not be surprising to identify new activities of nicorandil beyond those in the cardiovascular system. Indeed, the inhibitory effects induced by nicorandil on the overactive bladder in animal models,[12] proteinuria and glomerular injury in a model of diabetic nephropathy[13] and intraocular pressure in the anterior chamber of the eye of humans[14] have been demonstrated. Although the investigation of the effects induced by nicorandil in experimental models of pain is still too preliminary, it is warranted. Many compounds that release NO[15–17] and activate ATP-dependent K^+ channels,[18–20] and also nicotinamide,[21] a nicorandil metabolite, exhibit activities in different experimental models of pain. In addition, we have recently demonstrated that systemic administration of nicorandil inhibits the nociceptive response induced by formaldehyde in mice.[22]

In the present study, we synthesized the three isomers of nicorandil, which vary in the positions of the side chain that contains the $-ONO_2$ group (Fig. 1), and investigated their activities in an experimental model of nociceptive and inflammatory pain in mice.

Abbreviations: NO, nitric oxide; p.o., *per os*; T_{max}, maximum time; AUC_{0-inf}, area under curve (time 0 > infinite); CMC, carboxymethyl cellulose; NMR, nuclear magnetic resonance; C_{max}, maximum concentration; AUC_{0-t}, area under curve (time 0 > last time).

* Corresponding author. Tel.: +55 31 3409 6373; fax: +55 31 3409 5700.
E-mail address: adefatima@qui.ufmg.br (Â. de Fátima).

http://dx.doi.org/10.1016/j.bmc.2014.03.011
0968-0896/

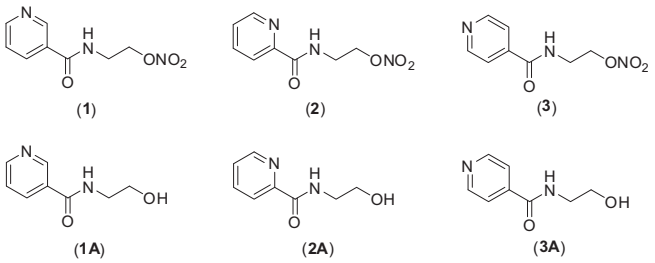


Figure 1. Chemical structures of nicorandil (**1**), *ortho*-nicorandil (**2**) and *para*-nicorandil (**3**) and their respective main metabolites *N*-(2-hydroxyethyl)nicotinamide (NHN) (**1A**), *N*-(2-hydroxyethyl)picolinamide (NHP) (**2A**) and *N*-(2-hydroxyethyl)isonicotinamide (NHI) (**3A**).

To further explore the structure-activity relationships, we determined some pharmacokinetic parameters of the three isomers, as well as the plasma concentrations of the corresponding main denitrated metabolites (Fig. 1) and also the plasma concentrations of nicotinamide and nitrite after the administration of each isomer in mice.

2. Material and methods

2.1. Chemical and reagents

All chemicals were obtained from commercial suppliers and used without further purification. Melting points were determined in a Gehaka PF 1500 apparatus and are uncorrected. ^{1}H nuclear magnetic resonance (NMR) spectra and ^{13}C NMR spectra were recorded on a Bruker Avance DPX/200. Chemical shift values (δ) were given in parts per million (ppm). Infrared (IR) spectra were recorded on a Spectro One Perkin Elmer. The elemental analyses were performed with a Perkin-Elmer apparatus.

2.2. Syntheses of nicorandil and its isomers

Nicorandil (**1**) and its positional isomers (**2** and **3**) were synthesized, as shown in Scheme 1. In summary, the corresponding esters were treated with ethanolamine under reflux, which furnished the hydroxylated precursors **1A** to **3A**. Compounds **1A**–**3A** were treated with fuming nitric acid at −5 °C, which lead to the nitrates **1** to **3**. All synthesized compounds were characterized by ^{1}H and ^{13}C NMR, infrared, melting point and elemental analysis; the data are in accordance with previous reports.[23,24]

2.3. Chemistry and biological assays

For the synthesis of the hydroxylated precursors, ethanolamine (1.5 mmol) was added slowly to the esters (1 mmol) at 55 °C and stirred for 3 h. The reaction mixture was stirred at room temperature for 15 h. The residue was purified by silica gel column chromatography (eluent/ethyl acetate/hexane 8:2) or recrystallized from ethyl acetate. The progress of the reaction was monitored by TLC.

2.3.1. *N*-(2-Hydroxyethyl)nicotinamide (1A)

(Yield 64%): mp 88.0 °C. IR (ATR): 3321, 3173, 2876, 1659, 1550, 1422, 1296, 1057, 1028, 702. ^{1}H NMR (200 MHz, DMSO-d_6): 3.34–3.39 (m, 2H), 3.57 (t, 2H, J = 5.8 Hz), 4.83 (br s, 1H), 7.42–7.50 (m, 1H), 8.19 (d, 1H, J = 8.0 Hz), 8.66–8.68 (m, 2H), 9.02 (s, 1H). ^{13}C NMR (50 MHz, DMSO-d_6): 42.3, 59.8, 123.4, 130.1, 135.0, 148.5, 151.7, 165.1. Anal. Calcd for $C_8H_{10}N_2O_2$. Calcd (%): C, 57.82; H, 6.07; N, 16.86. Found (%): C, 56.94; H, 6.03; N, 16.88.

2.3.2. *N*-(2-Hydroxyethyl)picolinamide (2A)

(Yield 92%): mp 36.0 °C. IR (ATR): 3364, 3297, 2932, 2876, 1645, 1569, 1436, 1365, 1298, 1060, 1044, 821, 752. ^{1}H NMR (200 MHz, $CDCl_3$): 3.60–3.68 (m, 2H), 3.84 (t, 2H, J = 5.0 Hz), 4.85 (br s, 1H), 7.32–7.41 (m, 1H), 7.78 (td, 1H, J = 7.6, 1.6 Hz), 8.10 (d, 1H, J = 7.8 Hz), 8.47 (d, 1H, J = 4.3 Hz), 8.64 (br s,1H). ^{13}C NMR (50 MHz, $CDCl_3$): 41.6, 60.8, 121.6, 125.7, 136.9, 147.6, 149.0, 164.6. Anal. Calcd for $C_8H_{10}N_2O_2$. Calcd (%): C, 57.82; H, 6.07; N, 16.86. Found (%): C, 57.78; H, 6.12; N, 16.85.

