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New pyrazole derivative 5-[1-(4-fluorphenyl) -1H-pyrazol-4-yl]-2H-tetrazole: Synthesis and assessment of some biological activities

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Highlights

Given pyrazole compounds' antipyretic, analgesic, and anti-inflammatory effects, we describe the synthesis and pharmacotoxicological evaluation of a new pyrazole derivative, 5-[1-(4-fluorphenyl)-1H-pyrazol-4-yl]-2H-tetrazole, as well as the antinociceptive, anti-inflammatory activity and involvement of the NO/cGMP pathway and ionic channels in its vasorelaxant effect. The blockade of voltage-dependent calcium channels could have also contributed to the vasorelaxant effect of the compound, which demonstrated a low toxicological profile promising antinociceptive, anti-inflammatory, and vasorelaxant effects.

Abstract

The molecular modification and synthesis of compounds is vital to discovering drugs with desirable pharmacological and toxicity profiles. In response to pyrazole compounds' antipyretic, analgesic, and anti-inflammatory effects, this study sought to evaluate the analgesic, anti-inflammatory, and vasorelaxant effects, as well as the mechanisms of action, of a new pyrazole derivative, 5-[1-(4-fluorphenyl)-1*H*-pyrazol-4-yl]-2*H*-tetrazole (FPPT). During the acetic acid-induced abdominal writhing test, treatments with FPPT reduced abdominal writhing, while during the formalin test, FPPT reduced licking times in response to both neurogenic pain and inflammatory pain, all without demonstrating any antinociceptive effects, as revealed during the tail flick test. FPPT also reduced carrageenan-induced paw edema and cell migration during the carrageenan-induced pleurisy test. As demonstrated by the model of the isolated organ, FPPT exhibits a vasorelaxant effect attenuated by Nω-nitro-L-arginine methyl ester, 1H-[1,2, 4]oxadiazolo[4,3-alpha]quinoxalin-1-one, tetraethylammonium or glibenclamide. FPPT also blocked $CaCl_2$-induced contraction in a dose-dependent manner. Suggesting a safe toxicity profile, FPPT reduced the viability of 3T3 cells at higher concentrations and was orally tolerated, despite signs of toxicity in doses of 2,000 mg/kg. Lastly, the compound's analgesic activity might be attributed to the involvement of the NO/cGMP pathway and K^+ channels observed in the vasorelaxant effect.

Keywords: Pyrazole derivatives, antinociceptive effect, anti-inflammatory effect, vasorelaxant effect, NO/cGMP pathway

Introduction

A natural physiological response to the removal of harmful agents and repair of lesioned tissues (1,2), inflammation is often accompanied by pain, typically as an adaptive alert mechanism that triggers appropriate protective responses to real or imminent injury (3). However, at a certain point, both pain and inflammation no longer serve the purpose of alerting and protecting the organism, yet continue to significantly affect the lifestyles of millions of people worldwide (4–6). Worse still, treating those symptoms with analgesics and anti-inflammatory drugs can cause side effects, primarily in the gastrointestinal tract. In response, developing drugs with strong therapeutic effects and weak side effects (7,8) remains crucial.

The pyrazole compound is a synthetic heterocycle with a five-membered ring, which includes two adjacent nitrogen atoms, three carbon atoms, and two double bonds (9,10). Since compounds with that chemical structure have demonstrated antimicrobial (11), antidepressant, anticonvulsant (12), antipyretic (13), analgesic, and anti-inflammatory effects (14,15), the molecular modification and synthesis of pyrazole derivatives could yield drug prototypes with desirable pharmacological aspects and minimal toxicity. To that end, the pyrazole derivative 5-[1-(3-fluorophenyl)-1*H*-pyrazol-4-yl]-2*H*-tetrazole (FPPT) was designed and synthesized from the commercially available pyrazole derivatives milrinone (**1**) and cilostazol (**2**).

Martins et al. (2013) (16) recently demonstrated the endothelium-independent relaxation of vascular smooth muscle 5-(1-(3-fluorophenyl)-1*H*-pyrazol-4)-2*H*-tetrazola (LQFM021), an analog of FPPT, which involved the activity of K^+ and Ca^{2+} channels, AC/cAMP, and GC/cGMP. In support, other studies have shown that the L-arginine/NO/cGMP pathway and K^+ and Ca^{2+} channels are involved in the mechanisms of antinociceptive activity in drugs such as morphine, dipyrone, and diclofenac (17–20). Opioids, for example, can inhibit neuronal activity by inhibiting voltage-dependent Ca^{2+} channels in neurons of the dorsal root ganglion and by activating K^+ channel rectifiers in postsynaptic neurons in the spinal cord, thereby promoting an antinociceptive effect (20, 21).

Considering the analgesic and anti-inflammatory activity of pyrazole compounds associated with ion channels, the aim of our study was to evaluate the antinociceptive, anti-inflammatory, and vasorelaxant effects of the new pyrazole derivative FPPT and, more particularly, investigate the involvement of the NO/cGMP/ pathway and both K^+ and Ca^{2+} channels in its vasorelaxant effect. We describe the synthesis and pharmacotoxicological evaluation of the new FPPT (**4**) heterocyclic derivative, originally designed as regioisomers from the LQFM021 (**3**) compound described by Martins et al. (2013) (16) and Florentino et al. (2015) (15), as illustrated in Figure 1A.

