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Original article

New pre-clinical evidence of anti-inflammatory effect and safety of a substituted fluorophenyl imidazole

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ABSTRACT

Acute Respiratory Distress Syndrome (ARDS) is an inflammatory condition with high mortality rates, and there is still no pharmacological approach with proven effectiveness. In the past few years, several imidazole small molecules have been developed to treat conditions in which inflammation plays a central role. In the present work, we hypothesize that a novel substituted fluorophenyl imidazole synthetized by our research group would present *in vivo* anti-inflammatory effect in an ARDS murine model induced by LPS. Results shows that the fluorophenyl imidazole has the ability to inhibit leukocyte migration to the bronchoalveolar lavage fluid and lung tissue of animals challenged intranasally with LPS. Furthermore, this inhibition is followed with reduction in myeloperoxidase activity, nitric oxide metabolites generation and cytokines (TNF-α, IL-6, IL-17, IFN-γ and IL-10) secretion. This effect is at least partly related to the capacity of the fluorophenyl imidazole in inhibit p38 MAPK and NF-κB phosphorylation. Finally, fluorophenyl imidazole showed no signs of acute oral toxicity in the toxicological protocol suggested by OECD 423. Taken together, the results shows that fluorophenyl imidazole is a promising prototype for the development of a novel anti-inflammatory drug in which p38 MAPK and NF-κB plays a pivotal role.

1. Introduction

Inflammation is still one of the main problems responsible for the medical burden in clinics and hospitals, as this phenomenon is involved in various pathological processes, taking many different forms *e.g.* metabolic syndromes [1], cancer [2], neurodegenerative conditions [3] and infectious diseases [4]. As more information is gathered about specific diseases, it is becoming apparent that inflammation is involved in many of these pathologies. The continuous pursuit for novel anti-inflammatory drugs, with the intention of produce safer and more efficient molecules for specific inflammatory conditions, is therefore essential [5,6].

In this scenario, Acute Respiratory Distress Syndrome (ARDS) is an inflammatory condition defined by acute hypoxemia and pulmonary cellular infiltration with diffuse alveolar injury following a trigging factor. To date, there is still no approved drug that can directly treat or resolve this complex pathology [7]. Mortality in patients with the severe form of ARDS is as high as 45%, and those who survive present a high risk of complications such as depression, post-traumatic stress disorder and cognitive decline [8]. Usually, ARDS is divided in two main phases: an exudative and a fibrotic phase. In the initial exudative phase, alveolar edema and cellular infiltration occur, mainly of neutrophils and macrophages. In conditions where the ARDS is indirectly caused by a septic process, activation of Toll-like receptor 4 (TLR4) by lipopolysaccharide (LPS) triggers an intracellular response in pulmonary resident cells that results in the activation of transcription factors, such as NF-κB, and mitogen-activated protein kinases, such as p38. Because of this, pro-inflammatory cytokines, enzymes and several inflammatory parameters are generated, activated and secreted [9,10]. Although several pharmacological approaches to ARDS have been

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developed in recent years, little success has been achieved in this regard [8,11].

Imidazoles are known for their multiple biological effects on the most varied experimental systems. In fact, there are several imidazole-derived molecules used by physicians around the world to treat pathologies such as cancer, fungal infections and hypertension. These polyvalent molecules are also being developed and tested to treat pathologies in which inflammation plays a pivotal role [12]. Our research group has recently developed and screened a series of tetra-substituted imidazoles *in vitro*, in J774 macrophages stimulated by LPS, and *in vivo* in an inflammatory model of carrageenan-induced pleurisy in mice. The results demonstrated that a substituted fluorophenyl imidazole was able to significantly inhibit all the inflammatory parameters accessed, an effect that was at least partly caused by the ability of this small molecule to inhibit the translocation of NF-κB to the cell nucleus after stimuli [13].

In the present work, we hypothesized that this fluorophenyl imidazole (FI) prepared by our research group would maintain its anti-inflammatory profile *in vivo* in an ARDS induced by LPS model, and we further investigated the possible influence of NF-κB and p38 MAPK phosphorylation. Moreover, we performed an acute oral toxicological test, according to the OECD 423 protocol, to determine the preliminary safety of this promising molecule.

2. Methods

2.1. Fluorophenyl imidazole synthesis

The substituted fluorophenyl imidazole, namely methyl 1-allyl-2-(4-fluorophenyl)-5-phenyl-1H-imidazole-4-acetate (purity > 99%, endotoxin free) (Fig. 1), was synthesized by our laboratory through a multicomponent reaction involving an azirine, a primary amine and an aldehyde [14]. The fluorophenyl imidazole (FI) was dissolved in a solution of sterile saline (0.9% NaCl) and 1% dimethyl sulfoxide (DMSO) to a final concentration of 2.5 mg/mL as a stock solution. These solutions were aliquoted and stored at -20 °C until the experiments.

2.2. Animals

Inflammatory and toxicological experiments were conducted in 6-week-old male Swiss mice weighing 25–30 g. The animals were housed at standardized conditions (room temperature at 20 ± 2 °C and alternating 12 h periods of light and dark) and were allowed free access to standard mouse chow and water. The procedures were approved by the Committee for Ethics in Animal Research of UFSC (6118110417 and 6363260718), and are in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH). All efforts were made to reduce the number of animals used, and the 3Rs principle were followed [15]. For procedures requiring anesthesia, the animals received xylazine (15 mg/kg) and ketamine (75 mg/kg) intraperitoneally. At the end of the experimental procedures, the animals were euthanized with an overdose of xylazine (45 mg/kg) and ketamine (225 mg/kg) and the samples were collected for further evaluations.

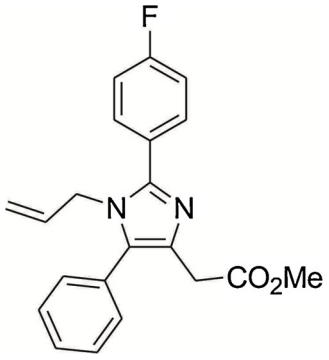

Fig. 1. Chemical structure of the tested tetrasubstituted imidazole, methyl 1-allyl-2-(4-fluorophenyl)-5-phenyl-1*H*-imidazole-4-acetate.