2.3.3. *N*-(2-Hydroxyethyl)isonicotinamide (3A)

(Yield 88%): mp 135.5 °C. IR (ATR): 3317, 3173, 3058, 2931, 1660, 1548, 1410, 1314, 1237, 1065, 1041, 1003, 816, 751. ^{1}H NMR (200 MHz, DMSO-d_6): 3.32–3.40 (m, 2H), 3.47–3.59 (m, 2H), 4.81 (t, 1H, J = 5.4 Hz), 7.76 (d, 2H, J = 4.8 Hz), 8.69-8.75 (m, 3H). ^{13}C NMR (50 MHz, DMSO-d_6): 42.3, 59.5, 121.3, 141.5, 150.1, 164.8. Anal. Calcd for $C_8H_{10}N_2O_2$. Calcd (%): C, 57.82; H, 6.07; N, 16.86. Found (%): C, 58.72; H, 6.04; N, 16.97.

The nitrated compounds were obtained by mixing fuming nitric acid (10 mmol) and the hydroxylated precursors (1 mmol) at −5.0 °C and stirring for 2 h. The reaction mixture was poured into a mixture of water and ice. The pH was adjusted to 6.0 by adding $CaCO_3$. The obtained solid was vacuum filtered and recrystallized in ethanol, which furnished a white solid in all cases.[23,24]

2.3.4. *N*-(2-Nytroxyethyl)nicotinamide (nicorandil; 1)

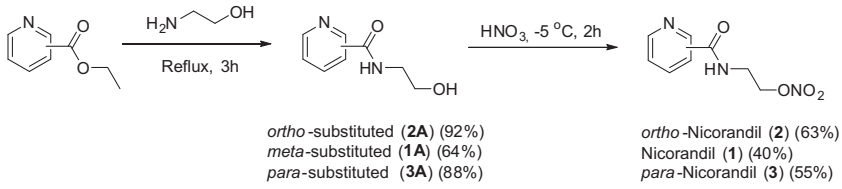
(Yield 40%): mp 91.0 °C. IR (ATR): 3241, 3073, 1717, 1627, 1590, 1554, 1372, 1361, 1319, 1286, 1012, 1000, 860, 824, 705. ^{1}H NMR (200 MHz, DMSO-d_6): 3.65 (q, 2H, J = 5.1 Hz), 4.67 (t, 2H, J = 5.1 Hz), 7.46–7.55 (m, 1H), 8.18 (d, 1H, J = 7.8 Hz), 8.71 (d, 1H, J = 4.4 Hz), 8.94–9.02 (m, 2H). ^{13}C NMR (50 MHz, DMSO-d_6): 36.8, 72.1, 123.5, 129.5, 135.0, 148.3, 152.0, 165.2. Anal. Calcd for $C_8H_9N_3O_4$. Calcd (%): C, 45.50; H, 4.30; N, 19.90. Found (%): C, 45.62; H, 4.31; N, 20.03.

2.3.5. *N*-(2-Nytroxyethyl)picolinamide (*ortho*-nicorandil; 2)

(Yield 63%): mp 62.5 °C. IR (ATR): 3391, 2956, 1664, 1630, 1520, 1275, 1012, 858, 752. ^{1}H NMR (200 MHz, DMSO-d_6): 3.64–3.74 (m, 2H), 4.69 (t, 2H, J = 5.1 Hz), 7.55–7.61 (m, 1H), 7.94–8.07 (m, 2H), 8.62 (d, 1H, J = 4.4 Hz), 9.08–9.10 (m, 1H). ^{13}C NMR (50 MHz, DMSO-d_6): 36.4, 72.2, 121.9, 126.6, 137.7, 148.3, 149.5, 164.3. Anal. Calcd for $C_8H_9N_3O_4$. Calcd (%): C, 45.50; H, 4.30; N, 19.90. Found (%): C, 45.45; H, 4.21; N, 19.86.

2.3.6. *N*-(2-Nytroxyethyl)isonicotinamide (*para*-nicorandil; 3)

(Yield 55%): mp 112 °C. IR (ATR): 3265, 1647, 1618, 1545, 1322, 1280, 1018, 877, 754. ^{1}H NMR (200 MHz, DMSO-d_6): 3.65 (q, 2H,



Scheme 1. Synthesis of nicorandil (**1**) and its isomers **2** and **3**.

J = 5.1 Hz), 4.67 (t, 2H, J = 5.1 Hz), 7.75 (d, 2H, J = 5.0 Hz), 8.73 (d, 2H, J = 5.0 Hz), 9.03 (br s, 1H). ^{13}C NMR (50 MHz, DMSO-d_6): 36.9, 72.0, 121.2, 140.9, 150.2, 165.1. Anal. Calcd for $C_8H_9N_3O_4$. Calcd (%): C, 45.50; H, 4.30; N, 19.90. Found (%): C, 45.67; H, 4.26; N, 20.05.

2.3.7. Drugs

Suspensions of nicorandil, its isomers and their corresponding metabolites were prepared in a 0.5% w/v carboxymethylcellulose (CMC; Sigma, USA) suspension in saline. The same vehicle was used to prepare a suspension with phenobarbital (Aventis Pharma, Brasil). The suspensions were administered *per os* (p.o.) in a volume of 4 mL/kg. The solution of 0.92% formaldehyde (Sigma, USA) was prepared in saline. The suspensions and solutions were prepared freshly before the experiments.

2.3.8. Animals

Female Swiss mice, which weighed 25–30 g, were used and had free access to food and water. The animals were kept in a room that had a 12 h light/12 h dark cycle and a temperature of 28 °C, which corresponds to the thermoneutral zone for rodents, for at least 3 days before the experiments to allow acclimatization. This study was approved by the Ethics Committee in Animal Experimentation of the Federal University of Minas Gerais (Protocol number 131/11), and all experiments were conducted according to the ethical guidelines for the investigation of experimental pain in conscious animals.[25]

2.3.9. Evaluation of the nociceptive response induced by formaldehyde

One hour after p.o. administration of the vehicle (CMC 0.5%, 4 ml/kg), nicorandil, its isomers (50, 100 and 150 mg/kg) or the main metabolites (equimolar doses to 50, 100 and 150 mg/kg of nicorandil), formaldehyde solution (0.92%, 20 μL) was injected subcutaneously (s.c.) into the dorsal surface of the right hindpaw of the animals. In one protocol, carried out after obtaining the pharmacokinetic data, *ortho*-nicorandil was administered 5 min before formaldehyde injection. Each mouse was placed under a transparent glass funnel (18 cm diameter, 15 cm-high) and the amount of time that the animal licked the injected paw was determined between 0 and 5 min (first phase) and 15 and 30 min (second phase) after the injection of formaldehyde.