Material and Methods

Analytical instruments

All melting points were determined with a Marte 284594 apparatus. Nuclear magnetic resonance (NMR) data were recorded using a spectrometer (AVANCE III, Bruker, Atibaia-SP, Brazil) operating at 500.13 MHz proton frequency. Samples for NMR measurements were prepared in $CDCl_3$ containing 1% tetramethylsilane (TMS) as an internal standard. Splitting patterns were d (doublet) and ddd (double double doublet). Infrared (IR) spectra were obtained with a Fourier transform (FT)-IR instrument (Spectrum 400N, Perkin–Elmer, Waltham, USA) in KBr plates. Microanalytical data were obtained with a spectrometric system (Spectrum BXII FT-IR, Perkin–Elmer) using a digital analytical scale (Gehaka, São Paulo, Brazil), whereas mass spectra were obtained with a mass spectrometer (Q-Exactive, Thermo Fisher Scientific, Bremen, Germany). The sample preparation for mass spectrometric analysis consisted of diluting 1 mg of each sample in 1 mL of methanol with 0.1% formic acid. The solution obtained was directly infused at a flow rate of 2 μL/min into the electrospray ionization source, whose conditions were a nebulizer gas pressure of 0.5–1.0 bar, a capillary voltage of 3.0 kV, and capillary transfer temperature of 250 °C. The progress of all reactions was monitored by thin-layer chromatography performed on 2.0 × 6.0-cm aluminium sheets precoated with silica gel 60 (Merck, Barueri-SP, Brazil) to a thickness of 0.25 mm. The developed chromatograms were viewed under ultraviolet light (254–265 nm) and treated with iodine vapor. For column chromatography, we used silica gel 70–230 mesh (Merck). Reagents and solvents were purchased from commercial suppliers.

Animals

Experiments were performed using female and male Swiss albino mice (25–30 g) and male Wistar rats (200–250 g) from the Central Animal House of the Federal University of Goiás, Goiânia, Brazil. Animals were kept in plastic cages at 22 ± 2 °C under a 12-h light–dark cycle with free access to pellet food and water in compliance with the International Guiding Principles for Biomedical Research Involving Animals. Animals were acclimatized for 7 d before experiments commenced. All experimental protocols were developed according to the principles of ethics and animal welfare designated by the Ethics Committee on Animal Experimentation. Experimental protocols were approved by the ethics committee of Federal University of Goiás (no. 137/2009, 017 and 020/2013). After each experiment, animals were anesthetized with the mixture of xylazine (10 mg/kg) and ketamine hydrochloride (100 mg/kg) administered intraperitoneally, prior to being euthanized by cervical dislocation (22).

Chemicals

Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, trypsin, ethylenediaminetetraacetic acid (EDTA), neutral red, carrageenan, dimethyl sulfoxide (DMSO), phenylephrine (PhE), acetylcholine (ACh), sodium nitroprusside, Nω-nitro-L-arginine methyl ester (L-NAME), tetraethylammonium (TEA), and 1H-[1,2, 4]oxadiazolo[4,3-alpha]quinoxalin-1-one (ODQ) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Acetic acid, ethanol, and glacial acetic acid were acquired from Vetec (Rio de Janeiro, RJ, Brazil), whereas naloxone chloridrate (Narcan®) and morphine (Dimorf®) were obtained from Cristalia (São Paulo, SP, Brazil). Dexamethasone and indomethacin were purchased from Prodome (Campinas, SP, Brazil), xylazine from Syntec (Cotia, SP, Brazil), and ketamine from König (Santana de Parnaíba, SP, Brazil). Acetone, formaldehyde, heparin, NaCl, and Türk solution were obtained from Isofar (Duque de Caxias, RJ, Brazil), Synth (Diadema, SP, Brazil), Hipolabor (Belo Horizonte, MG, Brazil), New Prov (Pinhais, PR, Brazil), respectively.

Syntheses

Synthesis of 1-(3-Fluorophenyl)-1H-pyrazole-4-carbonitrile (9)

To a heterogeneous mixture of $NH_2OH{\cdot}HCl$ (1.3 mmol) and NaI (4 mmol) in dimethylformamide (DMF) (4 mL), 1-(4-fluorophenyl)-1*H*-pyrazole-4-carbaldehyde (**8**) (1 mmol) was added and stirred at room temperature. The reaction mixture was heated at reflux temperature for 6 h and then cooled by pouring ice. The precipitate was vacuum filtered and dried, and the crude product was purified by chromatography using $CHCl_3$ as a mobile phase to provide 1-(4-fluorophenyl)-1*H*-pyrazole-4-carbonitrile (**9**) as a beige solid in an 84% yield, as Figure 2A shows, characterized as mp 162 °C, *Rf* = 0.65 (hexane:ethyl acetate, 7:3): IRmax (KBr) cm^{-1}: 3,132 (*v* C–H), 2,233 (*v* C–N), and 838 (*v* 1,3-C–F); ^{1}H NMR (500.13 MHz) $CDCl_3$ *δ*: 8.26 (1H, d, *J* = 0.6 Hz, H-5), 7.98 (1H, d, *J* = 0.6 Hz, H-3), 7.65 (1H, ddd, *J* = 9.3, 4.6, and 3.4 Hz, H-2'), 7.65 (1H, ddd, *J* = 10.3, 4.6, and 3.4 Hz, H-6'), 7.20 (1H, ddd, *J* = *9*.3, 8.3, and 3.4 Hz, H-3'), 7.20 (1H, ddd, *J* = 10.3, 9.3, and 3.4 Hz, H-5'); ^{13}C NMR (125.76 MHz) $CDCl_3$ *δ*: 162.0 (C-4'),143.3 (C-3), 135.0 (C-1'), 132.0 (C-5), 121.9 (C-2' and 6'), 116.7 (C-3' and 5'),112.8 (C–CN), 94.6 (C-4), and $[M+H]^+$ *m/z* of 188.06166 (error = 1.0 ppm) .