2.3. ARDS experimental design

The lung damage that occurs in ARDS was experimentally reproduced in mice by intranasal instillation of LPS (5 mg/kg) in sterile saline, as described by Szarka et al., [16] with modifications. Briefly, on the days of the experiments, the animals were randomly divided into different groups (n = 6 animals/group) and treated 1 h (pre-treatment) before lung injury induction. The groups consisted of:

(a) Sham control (S, healthy animal), animals pre-treated with vehicle (1% DMSO) per oral;

(b) Negative control (LPS, inflamed animal), animals pre-treated with vehicle (1% DMSO) per oral;

(c) Positive control (Dex, standard treatment animal), animals pre-treated with dexamethasone per oral (5 mg/kg);

(d) Experimental group (imidazole treatment animal), animals pre-treated with FI per oral (3–30 mg/kg). The doses were chosen based on previous studies developed by our laboratory and on the relevant bibliography, following log-scale distribution to optimize the results of the analysis [13,17].

After 1 h, the animals were anesthetized and received an intranasal dose of LPS (5 mg/kg, instilled at 0.025 mL in each nostril), except the sham group (S), which received an intranasal dose of sterile saline. The animals were observed and kept in the 45° inclined supine position until their recovery from the anesthetic protocol.

Twelve hours after the LPS challenge, the animals were euthanized with an anesthesia overdose (xylazine and ketamine) and the Broncho-Alveolar Lavage Fluid (BALF), and lung tissue samples were collected. BALF was collected through an incision in the trachea and cannulation of the lungs using a 22 G catheter. The lungs were washed three times with 1 mL of sterile PBS (phosphate-buffered saline) containing 1% citrate. The BALF and lung tissue samples were aliquoted in micro centrifuge tubes, cell counting was performed, and the samples were stored in a freezer at −80 °C until further evaluations.

2.4. Leukocyte count and capillary protein leakage

Total leukocytes were counted using a Neubauer chamber, after diluting the samples in Türk's solution (1:4). Cytospin slides of the BALF were stained with May-Grünwald-Giemsa for the differential count, which was performed under an oil immersion objective. The degree of capillary protein leakage (exudation) in the BALF samples was determined using the Lowry method, with bovine serum albumin as standard [18]. Total and differential leukocytes counts were expressed as cells x 10^5/mL, and exudation was expressed as μg/mL.

2.5. Myeloperoxidase activity

The analysis of myeloperoxidase (MPO) activity was performed as previously described by Bradley et al., [19]. BALF and lung tissue homogenate samples were centrifuged in a refrigerated centrifuge with HTAB (40.000 x g, 15 min at 4 °C), and the supernatant was immediately submitted to MPO activity analysis. MPO activity was estimated by means of colorimetric measurement in an ELISA plate reader (Organon Teknika®, Roseland, New Jersey, USA) at 450 nm by interpolation from the MPO (extracted from human leukocytes (Sigma-Aldrich®, St. Louis, Missouri, USA) standard curve (0.07–140 mUI/mL). The results were expressed as mUI/mL for BALF analysis and mUI/mg of tissue for lung tissue analysis.

2.6. Nitric oxide metabolites

Nitric oxide present in the BALF and lung tissue homogenate samples was measured using the Griess method and vanadium (III) chloride, to reduce nitrate to nitrite [20,21]. After centrifuging the BALF and lung tissue homogenates (300 g for 5 min), the colorimetric measurement was determined at 540 nm using an ELISA plate reader (Organon Teknika®, Roseland, New Jersey, USA). The amount of nitric oxide was determined indirectly by interpolation from the nitrite standard curve (0–100 μM), and the results were expressed in μM for BALF analysis and in μM/mg of tissue for lung tissue analysis.

2.7. Cytokines

The cytokines TNF-α, IL-6, IL-17, IFN-γ and IL-10 levels in the BALF were determined by flow cytometry (BD Biosciences FACSVerse flow cytometer®, San Jose, California, USA) using a commercial kit (Cytometric Bead Array (CBA) Mouse Th1/Th2/Th17 kit®, San Jose, California, USA). The data were analyzed using FCAP Array® software. The results were expressed in pg/mL.

2.8. Analysis of p38 MAPK and NF-κB phosphorylation

Lung tissue samples of mice were processed in a cell lysis buffer to extract the tissue intra cellular content. A commercial ELISA kit was used in accordance with the manufacturer's protocol to evaluate the phosphor-p65/total-p65 ratio (phospho-NF-κB, Invitrogen by Thermo Fisher®, Waltham, Massachusetts, USA; NF-κB p65 (total) Novex Life Technologies®, Carlsbad, California, USA) and the phospho-p38/total-p38 ratio (Phospho-p38α RD Systems®, Minneapolis, Minnesota, USA; p38 MAPK (total) Novex Life Technologies®, Carlsbad, California, USA) ratio. The quantity of total proteins was measured through the Lowry assay, and samples were adjusted to 60 μg of protein/well. An ELISA plate reader (Organon Teknika®, Roseland, New Jersey, USA) was used at 450 nm to evaluate the results. The results were expressed as phospho-p65/total-p65 ratio per milligram of protein and phospho-p38/total-p38 ratio per milligram of protein.

2.9. Histological analysis

Mice lungs were removed 12 h after LPS challenge, washed in PBS, and fixed in formalin solution 10% (v/v) for 48 h. The tissues were dehydrated in a series of ethanol solutions of increasing concentrations in graded series, followed by xylene, then included in paraffin and sliced into 3-μm sections (LEICA-Instruments® CM3050, Nussloch, Baden-Württemberg, Germany). The slices were stained with hematoxylin–eosin and analyzed under light microscopy (200x). The lung damage was graded according to previous studies described by Liu et al., [22], taking into account the degree of parenchymal distortion in the alveolar tissue. The scores used were as follows: 0) normal; 1) increased thickness in < 50% of interalveolar septa (IAS) due to edema and/or neutrophil infiltration; 2) increased thickness in > 50% of IAS; 3) increased thickness in > 50% of IAS and the presence of neutrophils within the alveolar space; and 4) consolidated infiltration of neutrophils with distortion of normal alveolar architecture. The mean score was reported for each microscope section.