2.3.10. Evaluation of the motor activity

The motor activity of the animals was evaluated in a rota-rod apparatus. The animals were trained on the apparatus at different occasions for 3 days before the experiment. On the day of the experiment, the animals were placed on a rota-rod (14 rpm) and the time they spent on it was measured. The cut-off time was 2 min. After the determination of the baseline values, the animals were treated with the vehicle (CMC 0.5%, 4 ml/kg), nicorandil, its isomers (150 mg/kg) or phenobarbital (40 mg/kg; positive control) and 1 h latter they were again tested in the apparatus.

2.3.11. Measurement of the plasma concentrations of nicorandil, its isomers and the main metabolites and nicotinamide

For the pharmacokinetic study, we used animals that were subjected to food restriction for 12 h. Nicorandil or its isomers were administered p.o. at a dose of 100 mg/kg. Each drug was administered to a group of 66 animals. At different times (5, 15, 30 and 45 min, 1, 2, 3, 4, 6, 8 and 10 h) after administration of the samples, a group of six animals was euthanized by decapitation. A blood sample from each animal and also from an additional group of six non-treated mice (control group) was collected in a heparinized tube and immediately centrifuged at 14,000 rpm for 10 min. Next, the plasma samples were transferred to plastic tubes and stored at −70 °C until analysis. The plasma concentrations of the compounds were determined by a LC–MS/MS method adapted from César et al.[26] HPLC–MS/MS analysis was performed on a Waters system (Milford, MA, U.S.A.), which was composed of a 1525 m binary pump, a 2777 sample manager, a TCM/CHM column oven and a Quattro LC triple quadrupole mass spectrometer that was equipped with an electrospray ion source. Calibration standards were prepared in plasma by adding known amounts of the test compounds to control plasma. Plasma samples (100 μL) were mixed with a 25 μL aliquot of internal standard solution (4 mg/mL procainamide in methanol). After agitation, the samples were extracted with 2 mL of ethyl acetate and methyl-*tert*-butyl ether (8:2) and centrifuged for 5 min at 3500 rpm. A 0.8 mL aliquot of the organic layer was evaporated to dryness and reconstituted in the mobile phase (320 μL). A 20 μL sample was injected into the LC–MS/MS system. Chromatographic separation was performed using a ShinPack C_{18} column (100 × 4.6 mm i.d.; 5 μm particle size) from Shimadzu (Kyoto, Japan), maintained at 30 °C. The mobile phase consisted of methanol and 2 mM aqueous ammonium acetate with 0.03% (v/v) formic acid (33:67 v/v), at a flow rate of 1 mL/min. For quantitation, the ion signal was recorded by selective reaction monitoring using the following transitions: m/z 212 → m/z 135 for nicorandil and its isomers, m/z 166 → m/z 106 for nicorandil metabolite and *ortho*-metabolite, m/z 166 → m/z 121 for *para*-metabolite, m/z 124 → m/z 80 for nicotinamide and m/z 236 → m/z 163 for procainamide.

2.3.12. Measurement of plasma concentrations of nitrite

Vehicle (CMC 0.5%), nicorandil or its isomers (100 mg/kg) were administered p.o. in animals fasted for 12 h. Each drug was administered to a group of 18 animals. At different times (15 min, 1 and 3 h) after the administration of nicorandil or its isomers, a group of six animals was anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg). A blood sample was collected from the abdominal artery of each animal, placed in a heparinized tube and immediately centrifuged at 14,000 rpm for 10 min. We used this method of blood collection to avoid hemolysis that could affect the nitrite colorimetric assay. Next, the plasma samples were transferred to plastic tubes and stored at −70 °C until analysis. Plasma samples were analyzed using a procedure based on the Griess reaction.[27] In summary, 60 μL of the Griess reagent (1% sulfanilamide and 0.1% napthylethylenediamine in 2.5% phosphoric acid) were added to 60 μL of the plasma samples. After 10 min at room temperature, the absorbance of the samples was measured at 540 nm. Blank plasma samples were also submitted to the same procedure.

2.3.13. Data collection and statistical analysis

The results, which are presented as the mean ± standard error mean (S.E.M.), were analyzed via one-way analysis of variance, which was followed by Newman–Keuls post-hoc test when the main effect was significant. A P <0.05 was considered significant.

3. Results

3.1. Synthesis and characterization of nicorandil and its isomers

The synthesis of nicorandil (**1**) and its isomers, *ortho*-nicorandil (**2**) and *para*-nicorandil (**3**), was accomplished using the reaction sequence shown in Scheme 1. The synthesis of nicorandil (**1**), *ortho*-nicorandil (**2**) and *para*-nicorandil (**3**) was completed by the nitration, which employed fuming nitric acid as a nitrating agent, of their respective precursors, **1A**, **2A** and **3A**. The IR spectra of **1A**, **2A** and **3A** displayed absorbances indicative of an –OH group (3321, 3364 and 3317 cm^{-1}, respectively), while these bands were absent in the IR spectra of nicorandil (**1**), *ortho*-nicorandil (**2**) and

para-nicorandil (**3**). The success of the nitration reactions was also validated by the presence of ^{1}H NMR signals corresponding to the methylene protons, $-CH_2-X$ (X = OH or ONO_2), present in these compounds. The ^{1}H NMR spectra displayed the signals at δ 3.57 (t, 2H) for (**1A**), δ 3.84 (t, 2H) (**2A**) and δ 3.47–3.59 (m, 2H) (**3A**) were shifted to δ 4.67 (t, 2H) for all nitrated compounds (nicorandil and its isomers). These data confirm the expected structures were successfully synthesized as such results are in agreement with those reported elsewhere.[23,24]

3.2. Effects induced by nicorandil, its positional isomers and main metabolites on the nociceptive response induced by formaldehyde

Previous (1 h) p.o. administration of nicorandil, at the doses of 3, 10 or 30 mg/kg, did not inhibit the nociceptive response induced by formaldehyde (data not shown). However, we observed that higher doses of nicorandil (50, 100 or 150 mg/kg) did inhibit this response in a dose-dependent manner. The first phase of the nociceptive response was inhibited by the three doses, while the second phase was inhibited by the doses of 100 and 150 mg/kg doses (Fig. 2A).

Next, we investigated whether the previous (1 h) p.o. administration of the two positional nicorandil isomers, *ortho*- (**2**) and *para*-nicorandil (**3**), at the doses of 50, 100 or 150 mg/kg, could also induce an antinociceptive effect. *ortho*-Nicorandil (**2**) inhibited both phases of the nociceptive response only at the highest dose (150 mg/kg) (Fig. 2B). Because the time needed to reach the peak concentration (T_{max}) for *ortho*-nicorandil (**2**) is shorter than those for nicorandil (**1**) and *para*-nicorandil (**3**), as will be described in the next session, we carried out another protocol to evaluate whether lower doses of *ortho*-nicorandil (**2**) could induce an antinociceptive effect when administered immediately before (5 min) the injection of formaldehyde. However, in this protocol, *ortho*-nicorandil (**2**) did not induce an antinociceptive effect (Fig. 3). The other positional isomer, *para*-nicorandil (**3**), at the doses of 100 and 150 mg/kg, inhibited only the second phase of the nociceptive response (Fig. 2C). To directly compare the effects induced by nicorandil (**1**) and its two positional isomers, we carried out another protocol in which the three compounds were administered at a fixed dose (100 mg/kg). Providing support to the previous results, the greatest inhibition of the nociceptive response was induced by nicorandil, followed by *para*-nicorandil (**3**), while *ortho*-nicorandil (**2**) was devoid of activity (Fig. 4).