Synthesis of 5-(1-(4-Fluorophenyl)-1H-pyrazol-4-yl)-2Htetrazole (4)

A mixture of 1-(4-fluorophenyl)-1*H*-pyrazole-4-carbonitrile (**9**) (2.0 g, 12.4 mmol), sodium azide (4.1 g,62 mmol), and ammonium chloride (3.35 g, 62 mmol) in 35 mL of DMF was heated at reflux temperature for 72 h. The reaction mixture was then poured into water and acidified to pH 5. The product was vacuum filtered and dried to provide 5-(1-(4-fluorophenyl)-1*H*-pyrazol-4-yl)-2*H*-tetrazole (**4**) as a beige solid in a 98% yield; as Figure 2B shows, it was characterized as having mp > 227 °C, *Rf* = 0.82 (ethyl ether:EtOH, 7:3): IRmax (KBr) cm^{-1}:3,135 (*v* C–H), 1,635 (*v* C=C), 1,212 (*v* C–F), 838 (*v* 1,4-C–F); ^{1}H NMR (500.13 MHz) DMSO-*d*6 *δ*: 9.09 (1H, d, *J* = 0.5 Hz, H-5), 8.28 (1H, d, *J* = 0.5 Hz, H-3), 7.93 (1H, ddd, *J* = 9.1, 4.7, and 3.5 Hz,H-2'), 7.94 (1H, ddd, *J*= =.5, 4.7, and 3.5 Hz, H-6'), 7.38 (1H, ddd, *J = 9*.1, 8.5, and 3.5 Hz, H-3'), 7.38 (1H, ddd, *J* = 10.5, 9.1, and 3.5 Hz, H-5'); ^{13}C NMR (125.76 MHz) DMSO-*d*6 *δ*: 160.7 (C-4'), 149.3 (C-1"), 139.5 (C-3), 135.5 (C-1'), 127.8 (C-5), 121.1 (C-2' and 6'), 116.7 (C-3' and 5'), 108.6 (C-4), and $[M+ H]^{+}$ of *m*/*z* 231.07854 (error = 1.5 ppm).

Antinociceptive activity

Acetic acid-induced abdominal writhing

Experimental groups of mice (*n* = 9) were treated orally (p.o.) with vehicle (10 mL/kg), FPPT (9, 18, or 36 mg/kg p.o.), or indomethacin (10 mg/kg) 60 min prior to the administration of 1.2% acetic acid solution (10 mL/kg, i.p.). The number of writhes produced in each group during the 30-min test was counted and expressed as *M* ± *SEM* (23,24).

Formalin-induced pain

Experimental groups of mice (*n* = 8) were treated with vehicle (10 mL/kg, p.o.), FPPT (36 mg/kg p.o.), indomethacin (10 mg/kg, p.o. - positive control for antinociceptive activity in the second phase) or morphine (5 mg/kg, s.c. - positive control for antinociceptive activity in the first and second phases). Either 60 min after p.o. treatment or 30 min after s.c. treatment, 20 µL of 3% formalin (in saline) was administrated into the plantar surface of the right hind paw. After being injected with the phlogistic agent, the mice were placed in an acrylic box over a mirror to enable unhindered observation of the formalin-injected paw for 30 min. Pain reaction time (i.e., licking time) was assessed during two phases: 0–5 min for neurogenic pain and 15–30 min for inflammatory pain, as described by Hunskaar and Hole (25). Results were expressed in seconds as *M* ± *SEM*.

Tail flick test

Performed as described by D'Amour et al. (1941) (26), the tail flick test gauges time taken to flick the tail (i.e., latency) during exposure to a heat source using an analgesimeter (Insight®, Ribeirão Preto, Brazil). Animals were divided into three experimental groups (n = 10): the vehicle-treated group (10 mL/kg, p.o.), the FPPT-treated group (36 mg/kg, p.o.), and the morphine-treated group (5.0 mg/kg, s.c. - positive control for antinociceptive activity). Latent reaction to pain was measured at -30, 0, 30, 60, 90, 120, 150, and 180 min of treatment with a cutoff set at 15 s. Results at all times were expressed in seconds as $M \pm SEM$.

Anti-inflammatory activity

Carrageenan-induced paw edema

Acute anti-inflammatory effect was evaluated by carrageenan-induced rat paw edema per the method of Passos et al. (2007) (27). Experimental groups of mice (n = 9) were treated orally with vehicle (10 mL/kg), FPPT (36 mg/kg), or indomethacin (10 mg/kg) 1 h before intraplantar injection of carrageenan 1% in the right paw. The left paw was used as a control and received saline (NaCl 0.9%) injection in the same volume. The edema was measured as the difference in the volume between the paws using a plethysmometer (Ugo Basile Co., Monvalle, Italy) at intervals of 0, 1, 2, 3, and 4 h following injection with the phlogistic agent.

Carrageenan-induced pleurisy

Groups of mice (n = 9) were treated orally with vehicle (10 mL/kg), FPPT (36 mg/kg), or dexamethasone (2 mg/kg, p.o). Roughly 1 h after treatment, the animals received an injection of 100 µL 1% carrageenan into the pleural cavity, and 4 h after the phlogistic agent was administered, the pleural exudate was collected with 1 mL of heparinized phosphate buffered saline (PBS). One aliquot was used to count the total number of leukocytes using Türk's solution in a Neubauer chamber (28,29).

Preparation of rat aortic rings

Rats' aortic rings free of fat and adherent connective tissues were cut into ring segments 4 mm long and suspended in 10-mL organ baths filled with modified Krebs–Henseleit solution (NaCl, 130 mM; $NaHCO_3$, 14.9 Mm; KCl, 4.7 mM; KH_2PO_4, 1.18 mM; $MgSO_4 \cdot 7H_2O$, 1.17 mM; $CaCl_2 \cdot 2H_2O$, 1.6 mM; glucose, 5.5 mM at pH 7.4). The preparation was maintained with continuous supply of 5% CO_2

and 95% O_2 mixture at 37 °C. The rings were passively stretched to a resting tension of 1.5 g and allowed to achieve equilibrium for 60 min; the bath fluid was changed every 15 min. Alterations in tension baseline were recorded by isometric transducers connected to a data acquisition system (World Precision Instruments, Sarasota, FL, USA). Endothelia were removed mechanically by gently rubbing a wet cotton swab through the luminal surface of the rings. Removed endothelia were examined by vascular contraction and relaxation response to phenylephrine (PhE, 10−6 M) and acetylcholine (ACH, 10−5 M), respectively (30).