2.10. Acute oral toxicity (OECD 423)

The acute oral toxicity study was conducted in compliance with OECD Guideline for the Testing of Chemicals n° 423 [23]. Although OECD 423 suggests the use of females due to their slightly greater sensitivity to toxicological evaluations, since our inflammatory experiments were conducted in male mice, we preferred to test the acute oral toxicity of fluorophenyl imidazole in male mice also, in order to continue the methodological reasoning.

A minimum number of animals was used in each step, as suggested by the stepwise procedure of the guideline. Due to the novelty of the FI and for animal welfare purposes, the OECD 423 guideline recommends a starting dose of 300 mg/kg. In each step, the animals were observed once during the first 30 min for clinical signs of toxicity and mortality, then periodically in the first 24 h (especially the first 4 h), and every day thereafter for 14 days. On day 14, mice were euthanized by an overdose of xylazine and ketamine and subjected to a gross pathological examination for signs of toxicity. Blood samples were also collected for blood count and leukogram.

Fluorophenyl imidazole at a 300 mg/kg dose was used as the starting dose (n = 3). Since there was no signs of toxicity or mortality at 300 mg/kg, the second group received the same dose as the next step, and in this second group no deaths or signs of toxicity were noted either (n = 3). The third group received a dose of 2000 mg/kg of FI, as suggested by the guideline (n = 3). Since no deaths or signs of toxicity were noted, the fourth group received the same dose of 2000 mg/kg (n = 3). Another group of animals were evaluated as control, receiving only the vehicle of fluorophenyl imidazole as treatment (n = 6).

2.11. Hematological parameters

Mouse blood samples were collected on day 14, immediately before euthanasia, and 0.5 M EDTA was used as anticoagulant (Synth®, Diadema, Sao Paulo, Brazil). The hematological parameters (Total WBC, lymphocytes, monocytes, granulocytes, red blood cells, HGB, HCT, MVC, MCH, MCHC and RDW) were accessed immediately after sample collection using a veterinarian automatic counter adjusted for mouse-specific parameters (Mindray®, BC-2800 Vet, Nanshan, Shenzhen, China).

2.12. Data analysis

The statistical analyses were conducted using the software GraphPad Prism 5.0 and SPSS 22. Parametric experimental results were expressed as mean ± standard error of the mean (S.E.M.). Data residuals were analyzed for normality using the Shapiro-Wilk test, and homoscedasticity using the Bartlett test. For the parametric homoscedastic data, we used one-way ANOVA followed by Tukey's post hoc test. Parametric heteroscedastic data were analyzed by one-way ANOVA-Welch followed by the Games-Howell post hoc test. Significance was set at $P < 0.05$.

3. Results

3.1. Fluorophenyl imidazole suppresses leukocyte infiltration and exudation in LPS-induced acute lung injury

The results shows that FI was able to inhibit, with statistical significance, the influx of leukocytes to the lungs of LPS challenged mice at 10 mg/kg (% inhibition: 41.5 ± 7.6) ($p < 0.001$) and 30 mg/kg (% inhibition: 49.2 ± 5.1) ($p < 0.001$) (Fig. 2 A). This effect is even conspicuous when only the neutrophils were analyzed, since this leukocyte migration was inhibited at doses of 3 mg/kg (% inhibition: 23.3 ± 4.3) ($p < 0.05$), 10 mg/kg (% inhibition: 84.9 ± 1.1) ($p < 0.001$) and 30 mg/kg (% inhibition: 87.9 ± 1.2) ($p < 0.001$) (Fig. 2 B). In relation to exudation, FI significantly inhibited this inflammatory parameter at 10 mg/kg (% inhibition: 43.7 ± 5.2) ($p < 0.01$) and 30 mg/kg (% inhibition: 56.9 ± 0.9) ($p < 0.001$) (Fig. 2C). The positive control used in the studies – dexamethasone, 5 mg/kg - inhibited total leukocyte (% inhibition: 51.2 ± 4.3) and neutrophil influx (% inhibition: 89.1 ± 1.1) to the lungs, and exudation (% inhibition: 63.1 ± 1.7), with significance ($p < 0.001$) (Fig. 2A–C).

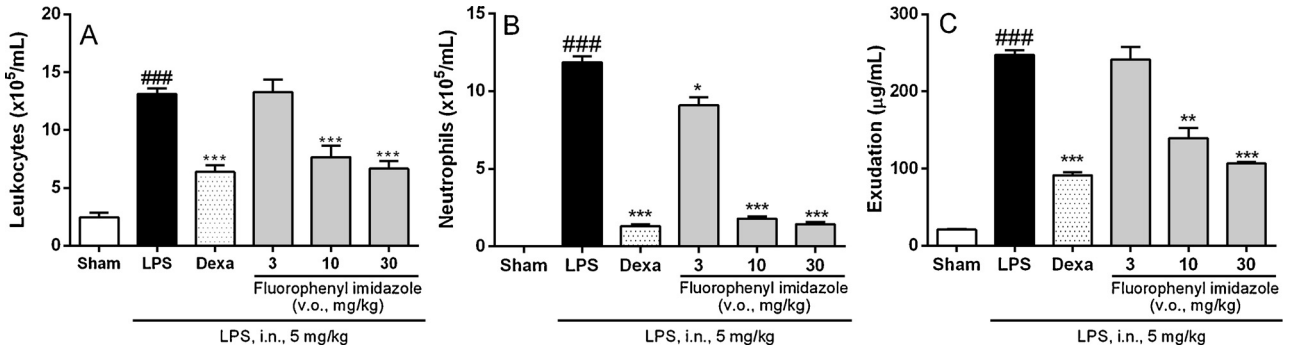

Fig. 2. Effect of fluorophenyl imidazole on leukocyte (A) and neutrophil (B) migration, and exudate concentration (C) in LPS-induced acute lung injury mice. Sham: animals treated with vehicle per oral 1 h before intranasal instillation with sterile saline; LPS: animals treated with vehicle per oral 1 h before intranasal instillation with sterile LPS; Dexa: animals treated with dexamethasone per oral (5 mg/kg) 1 h before intranasal instillation with sterile LPS; Fluorophenyl imidazole: animals treated with fluorophenyl imidazole per oral at doses of 3, 10 and 30 mg/kg, 1 h before intranasal instillation with sterile LPS.
Each group represents the mean ± standard error of the mean; n = 6/group.
###$p < 0.001$ compared to the Sham group.
*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to the LPS group.