As the main metabolites of nicorandil (**1**), *ortho*-nicorandil (**2**) and *para*-nicorandil (**3**) are NHN (**1A**), NHP (**2A**) and NHI (**3A**), respectively, we subsequently investigated whether the last three compounds could mediate the effect induced by nicorandil and its positional isomers. However, previous (1 h) p.o. administration of NHN (**1A**), NHP (**2A**) or NHI (**3A**), at doses equimolar to those of the respective parent compounds (50, 100 and 150 mg/kg), did not inhibit the nociceptive response induced by formaldehyde (Fig. 5).

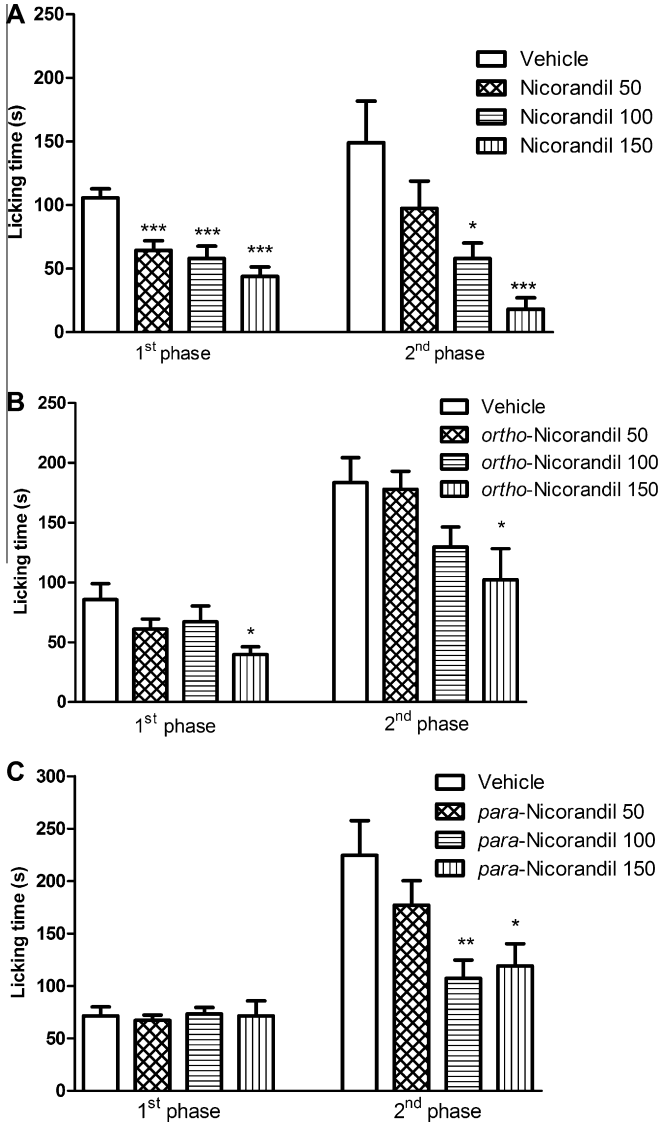


Figure 2. Nociceptive response induced by formaldehyde in mice previously (1 h) treated with different doses (50, 100 or 150 mg/kg, p.o.) of (A): nicorandil (**1**), (B): *ortho*-nicorandil (**2**) or (C): *para*-nicorandil (**3**). *, ** and *** indicate statistically significant difference compared to the vehicle-treated group (P <0.05, P <0.01 and P <0.001, respectively). n = 9.

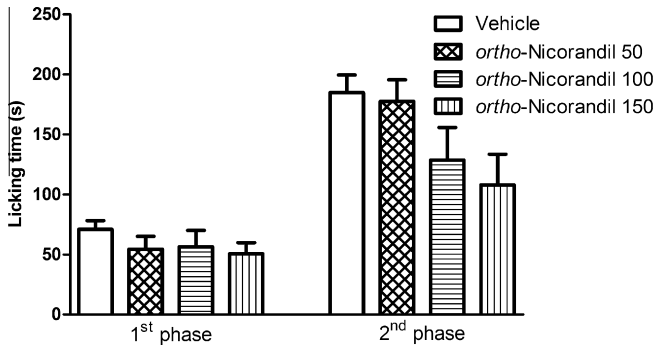


Figure 3. Nociceptive response induced by formaldehyde in mice previously (5 min) treated with *ortho*-nicorandil (**2**) (50, 100 or 150 mg/kg, p.o.) (n = 9).

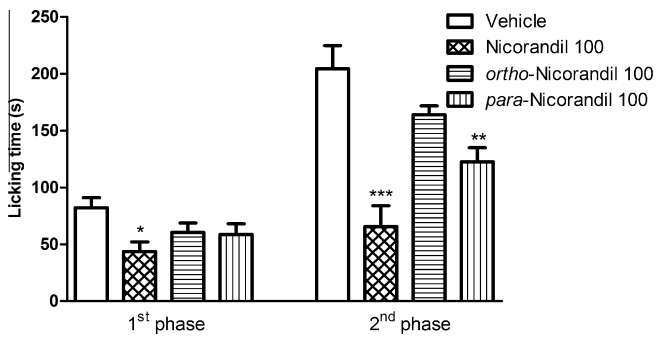


Figure 4. Nociceptive response induced by formaldehyde in mice previously (1 h) treated with nicorandil (**1**) or its isomers *ortho*-nicorandil (**2**) and *para*-nicorandil (**3**) (100 mg/kg, p.o.). *, ** and *** indicate statistically significant difference from vehicle-treated group (P <0.05, P <0.01 and P <0.001, respectively) (n = 9).