Effects of FPPT on nitric oxide pathways, prostaglandins, and ion channels

The vascular activity of FPPT was assessed prior to the incubation of the endothelium-intact rings with 100 µM L-NAME as the nitric oxide synthase inhibitor, 10 µM ODQ as the soluble guanylylcyclase inhibitor, 10 µM indomethacin as the COX inhibitor, 1 mM TEA as the nonspecific blocker of the voltage-dependent K^+ channel, or 10 µM GB as the blocker of adenosine triphosphate (ATP)-sensitive K^+ channels. After an incubation period of 30 min, aortas were contracted with 0.1 µM PhE prior to the construction of a concentration-response curve to FPPT. Results were expressed as percentage of the relaxation of the vascular contraction due to PhE. Endothelium-denuded rings were then incubated in Ca^{2+}-free high K^+ (60 mM) depolarizing Krebs–Henseleit solution. A cumulative concentration-response curve to $CaCl_2$ (10–100 µM) was evaluated in the presence of vehicle or FPPT. Results were expressed as percentages of the maximal response to KCl (120 mM).

Toxicological evaluation of FPPT

Cytotoxicity on BALB/c 3T3 cells

BALB/c 3T3-A31 fibroblasts were kindly donated by Dr. Mari Cleide Sogayar (Chemistry Institute, São Paulo University, SP, Brazil). The cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 100 mg/mL streptomycin and routinely grown as a monolayer in 75-cm^2 tissue culture flasks in a humidified atmosphere of 5% CO_2 in the air at 37 °C. Cell cultures were removed from the flasks using trypsinization (trypsin:EDTA solution, 0.025%:0.02%) when cells exceeded 50% confluence but had not reached 80% confluence (31).

To evaluate the cytotoxicity of FPPT on 3T3 cells, the neutral red uptake assay was performed as described by ICCVAM (2006) (32). Briefly, 3T3 fibroblasts (3×10^3 cells/well) were seeded in 96-well plates and incubated overnight. Afterward, cells were treated with eight different

concentrations of FPPT (0.01–1.4 mM) in complete medium and incubated for 48 h. Wells without cells received the complete culture medium with or without FPPT. After the exposure period, the supernatant was removed, and the cells were washed with 250 µL/well of prewarmed PBS followed by the addition of 250 µL of NR medium in each well. After 3 h of incubation, the NR medium was removed, the cells were carefully rinsed with 250 µL/well of prewarmed PBS, and 100 µL of NR desorption solution (50 ethanol:1 acetic acid:49 ultrapure water) was added to each well. Absorbance was measured at 550 nm. IC_{50} (mM) value—that is, the concentration that inhibited cell growth by 50% compared to the untreated group—was used to estimate the LD_{50} (mg/kg), or the lethal dose that causes death in 50% of animals, based on the equation of an established model (31)—namely, $\log (LD_{50}) = 0.545 \times \log (IC_{50}) + 0.757$.

Analysis of acute oral systemic toxicity in mice

The acute oral toxicity of FPPT was assessed by following OECD Guideline 423: Acute Toxic Class Method (2001) (33). Briefly, male and female mice (n = 3 per sex) were treated orally by gavage with a single dose (0.2 mL/mouse) of 2,000 mg/kg FPPT. Animals were observed during the first 10 min, 30 min, 1 h, 2 h, 4 h, 6 h, 12 h, and 24 h and daily thereafter through Day 14 post-treatment. Hippocratic screening was performed to investigate the animals' vocalizations, irritability, touch response, response to tail tightening, righting reflex, body tone, tremors, convulsions, straub, piloerection, cyanosis, salivation, and death. The intensities of the events were tabulated on a 5-point scale (i.e., *absent*, *minimal*, *little*, *moderate*, and *intense*). The safety of LD_{50} obtained was subsequently confirmed as recommended by OECD guidelines (33). The animals were euthanized 14 d after the experiments, at which point their abdominal cavities were opened to observe any macroscopic alterations.

Statistical analysis

Results are expressed as $M \pm SE$. Differences between two means were detected using Student's t test, whereas differences among more than two means were detected using one-way analysis of variance (ANOVA) with a post hoc Student–Newman–Keuls's test or two-way ANOVA with Bonferroni's post hoc test. Results were considered to be significant when $p < .05$ (34).

From data gathered during nociceptive and inflammatory tests, we calculated the percentage of the effect of FPPT or positive control in relation to control group values. The mean value of the control group was considered to be absolute, and differences in mean values were identified in other groups as a percentage relative to control group's mean value.

Results

Synthesis of FPPT (4)

As illustrated in Figure 1B, the synthetic route began with 1-(4-fluorophenyl)-1*H*-pyrazole (**5**) and proceeded through the classical method described by Finar and Godfrey (1954) (35). Chemoselective and regiospecific formylation of 1-(4-fluorophenyl)-1*H*-pyrazole (**7**) to 1-(4-fluorophenyl)-1*H*pyrazole-4-carbaldehyde (**8**) was performed under Duff's conditions (36). The synthesis of 1-(4-fluorophenyl)-1*H*-pyrazole-4-carbonitrile (**9**) was conducted via oxime formation with the reaction of 1-(4-fluorophenyl)-1*H*-pyrazole-4-carbaldehyde (**8**) with hydroxylamine, followed by in situ dehydration in the presence of sodium iodide and DMF at reflux temperature for 6 h to produce (**9**) a 90% yield (37). FPPT (**4**) was synthetized through a 1,3-bipolar cycloaddition between 1-(3-fluorophenyl)-1*H*-pyrazole-4-carbonitrile (**9**) and NaN_3 using NH_4Cl as the catalyst in DMF at reflux temperature for 48 h in a 98% yield (38). FPPT (**4**) was obtained in a 55% overall yield.