3.2. Fluorophenyl imidazole inhibits myeloperoxidase activity and nitric oxide secretion in BALF and lung tissue of ALI-induced mice

In addition to the observed inhibitory effect of FI in the migration of leukocytes and exudation of the LPS-challenged mice, this tetra-substituted imidazole inhibited the activity of MPO present in BALF at 3 mg/kg (% inhibition: 21.4 ± 3.9) ($p < 0.05$), 10 mg/kg (% inhibition: 65.9 ± 2.8) ($p < 0.001$) and 30 mg/kg (% inhibition: 67.4 ± 4.2) ($p < 0.001$). This effect was also noted in the lung tissues of the imidazole-treated animals at 3 mg/kg (% inhibition: 28.6 ± 5.6) ($p < 0.001$), 10 mg/kg (% inhibition: 51.3 ± 4.9) ($p < 0.001$) and 30 mg/kg (% inhibition: 60 ± 2.1) ($p < 0.001$) (Table 1). NO_x secretion was also significantly diminished in the BALF of ALI-induced mice at doses of 10 mg/kg (% inhibition: 50 ± 6.3) ($p < 0.001$) and 30 mg/kg (% inhibition: 65.3 ± 4.9) ($p < 0.001$), and this effect followed the same pattern in the lung tissue of these mice, with significant inhibition at 10 mg/kg (% inhibition: 41.5 ± 7.8) ($p < 0.001$) and 30 mg/kg (% inhibition: 48.7 ± 3.5) ($p < 0.001$) doses (Table 1).

As expected, dexamethasone significantly inhibited MPO activity in both BALF and lung tissue of mice (% inhibition: 66.1 ± 2.3 and 68.3 ± 2.1) ($p < 0.001$) as well as NO_x secretion (% inhibition: 61.5 ± 4.2 and 47.6 ± 3.3) ($p < 0.001$) (Table 1).

3.3. Fluorophenyl imidazole diminished pro-inflammatory cytokines secretion in BALF of ALI induced mice

FI was capable of reducing the secretion of pro-inflammatory cytokines at the BALF of LPS-challenged mice. The secretion of TNF-α was reduced in a significant manner only at the dose of 30 mg/kg (% inhibition: 61.1 ± 5.3) ($p < 0.001$). On the other hand, IL-6 was significantly reduced at the doses of 10 mg/kg (% inhibition: 71.7 ± 6.3) ($p < 0.001$) and 30 mg/kg (% inhibition: 76.6 ± 4.3) ($p < 0.001$). The same inhibitory pattern was observed in IL-17, which was significantly inhibited at the doses of 10 mg/kg (% inhibition: 55.7 ± 10.2) ($p < 0.01$) and 30 mg/kg (% inhibition: 74.7 ± 4.5) ($p < 0.001$). The same occurred with IFNγ, which was significantly reduced by FI at the doses of both 10 mg/kg (% inhibition: 24.2 ± 5.1) ($p < 0.05$) and 30 mg/kg (% inhibition: 53.4 ± 6.1) ($p < 0.001$). Although a tendency to a decline was observed in the IL-10 secretion in the BALF of FI treated mice, no statistical significance was detected ($p > 0.05$) (Table 2).

Dexamethasone was able to inhibit, with statistical significance, the secretion of all the tested cytokines (% inhibition - TNF-α: 74.1 ± 4.8; IL-6: 94.1 ± 4.2; IL-17: 75.7 ± 7.5; IFNγ: 91.7 ± 0.7 and IL-10: 46.9 ± 4.9) ($p < 0.05$) (Table 2).

3.4. Fluorophenyl imidazole inhibited p65 subunit (NF-κB) and p38 MAPK phosphorylation

In relation to the intracellular pathways, FI significantly reduced p65 phosphorylation on mice lungs when compared to the negative control at the doses of 10 mg/kg (% inhibition: 24.6 ± 3.8) ($p < 0.05$) and 30 mg/kg (% inhibition: 27.5 ± 6.3) ($p < 0.05$) (Fig. 3 A). The same phenomenon occurred for p38 MAPK, which was inhibited at the doses of 10 mg/kg (% inhibition: 20.6 ± 5.4) ($p < 0.05$) and 30 mg/kg (% inhibition: 32.2 ± 3.5) ($p < 0.05$)

Table 1
Fluorophenyl imidazole activity on myeloperoxidase (MPO) and nitric oxide metabolites (NO_x) in bronchoalveolar lavage fluid (BALF) and lung tissue of LPS-induced acute lung injury mice.

Group (treatment mg/kg)	Parameter			
	MPO in BALF (mUI/mL)	MPO in Lung Tissue (mUI/mg of protein)	NO_x in BALF (µM)	NO_x in Lung Tissue (µM/mg of protein)
Sham (vehicle)	2.39 ± 0.76	6.40 ± 0.79	14.90 ± 1.98	26.92 ± 5.04
LPS (vehicle)	146.79 ± 5.92	202.38 ± 4.77	76.07 ± 2.17	96.26 ± 4.91
Dexa (5 mg/kg)	49.74 ± 3.42 ***	64.12 ± 4.26 ***	29.29 ± 3.23 ***	50.41 ± 3.19 ***
FI (3 mg/kg)	115.35 ± 5.81 **	144.48 ± 11.28 ***	78.76 ± 1.92	97.20 ± 6.10
FI (10 mg/kg)	49.97 ± 4.12 ***	98.57 ± 9.96 ***	38.03 ± 4.77 ***	56.33 ± 7.55 ***
FI (30 mg/kg)	47.82 ± 6.26 ***	80.95 ± 4.37 ***	26.40 ± 3.78 ***	49.37 ± 3.35 ***

Sham: animals treated with vehicle per oral 1 h before intranasal instillation with sterile saline; LPS: animals treated with vehicle per oral 1 h before intranasal instillation with sterile LPS; Dexa: animals treated with dexamethasone per oral (5 mg/kg) 1 h before intranasal instillation with sterile LPS; FI (3, 10 and 30 mg/kg): animals treated with fluorophenyl imidazole per oral (3, 10 and 30 mg/kg) 1 h before intranasal instillation with sterile LPS. Each group represents the mean ± standard error of the mean; n = 5–6/group. **$p < 0.01$ and ***$p < 0.001$.