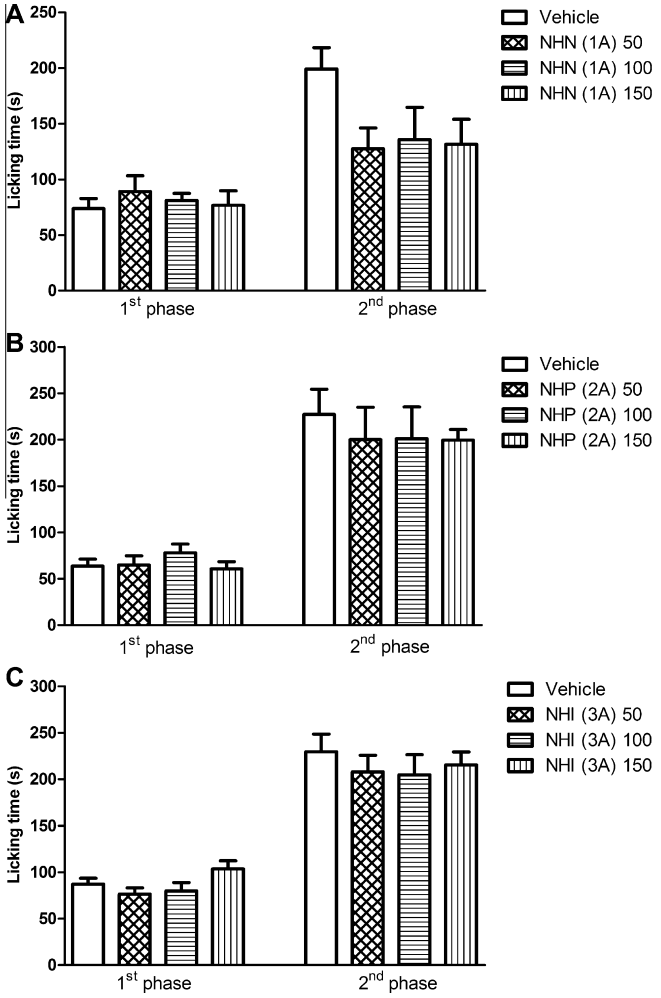


Figure 5. Nociceptive response induced by formaldehyde in mice previously (1 h) treated with (A) NHN (**1A**), (B) NHP (**2A**) or (C) NHI (**3A**) (50, 100 or 150 mg/kg, p.o.) ($n = 9$).

To eliminate motor incoordination or muscle relaxation as possible confounding effects to the antinociceptive activity of nicorandil and its two positional isomers, we evaluated the effect induced by previous (1 h) administration of the three compounds (150 mg/kg, p.o.) on the motor coordination of the animals. None of the compounds altered the time mice spent on the rota-rod apparatus, while phenobarbital (40 mg/kg) markedly reduced this parameter (Table 2).

3.3. Pharmacokinetic profiles of nicorandil and its two positional isomers

Pharmacokinetic parameters of nicorandil (**1**), its two positional isomers and their corresponding metabolites were calculated and

Table 2
Time spent by mice on the rotating rod after previous (1 h) p.o. treatment with nicorandil (**1**), *ortho*-nicorandil (**2**), *para*-nicorandil (**3**) (150 mg/kg) or phenobarbital (40 mg/kg)

Treatment	Time spent on the rotating rod (s)	
	Baseline	1 h
Vehicle	120 ± 0	119 ± 1
Nicorandil (**1**)	120 ± 0	120 ± 0
ortho-Nicorandil (**2**)	120 ± 0	120 ± 0
para-Nicorandil (**3**)	120 ± 0	120 ± 0
Phenobarbital	119 ± 1	26 ± 2[a]

[a] Indicates statistically significant difference compared to the vehicle-treated group ($P < 0.001$, respectively) ($n = 9$).

are shown in Table 1. The plots of the mean plasma concentration over time for all compounds are presented in Figure 6A. Nicorandil and its isomers were rapidly absorbed in the gastrointestinal tract, which was demonstrated by the T_{max} being less than 1 h. Plasma levels were low but still measurable 10 h after administration of all compounds. Nicorandil (**1**) and *para*-nicorandil (**3**) exhibited similar pharmacokinetic profiles, in spite of the greater bioavailability of *para*-nicorandil (**3**) compared to that of nicorandil, as indicated by the area under the curve (AUC) values. *ortho*-Nicorandil (**2**), when compared with the other two isomers, showed a distinct profile, characterized by a faster absorption, as well as a rapid biotransformation. This isomer was also rapidly eliminated from plasma and exhibited the lowest plasma concentration and bioavailability of the three compounds.

Figure 6B shows the plasma concentrations of the metabolites, NHN (**1A**), NHP (**2A**) and NHI (**3A**), after p.o. administration of nicorandil **(1)**, *ortho*-nicorandil (**2**) and *para*-nicorandil (**3**) (100 mg/kg), respectively. The plasma concentration–time curves of the major metabolites of nicorandil **(1)** and *para*-nicorandil (**3**) (**1A** and **3A**, respectively) reflected the metabolism of each parent compound. Similarly to the parent compounds, the metabolites of **1A** and **3A** exhibited similar profiles, with **3A** reaching higher concentrations when compared to **1A**. As expected, the formation of **2A**, the main metabolite of *ortho*-nicorandil (**2**), occurred at a lower extent, reflecting the lower absorption and rapid metabolism of the parent compound. Additionally, the plasma concentrations of nicotinamide did not change markedly after p.o. administration nicorandil or its two positional isomers (100 mg/kg; Fig. 6C).

As NO is known to be a metabolic product of nicorandil (**1**), we indirectly investigated its formation after administration of nicorandil (**1**), *ortho*-nicorandil (**2**) and *para*-nicorandil (**3**) by measuring the plasma concentrations of nitrite (NO_2^-), which is a primary, stable and non-volatile product. The plasma concentrations of nitrite were determined 15 min, 1 and 3 h after the p.o. administration of vehicle (CMC 0.5%, 4 ml/kg), **1**, **2** or **3** (100 mg/kg). Nicorandil (**1**) and *para*-nicorandil (**3**) induced similar increases in the plasma concentrations of nitrite. The highest concentrations were observed 15 min after the administration of the compounds and subsequently declined. On the other hand, the plasma concentrations of nitrite after the administration of *ortho*-nicorandil (**2**) did

Table 1
Pharmacokinetic parameters of nicorandil (**1**), its isomers *ortho*-nicorandil (**2**) and *para*-nicorandil (**3**) and corresponding metabolites (**1A**, **2A** and **3A**) (100 mg/kg) after p.o. administration of (**1**), (**2**) and (**3**) in mice ($n = 6$)