Analgesic activity

Acetic acid-induced abdominal writhing

In the acetic acid-induced abdominal writhing test, FPPT (9, 18, or 36 mg/kg, p.o.) reduced the number of writhes to 53.29 ± 4.8 in a dose-dependent manner (40% reduction, $p < .001$), 48.86 ± 1.6 (45% reduction, $p < .001$), and 35.86 ± 1.4 (60% reduction, $p < .001$), respectively (ANOVA: $F_{(4–35)} = 33.34$, $p < .0001$). The positive control indomethacin reduced the number of writhes to 51 ± 4.6 (42.6% reduction, $p < .001$) compared to the control group (number of writhes 88.9 ± 2.2) (Figure 3A).

Formalin-induced pain

In the formalin test, oral treatment with FPPT (36 mg/kg) showed antinociceptive activity compared with the control group in the first (ANOVA: $F_{(3,29)} = 44.16$, $p < .0001$) and second phases (ANOVA: $F_{(3,29)} = 42.17$, $p < .0001$) of the test. In the first phase, FPPT significantly reduced the licking time to 52.21 ± 2.8 s (30.9% reduction, $p < .01$) compared with the control group (licking time 75.57 ± 3.2 s), while in the second phase, the licking time was reduced to 76.29 ± 9.8 s (47% reduction, $p < .001$) compared with the control group (licking time 144 ± 6.7 s). In the group treated with morphine, licking time decreased significantly during both phases, to 6.63 ± 2.4 s (91.2% reduction, $p < .001$) and 2.07 ± 2.1 s (98.6% reduction, $p < .001$), respectively. Treatment with indomethacin

reduced only the second phase of the test to 83.22 ± 11.3 s (42.2% reduction, $p < .001$), as Figure 3B shows.

Tail flick test

Tail flick test results revealed that oral treatment with FPPT (36 mg/kg) did not elicit any significant antinociceptive activity compared to the group vehicle (two-way ANOVA, treatment factor: $F_{(2,210)} = 191.9$, $p > .05$). Under similar conditions, subcutaneous treatment with morphine (5 mg/kg, s.c. – opioid agonist) demonstrated significant antinociception at 30, 60, 90, 120, and 150 min post-treatment (data not shown).

Anti-inflammatory activity

Carrageenan-induced paw edema

The treatments with FPPT (36 mg/kg, p.o.) reduced the carrageenan-induced paw edema in a dose-dependent manner throughout the test period (two-way ANOVA, treatment factor: $F_{(2,135)} = 112.6$, $p < .0001$). FPPT showed a significant anti-edematogenic effect by reducing the edema to 106 ± 4.3 (19.7% reduction, $p < .001$), 106 ± 3.7 (25.9% reduction, $p < .001$), 102 ± 4.2 (25.5% reduction, $p < .001$), and 91 ± 3.8 µL (27.2% reduction, $p < .001$) at 1, 2, 3 and 4 h, respectively, compared with the control group (difference between paws: 132 ± 5.5, 143 ± 5.2, 137 ± 5.2, and 125 ± 2.7 µL, respectively). The group treated with indomethacin (10 mg/kg, p.o.), an anti-inflammatory positive control, exhibited decreased carrageenan-induced paw edema at 1, 2, 3, and 4 h post-treatment, as Figure 4A illustrates.

Carrageenan-induced pleurisy

In the carrageenan-induced pleurisy test, treatment with FPPT at a dose of 36 mg/kg (p.o.) reduced cell migration (ANOVA: $F_{(2,29)} = 36.15$, $p < .0001$). The number of leukocytes × 10^6 /mL dropped to 3.5 ± 0.3 (36.4% reduction, $p < .001$) compared with the group treated with the vehicle (5.5 ± 0.3 leukocytes × 10^6/mL). The group treated with dexamethasone (2 mg/kg) also demonstrated fewer leukocytes × 10^6 /mL to 1.9 ± 0.3 (65.5% reduction, $p < .001$) than the control group (Figure 4B).

Effects of FPPT on nitric oxide pathway, prostaglandin and ion channels

FPPT induced a concentration-dependent (1 μM–1 mM) vasodilation effect (maximal effect [Emax]: 93.29 ± 2.05) in rats' thoracic aortic rings with endothelia. The compound also induced a concentration-dependent (1 μM–1 mM) relaxation effect (Emax: 91.86 ± 2.27) in endothelium-denuded aortic rings (Figure 5), (two-way ANOVA, vessel factor: $F(3, 131) = 129.8$, $p < .0001$; FPPT concentration factor: $F(6, 131) = 126.1$, $p < .0001$). As Figure 6A shows, the incubation of each aortic ring with L-NAME or ODQ inhibited FPPT-induced vascular relaxation (two-way ANOVA, antagonist factor: $F(2, 105) = 66.56$, $p < .0001$; FPPT concentration factor: $F(6, 105) = 166.66$, $p < .0001$). Moreover, incubation of the aorta with TEA (Figure 6B) also inhibited FPPT's relaxant effect (two-way ANOVA, antagonist factor: $F(1, 54) = 27.36$, $p < .0001$; FPPT concentration factor: $F(6, 54) = 85.38$, $p < .0001$), as did GB in Figure 6C (two-way ANOVA, antagonist factor: $F(1, 56) = 37.76$, $p < .0001$; FPPT concentration factor: $F(6, 56) = 160.7$, $p < .0001$) or indomethacin in Figure 6D (two-way ANOVA, antagonist factor: $F(1, 77) = 21.78$, $p < .0001$; FPPT concentration factor: $F(6, 77) = 162.6$, $p < .0001$). Contractile response to $CaCl_2$ was blocked in a concentration-dependent manner by FPPT, as Figure 7 illustrates (two-way ANOVA, FPPT concentration factor: $F(3,160) = 55.77$, $p < .0001$; $CaCl_2$ concentration factor: $F(9,160) = 69.02$, $p < .0001$).

Evaluation of cytotoxicity on BALB/c 3T3 cells

After 48 h of exposure of FPPT, 3T3 cells showed less viability at high concentrations (0.35–1.4 mM), yielding an IC_{50} value of 0.302 mM, from which LD_{50} was estimated in 685 mg/kg as the initial dose of FPPT to be administered to mice for in vivo acute oral toxicity testing.