Table 2
Fluorophenyl imidazole activity upon TNF-α, IL-6, IL-17, IFNγ and IL-10 in bronchoalveolar lavage fluid (BALF) of LPS induced acute lung injury mice.

Group (treatment mg/kg)	TNF-α (pg/mL)	IL-6 (pg/mL)	IL-17 (pg/mL)	IFNγ (pg/mL)	IL-10 (pg/mL)
Sham	1.61 ± 0.44	0.46 ± 0.07	3.95 ± 0.96	0.19 ± 0.06	29.12 ± 4.23
LPS (5 mg/kg)	3691.00 ± 268.34	3063.85 ± 275.82	22.83 ± 1.57	1782.00 ± 126.58	136.98 ± 16.48
Dexa (5 mg/kg)	957.60 ± 178.45 ***	182.64 ± 128.31 ***	5.55 ± 1.70 ***	147.00 ± 13.25 ***	72.63 ± 6.80 *
FI (3 mg/kg)	3917.20 ± 343.35	2254.14 ± 215.86	23.85 ± 2.69	1830.00 ± 84.15	120.86 ± 11.52
FI (10 mg/kg)	2778.60 ± 354.74	866.75 ± 192.17 ***	10.11 ± 2.34 **	1350.00 ± 90.41 *	114.74 ± 13.25
FI (30 mg/kg)	1435.20 ± 195.38 ***	718.07 ± 133.13 ***	5.78 ± 1.03 ***	830.60 ± 109.63 ***	92.57 ± 9.36

Sham: animals treated with vehicle per oral 1 h before intranasal instillation with sterile saline; LPS: animals treated with vehicle per oral 1 h before intranasal instillation with sterile LPS; Dexa: animals treated with dexamethasone per oral (5 mg/kg) 1 h before intranasal instillation with sterile LPS; FI (3, 10 and 30 mg/kg): animals treated with fluorophenyl imidazole per oral (3, 10 and 30 mg/kg) 1 h before intranasal instillation with sterile LPS. Each group represents the mean ± standard error of the mean; n = 5–6/group. *p < 0.05, **p < 0.01 and ***p < 0.001.

(Fig. 3 B).

The positive control, dexamethasone, was able to inhibit with significance the phosphorylation of both p65 subunit (% inhibition: 37.3 ± 5.7) (p < 0.05) and p38 MAPK (% inhibition: 31.7 ± 7.3) (p < 0.05) (Fig. 3).

3.5. Fluorophenyl imidazole reduced lung loss of architecture of ALI induced lungs

The acute lung injury induction by LPS intranasal instillation provided a massive leukocyte infiltration with consequent loss of lung architecture. The pre-treatment with FI reduced this lung damage with statistical significance, at the doses of 10 mg/kg (% inhibition: 27.3 ± 6.3) (p < 0.05) and 30 mg/kg (% inhibition: 45.5 ± 7.7) (p < 0.001) (Fig. 4).

Dexamethasone treatment also significantly reduced the histological parameters studied (% inhibition: 45.4 ± 7.5) (p < 0.001) (Fig. 4).

3.6. Fluorophenyl imidazole did not show acute oral toxicity

The oral treatment with FI did not show visible signs of toxicity at any of the tested doses (300 and 2000 mg/kg) after administration. During the 14 days after the FI treatment, the animals gained weight normally, without any perceptive abnormality, and no significant changes when compared to the control group. After euthanasia, no macroscopic changes were observed in the gross pathological analysis. Moreover, no significant changes were observed in the relative organ weight (organ % of body mass) compared to the control group, which received vehicle as treatment (Table 3). In relation to the hematological parameters, no significant changes were perceived in the leukogram and blood count profile when FI treated animals were compared to those that received only vehicle (Table 4).

4. Discussion

Since it was first described by Ashbaugh et al., [24] in 1967, ARDS has suffered several updates and has been the focus of different research groups across the world. There is no doubt that progress has been made regarding the specific approach related to this inflammatory phenomenon. However, the mortality rates are still higher than expected. There is not yet an effective pharmacological therapy, and supportive approaches are still one of the only tools that seem to increase patient survival rates [8,25]. In this context, we decided to test a promising new imidazole synthesized in our laboratory, in an *in vivo* experimental model that mimics some ARDS inflammatory events.

The inflammatory process is one of the main causes of high mortality rates in patients with ARDS. Although inflammation is a protective phenomenon aimed at preservation of the host, the imbalanced and disharmonious activation of inflammation causes serious and harmful events, either systemic or in the local tissue. ARDS is developed by direct lung-injuries, such as pneumonia, aspiration of gastric content or pulmonary contusion, or indirect lung-injuries, such as sepsis, non-thoracic trauma or major burn injuries [26]. The resident alveolar macrophages, once activated by a trigging factor, recruits neutrophils