Pharmacokinetic parameters	Nicorandil and isomers			Metabolites		
	1	**3**	**2**	**1A**	**3A**	**2A**
C_{max} (μg/mL)	42.63 ± 9.58	49.44 ± 7.42	30.77 ± 7.24	9.46 ± 1.42	11.80 ± 0.66	2.64 ± 0.58
T_{max} (h)	0.67 ± 0.26	0.88 ± 0.63	0.11 ± 0.07	2.83 ± 0.75	2.00 ± 0.52	0.31 ± 0.23
$AUC_{0\text{-}t}$ (μg h/mL)	121.18 ± 10.63	160.40 ± 23.49	10.11 ± 1.39	41.12 ± 3.22	41.56 ± 2.36	2.69 ± 0.67
$AUC_{0\text{-}inf}$ (μg h/mL)	122.64 ± 10.84	160.44 ± 23.50	10.14 ± 1.40	41.97 ± 3.04	41.69 ± 2.28	2.70 ± 0.66
$t_{1/2}$ (h)	1.5 ± 0.4	0.76 ± 0.10	1.1 ± 0.2	1.4 ± 0.3	1.1 ± 0.4	1.2 ± 0.3

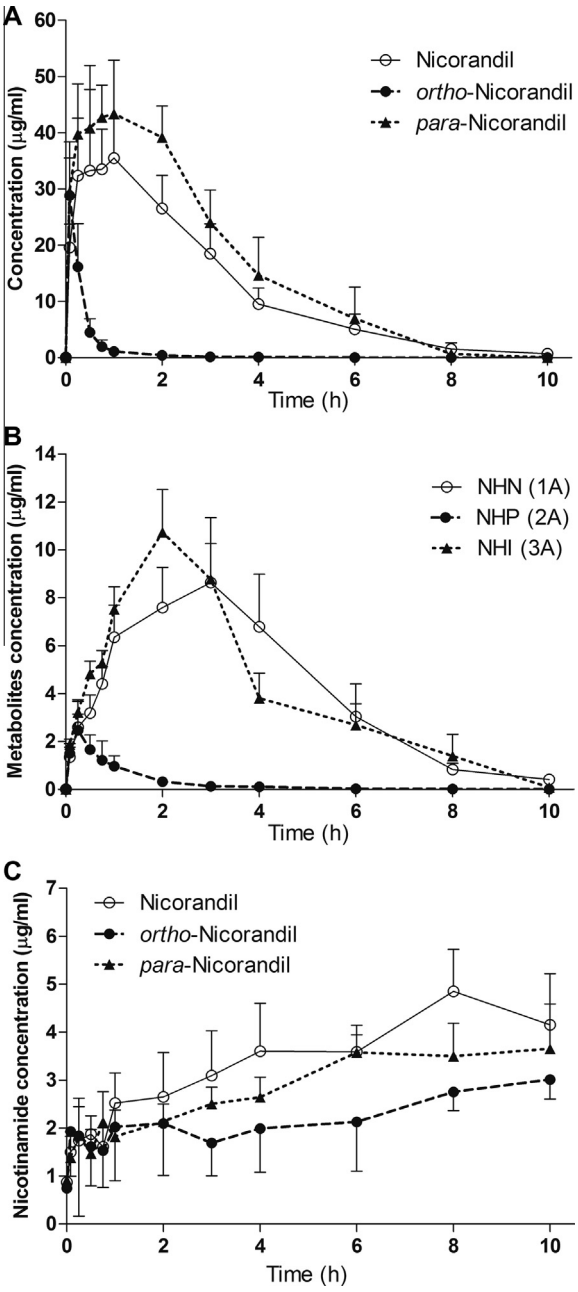


Figure 6. Plasma concentrations of nicorandil (**1**) and its isomers *ortho*-nicorandil (**2**) and *para*-nicorandil (**3**) (A), the corresponding metabolites NHN (**1A**), NHP (**2A**) and NHI (**3A**) (B) or nicotinamide (C) after p.o. administration of **1**, **2** or **3** (100 mg/kg) ($n = 6$).

not statistically differ from those exhibited by the group treated with vehicle (Fig. 7).

4. Discussion

Positional isomerism may markedly affect the pharmacological properties of a given compound.[28–30] In the present study, we investigated the effects induced by the systemic administration of nicorandil and its two positional isomers on the nociceptive response induced by formaldehyde in mice, an experimental model of nociceptive and inflammatory pain. We demonstrated that the three isomers exhibit an antinociceptive activity, inhibiting mainly the second phase of this nociceptive response. These results provide additional support for the antinociceptive activity of

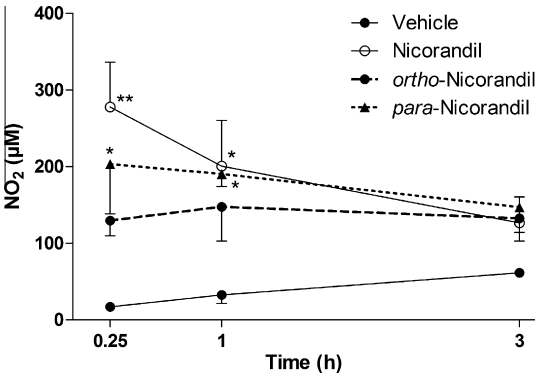


Figure 7. Plasma concentrations of nitrite after p.o. administration of nicorandil (**1**), *ortho*-nicorandil (**2**) or *para*-nicorandil (**3**) (100 mg/kg). * and ** indicate significant difference from vehicle-treated group ($P < 0.05$ and $P < 0.01$, respectively) ($n = 7$).

nicorandil, which we recently demonstrated,[22] and expand this activity to its two positional isomers.

The nociceptive response induced by formaldehyde involves inflammatory, neurogenic and central nervous system components, clearly characterized by a biphasic profile. The first phase begins immediately after the injection of formaldehyde and involves direct activation by the nociceptive stimulus of $TRPA_1$ and $TRPV_1$ channels in the nociceptors.[31,32] The second phase begins 15 to 20 min after the injection of formaldehyde and lasts for 20 to 60 min. This phase is related to an increased excitability of dorsal horn neurons and the production of multiple inflammatory mediators, which lead to the sensitization of nociceptors.[33,34]

Nicorandil (**1**) and its isomers, *ortho*-nicorandil (**2**) and *para*-nicorandil (**3**), inhibited mainly the second phase of the nociceptive response, although to different extents as nicorandil **(1)** exhibited the highest activity, while *ortho*-nicorandil (**2**) was the least active. Regarding the first phase, the compounds induced a less marked effect, with nicorandil **(1)** and *ortho*-nicorandil (**2**) partially inhibiting the response and *para*-nicorandil (**3**) devoid of activity. As the pharmacokinetic data demonstrated that *ortho*-nicorandil (**2**) was immediately absorbed after p.o. administration and rapidly removed from the blood, we investigated whether the reduced activity of this compound during the second phase of the nociceptive response could be related to its peculiar pharmacokinetic profile. However, the administration of this compound immediately before formaldehyde did not significantly inhibit the nociceptive response. Thus, the reduced or absent antinociceptive effect of *ortho*-nicorandil (**2**) cannot be attributed to its possible fast clearance. Rather, the results indicate an intrinsic lack of activity of compound **2** in the pain model used in the present study.