Analysis of acute oral sytemic toxicity in mice

Following exposure to a single FPPT dose of 2,000 mg/kg, both male and female mice showed moderate body tremors and mild ptosis in the first 30 min. Until the second hour of observation, the animals remained slightly lethargic, shrunken, and contracted in terms of body tone, with slightly bristled hair. Afterward, no signs of toxicity were observed, nor was any mortality, during the 14 d of study. Necropsy showed no macroscopic alterations in the organs of mice. As such, FPPT was classified to be in Category 5 of the Harmonized Classification System for Chemical Substances and Mixtures (GSH) and LD_{50} determined to be 2,000 mg/kg > LD_{50} < 5,000 mg/kg.

Discussion

Previous reports have shown that pyrazole derivatives constitute an important class of chemical compounds with a broad spectrum of biological activities, including analgesic, anti-inflammatory (39,40), antipyretic (13), antimicrobial (11), vasorelaxant (16), antidepressant, and anticonvulsant effects (12). With that knowledge, we performed classical *in vivo* and *in vitro* assays to evaluate the new pyrazole derivative FPPT's antinociceptive, anti-inflammatory, and vasorelaxant effects, as well as the involvement of the NO/cGMP pathway and both K^+ and Ca^{2+} channels in its vasorelaxant effect. We also performed a toxicological analysis of FFPT.

The acetic acid-induced writhing test was performed in mice to evaluate any analgesic effect. Local irritation provoked by intraperitoneal injection of acetic acid triggers the liberation of various chemical mediators, including bradykinin, substance P, prostaglandins, and cytokines (e.g., IL-1β, TNF-α, and IL-8) (41). In that model, the oral administration of FPPT (9, 18, or 36 mg/kg) reduced the number of writhes in a dose-dependent manner. Despite the satisfactory sensitivity of the model to antinociceptive agents, the suppression of abdominal writhing response by muscle relaxants, antihistamines, neuroleptics, and other drugs made acetic acid-induced writhing an unspecific animal model of nociception (42).

To confirm the analgesic effect suggested in the writhing test, we also performed a formalin test that identified two distinct phases of pain (43). The first phase, known as the neurogenic phase, starts immediately after the intraplantar injection of formalin and is characterized by direct stimulation of nociceptive fibers and the release of preformed mediators such as serotonin, substance P, kinins, histamine, and calcitonin gene-related peptide (25,43,44), whereas the second phase, or the persistent inflammatory phase, is associated with the formation or release of inflammatory mediators such as cytokines, eicosanoids, and kinins (25,43,45). The time that animals spent licking their paws (i.e., reactivity time to formalin-induced pain) in both phases of the test decreased in the group of mice treated with FPPT in a dose of 36 mg/kg. According to Tjolsen et al. (1992) (43), a distinct biphasic nociception induced by formalin allows that model of nociception to advantageously discriminate central and peripheral components of pain. Shibata et al. (1989) (44) have shown that central acting analgesics such as narcotics (e.g., morphine) can reduce both phases of nociception, whereas peripherally acting drugs such as steroids (e.g., hydrocortisone and dexamethasone) and non-steroidal anti-inflammatory drugs (e.g., indomethacin) primarily suppress the late phase of nociception. However, data obtained regarding FPPT in the formalin test are not sufficiently distinct to indicate whether their antinociceptive effect is centrally mediated.

To study centrally mediated antinociceptive action, we conducted the tail flick test, which involves a spinal reflex, yet is subject to supraspinal influences. The reflex has been reported to be

sensitive to opioid-like and analgesic drugs with central action (46,47). The effective dose of the pyrazole derivative in the formalin test did not change the latency to thermal stimulation in the tail flick test. The reference drug morphine, an opioid receptor agonist, induced a significant increase in latency to thermal stimulation, as anticipated. Such results suggest that FPPT's mechanism of antinociceptive action does not involve central mechanisms.

To investigate the anti-inflammatory effect of FPPT, paw edema was induced via carrageenan, a complex group of polysaccharides formed by repeating galactose-related monomers (48). The development of paw edema in mice following carrageenan injection has been characterized by multiple inflammatory mediators and exudate of inflammation (49). The first 2 h after carrageenan injection predominantly has been shown to involve the release of proinflammatory agents such as histamine, serotonin, and bradykinin, while subsequent hours can entail the release of prostaglandins (50). In our study, FPPT and indomethacin significantly reduced the carrageenan-induced paw edema throughout the test period.

We performed the carrageenan-induced pleurisy test to confirm the anti-inflammatory effect of FPPT. This acute inflammation model is characterized by the accumulation of exudate and a rapid influx of polymorphonuclear cells, followed by mononuclear cell infiltration in the inflamed pleural cavity, thereby allowing the quantification of migrated cells (28,51,52). In that test, treatment with FPPT at a dose of 36 mg/kg significantly reduced cell migration, thereby confirming the anti-inflammatory effect of FPPT in the second phase of the formalin-induced pain and the carrageenan-induced paw edema tests.

Our results showed an endothelium-independent vasorelaxant effect of FPPT in the isolated organ model, as consistent with an earlier study on LQFM021 (16), an analog of FPPT. The vascular relaxation effect of FPPT, which showed that the compound is a vasodilator, excludes a false positive effect on the redution of edema and cell migration that could be associated with a vasoconstrictive property.

Nitric oxide (NO) is involved in multiple cellular functions and recognized as a major physiological regulator of vascular tone (53,54). NO is also the chief activator of soluble guanylate cyclase, an enzyme that synthesizes guanosine-3-5-cyclic monophosphate, the level of which is regulated by specific phosphodiesterases (PDE). Several PDE inhibitors have been developed for use as therapeutic agents since they increase cyclic nucleotide levels by blocking PDE function to enhance NO-cGMP signalization (55).