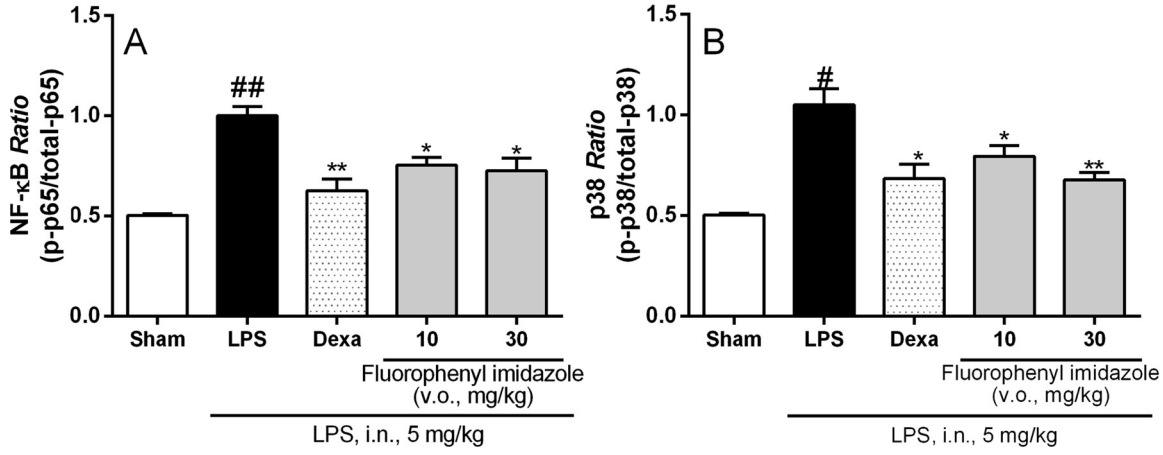

Fig. 3. Effect of Fluorophenyl imidazole on p65 NF-κB ratio (A) and p38 MAPK ratio (B) in LPS induced acute lung injury mice.
Sham: animals treated with vehicle per oral 1 h before intranasal instillation with sterile saline; LPS: animals treated with vehicle per oral 1 h before intranasal instillation with sterile LPS; Dexa: animals treated with dexamethasone per oral (5 mg/kg) 1 h before intranasal instillation with sterile LPS; Fluorophenyl imidazole: animals treated with fluorophenyl imidazole per oral at doses of 10 and 30 mg/kg, 1 h before intranasal instillation with sterile LPS.
Each group represents the mean ± standard error of the mean; n = 6/group.
#p < 0.05 and ##p < 0.01 compared to the Sham group.
*p < 0.05 and **p < 0.01 compared to the LPS group.

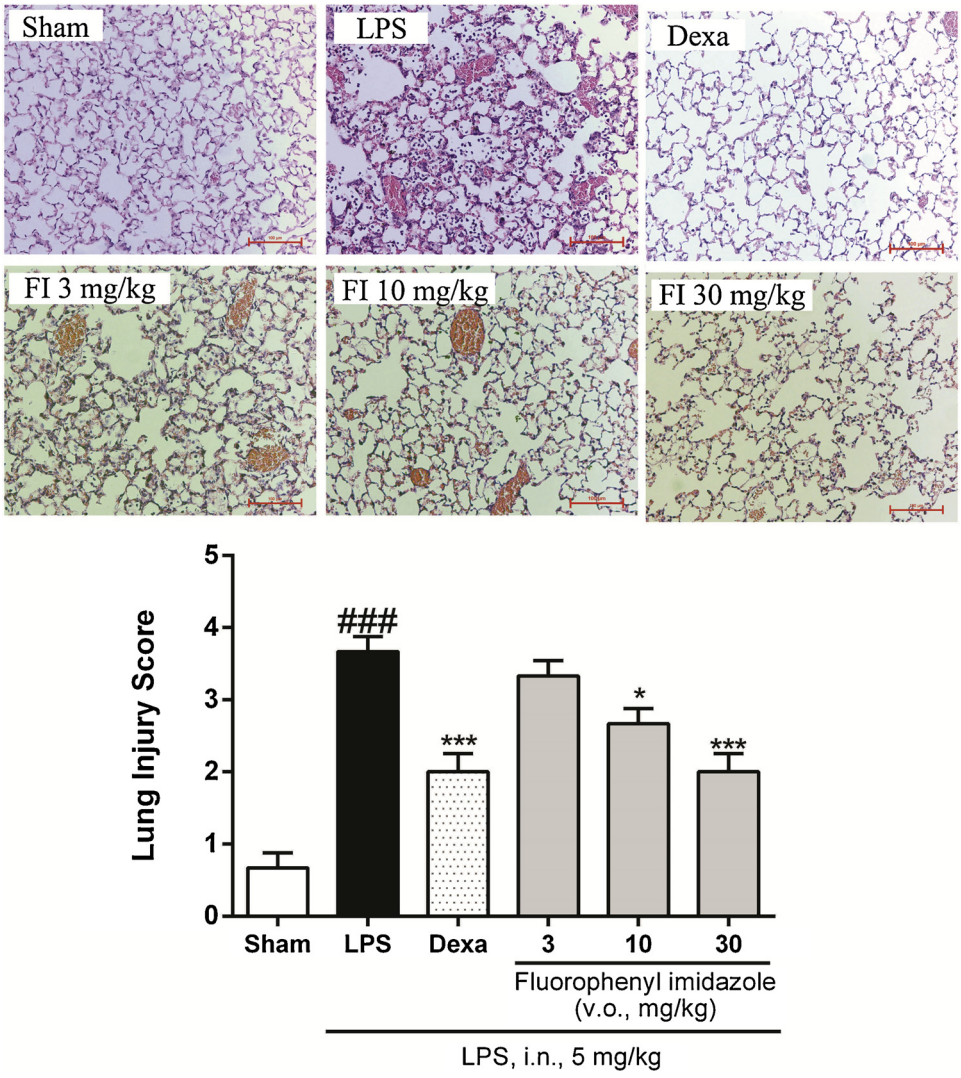

Fig. 4. Effect of fluorophenyl imidazole on lung architecture in LPS induced acute lung injury mice.
Sham: animals treated with vehicle per oral 1 h before intranasal instillation with sterile saline; LPS: animals treated with vehicle per oral 1 h before intranasal instillation with sterile LPS; Dexa: animals treated with dexamethasone per oral (5 mg/kg) 1 h before intranasal instillation with sterile LPS; FI (3, 10 and 30 mg/kg): animals treated with fluorophenyl imidazole per oral (3, 10 and 30 mg/kg) 1 h before intranasal instillation with sterile LPS.
Each group represents the mean ± standard error of the mean; n = 6/group.
###p < 0.001 compared to the Sham group. *p < 0.05 and ***p < 0.001 compared to the LPS group.