As nicorandil (**1**), *ortho*-nicorandil (**2**) and *para*-nicorandil (**3**) inhibit mainly the second phase of the nociceptive response induced by formaldehyde, their profiles resemble those of anti-inflammatory drugs, such as indomethacin[35] and diclofenac.[36] In addition, it is unlikely that the effects induced by nicorandil **(1)**, *ortho*-nicorandil (**2**) and *para*-nicorandil (**3**) on the nociceptive behavior resulted from a muscle relaxing effect or loss of motor coordination, because these compounds did not reduce the time that the animals spent on the rotating rod.

As previously described,[4] the metabolism of nicorandil initially involves a denitration process, which leads to the formation of **1A** and the release of NO. In the present study, we also observed the quick formation of **1A** after administration of nicorandil **(1)**. The highest concentration of this metabolite was observed 3 h after administration of nicorandil **(1)** and subsequently declined. Similarly, there was also an increase in the plasma concentrations of nitrite, which indirectly indicates the release of NO. After p.o.

administration of the *para* isomer **3**, the formation of the corresponding denitrated metabolite (**3A**) occurred in a similar manner to the formation of **1A** after the administration of nicorandil **(1)**. The highest concentration of **3A**, which had a magnitude similar to that of **1A**, was observed 1 h after the administration of *ortho*-nicorandil (**2**), and subsequently declined. We also observed that parallel to the increase of the plasma concentrations of **3A**, there was an increase of the plasma concentrations of nitrite. These results indicate that moving the side chain with the $-ONO_2$ group to the *para* position results in a compound **3** that retains its ability to release NO. However, *ortho*-nicorandil (**2**) exhibits a different profile when compared to nicorandil **(1)** and *para*-nicorandil (**3**). The highest concentration of the corresponding denitrated metabolite **2A** occurred earlier (0.5 h) when compared to those of **1A** and **3A** and had a much lower magnitude, declining thereafter. As expected, the reduced formation of **2A** was associated with a decreased formation of nitrite. The pharmacokinetic profile of **2A** is very different compared to **1A** and **3A** and indicates that its stability is reduced, which contributes to the lower plasma concentration and rapid clearance. Additionally, we observed that the plasma concentrations of nicotinamide barely changed after the administration of nicorandil or its two positional isomers. Although nicotinamide exhibits an antinociceptive activity,[21] the doses that induce an effect in the model of nociceptive response induced by formaldehyde are much higher than those of nicorandil (**1**). Altogether, these results clearly indicate that the main metabolites of nicorandil (**1**) and its positional isomers are the denitrated compounds and that their effects are not mediated by nicotinamide.

The pharmacokinetic profiles of nicorandil and its two positional isomers provided the rationale to investigate the activity of their respective denitrated metabolites, as they were rapidly formed after the administration of the parent compounds. Therefore, we also investigated the effects induced by equimolar doses of **1A**, **2A** and **3A**, which are the main metabolites of nicorandil **(1)**, *ortho*-nicorandil (**2**) and *para*-nicorandil (**3**), respectively. However, in contrast to the parent compounds, none of the denitrated metabolites exhibited antinociceptive activity in the pain model used in the present study. Among these metabolites, only **1A** has been investigated to date and it has been suggested that it may be a putative contributor to the vasodilator activity of nicorandil.[7]

Parallel to the formation of the denitrated metabolites, there were similar increases in the plasma concentrations of nitrite, an oxidation product of NO, after the administration of nicorandil **(1)**, *ortho*-nicorandil (**2**) and *para*-nicorandil (**3**). The formation of nitrite indirectly indicates the formation of NO, which is associated with the denitration process of the three compounds. NO is rapidly degraded to nitrite and nitrate and measurement of these two metabolites is used to indirectly determine the production of NO.[37,38] As it has been demonstrated that NO-donors induce antinociceptive and anti-hyperalgesic effects in different experimental models of pain,[39–41] it is proposed that the release of NO may mediate the antinociceptive activity of nicorandil (**1**) and its isomers. Curiously, nicorandil and *para*-nicorandil (**3**), compounds that increased the plasma concentrations of nitrite to a greater extent, induced a greater antinociceptive effect when compared to *ortho*-nicorandil (**2**). Altogether, these results indicate that the release of NO is a putative mechanism that may mediate the activity of these compounds.

5. Conclusions

Besides the discovery of new drugs, a promising approach is the assessment of new pharmacological applications for clinically approved drugs, with proved safety and well established toxicological profile. We demonstrated that nicorandil, which is used in many countries as a vasodilator, exhibits a marked antinociceptive activity, increasing the number of NO-releasing compounds that are potential analgesics. In addition, structural changes in drugs with well-established pharmacologic activities may alter their pharmacokinetic or pharmacodynamic properties and improve their therapeutic efficacy. We demonstrated that the *ortho*- (**2**) and *para*-substituted (**3**) isomers of nicorandil (**1**) exhibited distinct pharmacodynamic and pharmacokinetic properties when compared to nicorandil. An important structural feature for the antinociceptive activity seems to be the side chain in the *meta* position of the pyridine ring, since nicorandil was the most effective isomer.

Author contribution

I.C.C., A.M.G., R.R.M., M.O.A., M.M.G.B.D., R.R.M., G.A.P., J.R.A.S., D.A.S. and M.M.C. were responsible for the pharmacological assay. D.P.A., F.C.O. and A.D.F. were responsible for the design, synthesis and characterization of the nicorandil, its analogs and metabolites.

Acknowledgment

We thank the Brazilian agencies Fundação de Amparo à Pesquisa do Estado de Minas Gerais, Conselho Nacional de Desenvolvimento Científico e Tecnológico and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior for financial support. A.D.F. is recipient of research fellowship from CNPq.

Supplementary data

Supplementary data (the NMR spectra for all the synthesized compounds) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2014.03.011.