To evaluate the involvement of NO in FPPT's vasorelaxant effect, isolated aortic rings were incubated with L-NAME—that is, the inhibitor of endothelial nitric oxide synthase—or ODQ, a

guanylate cyclase inhibitor. Both L-NAME and ODQ blocked the effect of FPPT, thereby suggesting the involvement of the NO/cGMP pathway.

To investigate the involvement of K^+ channels on vasorelaxant effect of FPPT, aortic rings were incubated with TEA, a nonselective inhibition of voltage-dependent K^+ channels. Incubation with TEA blocked FPPT's vasorelaxant effect, while incubation with glibenclamide, a blocker of ATP-sensitive K^+ channels, attenuated the compound's vasorelaxant effect, thereby showing the involvement of K^+ channels on its vasorelaxant effect.

Studies have also shown that the activation of the L-arginine-NO-cGMP pathway K^+ channel is also involved in the antinociceptive effect of certain drugs. L-arginine is a substrate that NOS uses to produce NO, which subsequently activates guanylate cyclase, thereby stimulating the synthesis of cGMP that can interact with K^+ channels and promote its opening. The opening of K^+ channels leads to the hyperpolarization of the cell membrane and prevents the generation of nociceptive stimuli (56,57). It has been reported that the blockade of K^+ channels can moreover reduce the analgesic effect (18,58).

As the literature shows, several drugs used to treat pain or inflammation have exhibited the participation of NO/cGMP, prostanoids, and ionic channels in their mechanisms of action (17,59). Dipyrone, diclofenac, ketorolac, ketamine, and morphine are classic examples of analgesics that act via the NO/cGMP pathway (18,60,61), and several preclinical studies seeking new substances with antinociceptive and anti-inflammatory activities have revealed the involvement of NO/cGMP and K^+ channels (62–64).

Considering that LQFM021, an analog of FPPT, was able to inhibit PDE_3 and produce vasorelaxant effects similar to the tested compound in a docking assay (16), as well as showed an antinociceptive effect in the first phase of the formalin test, an effect blocked by NO/cGMP/K^+ channel pathway inhibitors (15), results for FPPT in the formalin test and isolated aortic rings assay support the hypothesis that the antinociceptive effect of FPPT involves those pathways, along with its anti-inflammatory effect demonstrated during edema and pleurisy tests.

Other than NO, vasoactive substances such as prostanoids are important modulators of vascular tone. Their production is directly related to the availability of arachidonic acid substrate and enzyme cyclooxygenases. Prostacyclin (PGI2) is a member of the prostanoids group that in blood vessels performs an important vasodilatory function that overrides the vasoconstrictive function of thromboxane A_2 (65,66). Therefore, to evaluate the involvement of prostanoids in FPPT's vascular effect, aortic rings were incubated with indomethacin, a known cyclooxygenase inhibitor. The rightward shift of the dose-effect curve demonstrates the blockade of FPPT due to indomethacin, which, as a result, requires additional study to ascertain the involvement of COX in pyrazole

derivative's biological activities. At the same time, its effect is highly similar to that of other pyrazole derivatives reported in the literature (16,67).

Other findings in our study demonstrate that FTTP can alter the contractile effect of a cumulative addition of $CaCl_2$ in aortic rings kept under depolarising conditions. That result suggests the involvement of voltage-sensitive Ca^{2+} channels at the plasmatic membrane. The blockade of voltage-dependent Ca^{2+} channels have been implicated in the mechnisms of antinociceptive and anti-inflammatory actions of some drugs (20,68,69). FPPT attenuated the contractile effect of a cumulative addition of $CaCl_2$ in the aorta maintained in depolarizing conditions. The attenuation of the contractile effect indicates that FPPT's vasorelaxant effect could involve a blockade of Ca^{2+} channels independent of K^+ channels or NO, if not both.

In higher concentrations (0.35–1.4 mM), FPPT promoted the reduction of 3T3 basal cells' viability. The acute oral toxicity of FPPT was assessed according to OECD Guideline 423: Acute Toxic Class Method (2001), a method that uses predefined doses and the results of which allow a substance to be ranked and classified according to the Globally Harmonized System for the classification of chemicals that cause acute toxicity. The principle of the test is that, based on a stepwise procedure using a minimum number of animals per step, sufficient information can be obtained about the acute toxicity of the tested substance to enable its classification (33). In that sense, FPPT was orally tolerated well in a dose of 2,000 mg/kg despite some signals of toxicity 2 h after exposure; it can thus be classified at GSH Category 5, which includes substances with a low acute toxicity hazard. Our findings are similar to those of its synthetic analog (16).

Conclusion

In all, the new pyrazole derivative FPPT showed a low toxicological profile according to OECD Guideline 423: Acute Toxic Class Method and was classified in Category 5 of the Harmonized Classification System for Chemical Substances and Mixtures, with LD50 determined to be 2,000 mg/kg > LD50 < 5,000 mg/kg. The antinociceptive effect of the compound was observed in an acetic acid-induced abdominal writhing test and in a formalin-induced pain test. FPPT also reduced the edema formation and cell migration in a pleurisy test, thereby confirming its anti-inflammatory effect. It furthermore demonstrated a vasorelaxant effect, whose reduction by indomethacin, L NAME, ODQ, TEA, and GB suggests the involvement of NO/cGMP and K^+ channels in the effect. That pathway might also modulate the antinociceptive effect, while the blockade of voltage-dependent calcium channels could have contributed to the vasorelaxation effect.

From the perspective of developing new drugs, additional pharmacological tests that evaluate the role of NO in the anti-inflammatory effect of FPPT should be conducted, as well as studies that evaluate the hypotensive or antihypertensive effect of FPPT in rats. Performing such preclinical toxicity tests can better ensure the safe pharmacology of this new compound.

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Conflict of interest statement

The authors declare no conflict of interest.