Table 3
Fluorophenyl imidazole effect on body mass and relative organ mass in mice exposed to the acute oral toxicity protocol (OECD 423).

Group (treatment)	Parameter							
	Initial Body Mass (g)	Final Body Mass (g)	Heart (% b.m.)	Lung (% b.m.)	Right Kidney (% b.m.)	Left Kidney (% b.m.)	Spleen (% b.m.)	Liver (% b.m.)
Control (vehicle)	26.80 ± 0.34	36.78 ± 0.11	0.47 ± 0.01	0.79 ± 0.01	0.98 ± 0.01	0.91 ± 0.02	0.53 ± 0.01	5.66 ± 0.29
FI (300 mg/kg)	27.27 ± 0.21	36.18 ± 0.39	0.43 ± 0.01	0.76 ± 0.01	0.93 ± 0.01	0.86 ± 0.02	0.49 ± 0.02	5.67 ± 0.25
FI (2000 mg/kg)	27.00 ± 0.36	37.75 ± 0.42	0.45 ± 0.01	0.77 ± 0.02	0.96 ± 0.02	0.85 ± 0.02	0.53 ± 0.02	5.96 ± 0.21

Control: (healthy animal), animals treated with vehicle per oral and observed for 14 days thereafter; FI (300 mg/kg): (imidazole treatment animal), animals treated with fluorophenyl imidazole per oral (300 and 2000 mg/kg) and observed for 14 days thereafter. Each group represents the mean ± standard error of the mean; n = 6/group.

and circulating macrophages to the lungs in order to eradicate the damaging cause. These migrated leukocytes are involved in the activation and secretion of a vast array of mediators, including proteases, reactive oxygen species, eicosanoids, phospholipids and cytokines, which perpetuate the inflammatory condition. The consequence of this stressful environment is the death of Type 2 epithelial cells, which in homeostatic conditions, are responsible for maintaining the lung functions by synthetizing and secreting pulmonary surfactants and controlling lung fluid [27].

The nasal instillation of LPS in mice causes a massive leukocyte migration to the lungs, a feature also observed in patients with ARDS. In a recent study, our research group identified the promising anti-inflammatory effect of FI in a pleurisy-induced carrageenan murine model. In that study, mice intraperitoneally pre-treated with FI showed a significant decrease in leukocyte migration to the pleural cavity after the intrapleural carrageenan challenge [13]. In the present study, the same leukocyte migration inhibitory pattern was observed in the BALF and lungs of mice, even when we modified the route of administration of the tested molecule. This was no surprise, given that the chemical structure of FI presents no violations to any of the Lipinski's rule of five [28].

Williams and Maier, [29] showed that ketoconazole, a well-known imidazole, has the ability to inhibit the production of LTB4, TXB2, and PCA by rabbit alveolar macrophages after endotoxin or calcium

Table 4
Fluorophenyl imidazole effect on leukogram and blood count in mice exposed to the acute oral toxicity protocol (OECD 423).

Group (treatment)	Parameter										
	WBC (x 10^9/L)	Lymphocytes (x 10^9/L)	Monocytes (x 10^9/L)	Granulocytes (x 10^9/L)	RBC (x10^{12}/L)	HGB (g/L)	HCT (%)	MCV (fL)	MCH (pg)	MCHC (g/L)	RDW (%)
Control (vehicle)	12.08 ± 0.51	9.46 ± 0.28	0.36 ± 0.02	2.26 ± 0.27	7.57 ± 0.27	122.4 ± 4.4	42.58 ± 1.79	56.24 ± 0.39	16.14 ± 0.14	287.4 ± 3.88	12.78 ± 0.42
FI (300 mg/kg)	13.52 ± 0.42	10.33 ± 0.27	0.35 ± 0.02	2.83 ± 0.15	7.02 ± 0.21	120.0 ± 3.1	44.00 ± 1.72	62.55 ± 0.96	17.07 ± 0.39	274.0 ± 8.91	13.12 ± 0.29
FI (2000 mg/kg)	11.48 ± 0.45	8.73 ± 0.52	0.35 ± 0.03	2.40 ± 0.23	7.18 ± 0.32	112.7 ± 5.3	39.53 ± 1.75	55.13 ± 0.47	15.57 ± 0.10	284.3 ± 1.85	12.15 ± 0.23

Control: (healthy animal), animals treated with vehicle per oral and observed for 14 days thereafter; FI (300 mg/kg): (imidazole treatment animal), animals treated with fluorophenyl-imidazole per oral (300 and 2000 mg/kg) and observed for 14 days thereafter. Each group represents the mean ± standard error of the mean; n = 6/group.

ionophore stimulation. The authors suggests that this effect may be of benefit in preventing ARDS by minimizing neutrophil infiltration to the lungs. More recently, ketoconazole was tested by Thompson [30], and did not maintain the efficacy in clinical trials that was shown in the preclinical study phase, although the author gives reassurance that it is indeed a safe drug. Even so, there is increased interest in imidazole derived molecules with anti-inflammatory effect that may be of benefit for patients with inflammatory conditions [12]. An example of this is epiisopiloturine, an imidazole alkaloid that was demonstrated by Silva et al. as having anti-inflammatory action [31], and that also showed inhibitory activity on granulocyte migration to the colon of trinitrobenzenesulfonic acid induced colitis in rats, in a Crohn's disease model [32].

The massive leukocyte migration to the lungs of mice after LPS instillation triggers another common feature of ARDS: pulmonary edema. The results demonstrate that FI was able to reduce exudation in the lungs of mice challenged with LPS. This result was no surprise, since neutrophils are the main factor responsible for the production and secretion of inflammatory mediators that disrupt both epithelial and endothelial lung barriers and cause loss of lung architecture. The results of this disruption are fluid extravasation to the lungs, with consequent hypoxia and respiratory failure. It has been shown that the percentage of neutrophils in the patient's lungs is directly correlated with the alveolar-arterial partial pressure of oxygen (PO_2) alterations in ARDS pulmonary edema [33].