References and notes

1. Eremenko, L. T.; Nesterenko, D. A.; Garanin, V. A.; Kosilko, V. P. *Russ. J. Appl. Chem.* **2009**, *82*, 1776.
2. The IONA Study Group *Lancet* **2002**, *359*, 1269.
3. Markham, A.; Plosker, G. L.; Goa, K. L. *Drugs* **2000**, *60*, 955.
4. Simpson, D.; Wellington, K. *Drugs* **2004**, *64*, 1941.
5. Taira, N. *Am. J. Cardiol.* **1989**, *63*, J18.
6. Sakai, K.; Saito, K. *Fundam. Clin. Pharmacol.* **1998**, *12*, 492.
7. Sakai, K.; Saito, K. *Fundam. Clin. Pharmacol.* **1999**, *13*, 27.
8. Geiger, J.; Zou, A. P.; Campbell, W. B.; Li, P. L. *Hypertension* **2000**, *35*, 397.
9. Nussler, A. K.; Billiar, T. R. *J. Leukoc. Biol.* **1993**, *54*, 171.
10. Da Silva-Santos, J. E.; Santos-Silva, M. C.; Cunha, F. Q.; Assreuy, J. *J. Pharmacol. Exp. Ther.* **2002**, *300*, 946.
11. Ocaña, M.; Del Pozo, E.; Baeyens, J. M. *Eur. J. Pharmacol.* **1993**, *230*, 203.
12. Kamiyama, Y.; Muto, S.; Masuda, H.; Ide, H.; Ishizuka, N.; Saito, K.; Horie, S. *BJU Int.* **2008**, *101*, 360.
13. Tanabe, K.; Lanaspa, M. A.; Kitagawa, W.; Rivard, C. J.; Miyazaki, M.; Klawitter, J.; Schreiner, G. F.; Saleem, M. A.; Mathieson, P. W.; Makino, H.; Johnson, R. J.; Nakagawa, T. *Am. J. Physiol. Renal Physiol.* **2012**, *302*, F1151.
14. Chowdhury, U. R.; Bahler, C. K.; Hann, C. R.; Chang, M.; Resch, Z. T.; Romero, M. F.; Fautsch, M. P. *Invest. Ophthalmol. Vis. Sci.* **2011**, *52*, 6435.
15. Bhandari, S. V.; Dangre, S. C.; Bothara, K. G.; Patil, A. A.; Sarkate, A. P.; Lokwani, D. K.; Gore, S. T.; Deshmane, B. J.; Raparti, V. T.; Khachane, C. V. *Eur. J. Med. Chem.* **2009**, *44*, 4622.
16. Keeble, J. E.; Moore, P. K. *Br. J. Pharmacol.* **2002**, *137*, 295.
17. Romero-Sandoval, E. A.; Curros-Criado, M. M.; Gaitan, G.; Molina, C.; Herrero, J. F. *CNS Drug Rev.* **2007**, *13*, 279.
18. Asano, T.; Dohi, S.; Iida, H. *Anesth. Analg.* **2000**, *90*, 1146.
19. Rodrigues, A. R.; Duarte, I. D. *Br. J. Pharmacol.* **2000**, *129*, 110.
20. Yamazumi, I.; Okuda, T.; Koga, Y. *Jpn. J. Pharmacol.* **2001**, *87*, 268.
21. Godin, A. M.; Ferreira, W. C.; Rocha, L. T.; Seniuk, J. G.; Paiva, A. L.; Merlo, L. A.; Nascimento, E. B., Jr.; Bastos, L. F.; Coelho, M. M. *Pharmacol. Biochem. Behav.* **2011**, *99*, 782.
22. Dutra, M. M.; Godin, A. M.; César, I. C.; Nascimento, E. B., Jr.; Menezes, R. R.; Ferreira, W. C.; Soares, D. G.; Seniuk, J. G. T.; Araujo, D. P.; Bastos, L. S.; Pianetti, G. A.; Fátima, A.; Machado, R. R.; Coelho, M. M. *Pharmacol. Biochem. Behav.* **2013**, *106*, 85.
23. Paixão, D. A.; Guilardi, S.; Fátima, A.; Araujo, D. P.; Oliveira, F. C. *Acta Crystallogr.* **2011**, *E67*, o2816.

24. Jianyu, J.; Qingyun, H.; Xia, C.; Qinghua, L.; Dongmei, Z. *Chin. J. Med. Chem.* **1990**, *1*, 75.
25. Zimmermann, M. *Pain* **1983**, *16*, 109.
26. Cesar, I. C.; Bastos, L. F.; Godin, A. M.; Coelho, M. M.; Araujo, D. P.; Fatima, A.; Guidine, P. A.; Pianetti, G. A. *J. Mass Spectrom.* **2011**, *46*, 1125.
27. Sun, J.; Zhang, X.; Broderick, M.; Fein, H. *Sensors* **2003**, *3*, 276.
28. Raz, A. *Biochem. Pharmacol.* **2002**, *63*, 343.
29. Kashfi, K.; Borgo, S.; Williams, J. L.; Chen, J.; Gao, J.; Glekas, A.; Benedini, F.; Del Soldato, P.; Rigas, B. *J. Pharmacol. Exp. Ther.* **2005**, *312*, 978.
30. Hua, A.; Mackenzie, G. G.; Rigas, B. *Int. J. Oncol.* **2009**, *35*, 837.
31. McNamara, C. R.; Mandel-Brehm, J.; Bautista, D. M.; Siemens, J.; Deranian, K. L.; Zhao, M.; Hayward, N. J.; Chong, J. A.; Julius, D.; Moran, M. M.; Fanger, C. M. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 13525.
32. Tian, L. J.; Du, Y. R.; Xiao, Y.; Lv, Z. M.; Yu, Y. Q.; Cui, X. Y.; Chen, J. *Acta Physiol. Sin.* **2009**, *61*, 404.
33. Hunskaar, S.; Hole, K. *Pain* **1987**, *30*, 103.
34. Tjolsen, A.; Berge, O. G.; Hunskaar, S.; Rosland, J. H.; Hole, K. *Pain* **1992**, *51*, 5.
35. Morgan, C. V.; Babbedge, R. C.; Gaffen, Z.; Wallace, P.; Hart, S. L.; Moore, P. K. *Br. J. Pharmacol.* **1992**, *106*, 493.
36. Leon-Reyes, M. R.; Castaneda-Hernandez, G.; Ortiz, M. I. *J. Pharm. Pharm. Sci.* **2009**, *12*, 280.
37. Bush, P. A.; Gonzalez, N. E.; Griscavage, J. M.; Ignarro, L. J. *Biochem. Biophys. Res. Commun.* **1992**, *185*, 960.
38. Moriel, E. Z.; Gonzalez-Cadavid, N.; Ignarro, L. J.; Byrns, R.; Rajfer, J. *Urology* **1993**, *42*, 551.
39. Duarte, I. D.; Lorenzetti, B. B.; Ferreira, S. H. *Eur. J. Pharmacol.* **1990**, *186*, 289.
40. Soares, A. C.; Leite, R.; Tatsuo, M. A.; Duarte, I. D. *Eur. J. Pharmacol.* **2000**, *400*, 67.
41. Sousa, A. M.; Prado, W. A. *Brain Res.* **2001**, *897*, 9.