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Figure Legends

Figure 1. Structural design and synthetic route. (A) Structural design concept of new 5-(1-(4-fluorophenyl)-1H-pyrazol-4-yl)-1H-tetrazole (4) and (B) synthetic route for the preparation of 5-(1-(4-fluorophenyl)-1H-pyrazol-4-yl)-1H-tetrazole (4).

Figure 2. Analytical data. (A) ESI (+) FT-*Orbitrap* MS spectra obtained for 1-(4-fluorophenyl)-1*H*-pyrazole-4-carbonitrile. (B) ESI (+) FT-*Orbitrap* MS spectra obtained for 5-(1-(4-fluorophenyl)-1H-pyrazol-4-yl)-2H-tetrazole.

Figure 3. Antinociceptive effect of FPPT. (**A**) The animals treated with FPPT at doses 9, 18 or 36 mg/kg p.o., reduced significantly the number of acetic acid-induced writhing in mice (n= 9) in dose-dependent manner. Indomethacin (10 mg/kg p.o.) was used as a positive control. (ANOVA: $F_{(4\text{-}35)}$ = 33.34, $P < 0.0001$). (**B**) FPPT at dose 36 mg/kg p.o., reduced significantly the licking time (s) in the first phase and second phase of formalin test in mice (n= 8). Indomethacin (10 mg/kg, p.o.) and morphine (5 mg/kg, s.c.) were used as positive controls. [First phase (ANOVA: $F_{(3,29)}$ = 44.16, $P <$ 0.0001) and the second phase (ANOVA: $F_{(3,29)}$ = 42.17, $P < 0.0001$) of the test]. This results are presented as mean ± S.E.M. for each experimental group. **P< 0.01; ***P < 0.001 compared with control group treated with vehicle (10 mL/kg p.o.), acoording to ANOVA followed by Student-Newman-Keuls' test.

Figure 4. Anti-inflammatory effect of FPPT. (**A**)The treatmet with FPPT (36 mg/kg p.o.) reduced significantly the edema induced by carrageenan in mice (n = 10) in all hours of test. Indomethacin (10 mg/kg p.o.) was used as positive control. The results are presented as mean ± S.E.M. of the difference between the paws, in µL. ***P < 0.001 compared with control group, acoording to two-way ANOVA followed by Bonferroni's test. (Two-way ANOVA, treatment factor: $F_{(2,135)}$ = 112.6, $P <$ 0.0001). (**B**) FPPT at dose 36 mg/kg p.o. also reduced significantly the number of leukocytes migrated

for the pleural cavity of mices (n = 10) in carrageenan-induced pleurisy test. Dexamethasone (2 mg/kg p.o.) was used as positive control. The results are presented as mean ± S.E.M. of the number of leukocytes migrated. ***P < 0.001 compared with control group, acoording to ANOVA followed by Student-Newman-Keuls' test (ANOVA: $F_{(2,29)}$ = 36.15, *P* < 0.0001).

Figure 5. Vasorelaxant effect of compound FPPT in thoracic aorta of rats (n=6). Cumulative concentration-response curves for FPPT (1 µM to 1 mM) in rats aortic precontracted with phenylrphrine (0.1 µM) in endothelium-intact aortic rings (E+) or endothelium-denuded (E−) aortic rings. **P< 0.01; ***P < 0.001 compared with control group, acoording to two-way ANOVA followed by Bonferroni's test. (Two-way ANOVA, vessel factor: $F_{(3, 131)}$= 129.8, P < 0.0001; FPPT concentration factor: $F_{(6, 131)}$= 126.1, P < 0.0001).

Figure 6. Cumulative concentration-response curves for FPPT (1 µM to 1 mM) in rats aortic rings (n=6) with intact endothelium precontracted with phenylrphrine (0.1 µM) in the presence of (**A**) Nω-nitro-L-arginine methyl ester (L-NAME) a nitric oxide synthase inhibitor, 100 µM or 1H-[1,2,4]oxadiazolo[4,3-alpha]quinoxalin-1-one (ODQ) a guanylylcyclase inhibitor, 100 µM (Two-way ANOVA, antagonists factor: $F_{(2, 105)}$=66.56, *P* < 0.0001; FPPT concentration factor: $F_{(6, 105)}$=166.66, *P* < 0.0001); (**B**) Tetraethylammonium (TEA) voltage-dependent K^+ specific channel blocker, 1 µM (Two-way ANOVA, antagonists factor: $F_{(1, 54)}$=27.36, *P* < 0.0001; FPPT concentration factor: $F_{(6, 54)}$=85.38, *P* < 0.0001); (**C**) Glibenclamide (GB) a seletive inhibitor of channels $K_{ATP,}$ 100 µM (Two-way ANOVA, antagonists factor: $F_{(1, 56)}$=37.76, *P* < 0.0001; FPPT concentration factor: $F_{(6, 56)}$=160.7, *P* < 0.0001); or (**D**) Indomethacin (INDO) a cyclooxygenase inhibitor, 10 µM (Two-way ANOVA, antagonists factor: $F_{(1, 77)}$=21.78, *P* < 0.0001; FPPT concentration factor: $F_{(6, 77)}$=162.6, *P* < 0.0001). **P< 0.01; ***P < 0.001 compared with control group, acoording to two-way ANOVA followed by Bonferroni's test.

Figure 7. Effect of FPPT (0.1, 0.3 and 1 mM) on the contractile responses elicited by incremental addition of Ca^{2+} (10 µM to 0.1 mM) to aortic rings depolarized by KCl (60 mM) in a Ca^{2+}-free solution.

Contractile responses are expressed as the percentage of maximum contraction evoked by KCl (120 mM) (n=6). Contractile responses are expressed as the percentage of maximum contraction evoked by KCl (120 mM) (n=6). **P<0.01; ***P < 0.001 compared with control group, acoording to two-way ANOVA followed by Bonferroni's test. (Two-way ANOVA, FPPT concentration factor: $F_{(3,160)}$=55.77, *P* < 0.0001; $CaCl_2$ concentration factor: $F_{(9,160)}$=69.02, *P* < 0.0001).