As with any other pathological process in which neutrophils are the main factor associated with the tissue damage, in ARDS the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) by these leukocytes play and important part in the harmful phenomena caused by inflammation [34]. The results described in Section 3.2 shows that in ARDS-induced animals pre-treated with FI, the MPO activity and NOx secretion concentrations were decreased, both in BALF and lung tissues. MPO is an enzyme present in primary azurophilic granules of neutrophils, and is responsible for the transformation of hydrogen peroxide into hypochlorous acid, which in turn, has a bactericidal effect [35]. NO is a soluble gas that participates in several physiological and homeostatic processes. However, it also has a protective role when leukocytes and endothelial cells secrete this mediator in high amounts, and it reacts with superoxide molecules to form peroxynitrite, another powerful bactericidal molecule [36]. Although both of these mediators are important in the host defense against foreign molecules, their overexpression will certainly result in tissue damage and consequent loss of function by the harmed tissue [36,37]. The notable reduction in both MPO and NO_x described in the results section are presumably a consequence of the inhibition of leukocyte migration to the lungs of FI pretreated LPS-challenged mice, since activated neutrophils and macrophages are among the main sources of activated MPO and NO production, which in turn, is directly correlated with damage caused by oxidative stress [38,39].

The signaling process that recruits immune cells to the injury site relies mainly on the production and secretion of small proteins known as cytokines. Different harmful stimuli require specific cells to engage, and this cellular recruitment specificity is partly mediated by distinct cytokines [40]. For instance, IL-6 and TNF-α are directly involved in acute inflammatory conditions. Furthermore, both of these cytokines are biomarkers for ARDS in experimental and clinical routines [9]. The results show that FI pretreatment significantly diminished the secretion of these cytokines. This corroborates the decrease in leukocyte migration to the lungs of mice, since TNF-α is one of the main priming factors that triggers neutrophil adhesion, and IL-6 is an optimizer of neutrophil chemotaxis [41,42]. INFγ was also diminished in the BALF of FI pretreated animals. This cytokine acts as an immunomodulator, and as such, it may have pro-inflammatory or anti-inflammatory roles, depending on the specific condition in which is released. In the lung injury context, IFNγ participates in the upregulation of the tissue factor expressed by the alveolar epithelial cells. Lower concentrations of IFNγ

result in lower expression of tissue factor, which in turn, is directly correlated with a better prognostics to patients with sepsis-induced ARDS [43–45]. The same applies to IL-17, which was also inhibited by the FI treatment. This cytokine is crucial to neutrophil recruitment after infectious injuries, by promoting chemokine production. As stated earlier, this exacerbated response may have an important protective role, but at the cost of tissue damage. Furthermore, IL-17 is associated with alveolar inflammation and poor outcomes in ARDS patients [46]. IL-10 was the only cytokine that was not inhibited with statistic significance by FI treatment, although an inhibitory tendency can be clearly noted in the concentration of this cytokine in BALF. This inhibitory tendency may be caused by the decreased leukocyte recruitment, since neutrophils are significant producers of IL-10 during septic conditions, and the intranasal instillation of LPS in the present model may have led to a septic condition once it reached the circulation [47].

Transcription factors and intracellular proteins are known controllers of biological phenomena, and the inflammatory process is no exception. Once a pathogen associated molecular pattern (PAMP) or a damage associated molecular pattern (DAMP) stimulates a specific receptor, an intracellular chain of phosphorylation is triggered, and the inflammatory orchestra begins. One of the main transcription factors involved in inflammation known nowadays is NF-κB. Once this pathway is activated, the phosphorylation of intracellular proteins leads to the degradation of IκB, an inhibitory protein of the dimer p65/p50 (NF-κB). When the p65/p50 is detached from IκB, it translocates to the nucleus, where it begins its physiological function by promoting the generation, activation and release of inflammatory mediators, such as cytokines and enzymes [48,49]. Our previous work has already shown that FI has the ability to inhibit the translocation of NF-kB to the nucleus of LPS-stimulated J774 macrophages [13]. These results show that this *in vitro* pattern of inhibition response on NF-κB is maintained *in vivo*.

p38 MAPK are intracellular proteins that exert regulatory activity on cytokines and cell immune proliferation/survival. As a result, these proteins may have a key role in the initiation and further development of inflammatory diseases [50,51]. This MAPK has been the target of several clinical trials, having shown promising results in preclinical studies [52–54]. Furthermore, p38 MAPK inhibitors available on the market nowadays are mostly imidazole derived small molecules, such as SB202190, SB203580 and PD169316. Our results show that FI inhibited p38 MAPK phosphorylation *in vivo*, with statistical significance. These findings corroborate a research trend towards the use of imidazolic compounds, specially tri- and tetrasubstituted imidazole small molecules, to inhibit the phosphorylation and consequent activation of p38 MAPK [12,55–57].

Finally, the OECD 423 guideline for Testing of Chemicals regarding their acute oral toxicity is a useful method for screening safe molecules. It is a preliminary result concerning the safety of molecules, but it guides the next steps regarding chronic toxicity tests (repeated doses). In addition to the anti-inflammatory results, FI showed no signs of acute toxicity at doses 10 times higher than those used in the ARDS inflammatory experiments. According to OECD 423, FI is considered an unclassified chemical compound by the Globally Harmonized System (GHS), since no animals died or showed signs of toxicity during the protocols. Furthermore, the LD_{50} cut-off indicated by the OECD algorithm is higher than 5000 mg/kg of body weight.

5. Conclusion

FI shows an important anti-inflammatory profile in a specific pulmonary inflammatory protocol *in vivo*. This anti-inflammatory effect is probably caused by the ability of this tetrasubstituted imidazole to inhibit important intracellular inflammatory pathways, such as p38 MAPK and NF-κB. The consequence of this inhibition is the decrease in the secretion/activation of inflammatory mediators (cytokines, nitric oxide, and myeloperoxidase), leading to the decline of leukocyte migration and mitigation of lung injury and loss of pulmonary architecture. Moreover, this small molecule showed preliminary oral safety in terms of its acute toxicity. However, further studies are needed to investigate the chronic toxicity of this promising molecule. Additionally, the exact effect of this molecule on lung function, the effect in post-treatment, and the exact mechanism of action, still need to be elucidated.

Conflict of interest

The authors declare no conflict of interest.

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