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HEALTH SCIENCES

Milonine attenuates the lipopolysaccharide-induced acute lung injury in mice by modulating the Akt/NF-κB signaling pathways

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Abstract: Acute lung injury is an inflammation that triggers acute respiratory distress syndrome with perialveolar neutrophil infiltration, alveolar-capillary barrier damage, and lung edema. Activation of the toll-like receptor 4 complex (TLR4/MD2) and its downstream signaling pathways are responsible for the cytokine storm and cause alveolar damage. Due to the complexity of this pulmonary inflammation, a defined pharmacotherapy has not been established. Thus, this study evaluated the anti-inflammatory potential of milonine, an alkaloid of *Cissampelos sympodialis* Eichl, in an experimental model of lung inflammation. BALB/c mice were lipopolysaccharide-challenged and treated with milonine at 2.0 mg/kg. Twenty-four hours later, the bronchoalveolar fluid, peripheral blood, and lungs were collected for cellular and molecular analysis. The milonine treatment decreased the cell migration (mainly neutrophils) to the alveoli, the pulmonary edema, and the cytokine levels (IL-1β, IL-6, TNF-α). The systemic IL-6 level was also reduced. The milonine docking analysis demonstrated hydrophobic interaction at TLR4/MD2 groove with Ile124 and Phe126 amino acids. Indeed, the alkaloid downregulated the kinase-Akt and NF-κB through TLR4/MD2. Therefore, milonine is an effective inflammatory modulator being a potential molecule for the treatment of lung inflammation.

Key words: Acute lung injury, cytokines, morphinan alkaloid, neutrophils, Akt/NF-κB.

INTRODUCTION

Acute lung injury (ALI) and its most severe form, acute respiratory distress syndrome (ARDS), are inflammatory lung diseases that cause respiratory failure. Their development of ALI/ARDS may be due to a primary infection, which is the case of pneumonia bacterial, viral or fungal or an indirect lung infection being more common in severe sepsis. The pathological characteristic of these diseases is the non-hydrostatic pulmonary edema formation, rich in proteins and inflammatory cells, which leads to a condition of refractory hypoxemia, affecting lung compliance and compromising the elimination of carbon dioxide by the lung (Rezoagli et al. 2017, Ware & Matthay 2000).

Lipopolysaccharide (LPS) is an endotoxin found in gram-negative bacteria widely used in studies with mice to induce ALI. The toll-like receptor 4 (TLR4), present in many immune cells, plays a key role in innate immunity by recognizing pathogen-associated molecular patterns (PAMPs) such as LPS, through the myeloid differentiating factor 2 (MD2) complexed with TLR4 (TLR4/MD2) (De Nardo 2015, Mazgaeen

& Gurung 2020). Thus, the TLR4/MD2 activation occurs when the LPS is transferred to a hydrophobic groove of the MD2, which leads to the TLR4 homodimerization (Park et al. 2012) triggering off the pro-inflammatory signal by activation of phosphatidylinositol-3-kinase (PI3K) and protein kinase B (Akt or PKB) (Jiang et al. 2005). The kinase Akt acts on the nuclear factor-κB (NF-κB) signaling pathway to induce the production of large amounts of cytokines such as interleukin 6 (IL-6), tumor necrosis factor α (TNF-α) and IL -1β (Yum et al. 2001, Strassheim et al. 2004, Nie et al. 2019), promoting the progression of ALI. Thus, the blockage of the TLR4/MD2-mediated NF-κB signaling pathway can attenuate the LPS-induced ALI.

Although it has been advancing in the ALI/ARDS pathophysiology studies, the effectiveness of therapeutic approaches is still limited. The rate mortality of patients with ALI remains high, around 40-60%, and a definitive pharmacological treatment has not been established (Bellani et al. 2016). In this scenario, pharmacological treatments must be developed to improve clinical results and reduce the mortality rate of patients.

Milonine, an 8,14 dihydromorfinandeinonic alkaloid (Figure 1), belongs to the morphinan class as morphine, codeine, and sinomenine. Milonine was isolated and identified from the leaves of *Cissampelos sympodialis* Eichl, a plant popularly used in northeastern Brazil to treat inflammatory diseases as asthma (de Freitas et al. 1995a, Barbosa-Filho et al. 1997). In cytotoxic studies, using hepatocytes, fibroblasts, and Vero lineages, the IC50 varied between 100-400 μm (Melo et al. 2003, Santos et al. 2019). Besides, i*n vivo* studies demonstrated vasorelaxant activity of milonine mediated, in part, by the endothelium, through the nitric oxide-cGMP pathway and opening K^+ channels, allowing to

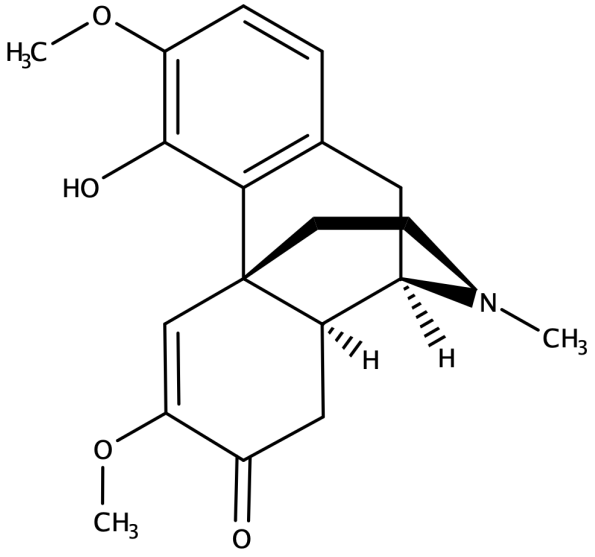

Figure 1. Three-dimensional structure of milonine.

reduce blood pressure in normotensive rats (Cavalcante et al. 2011). Experimental models of acute inflammation showed that the milonine treatment inhibited the mast cell degranulation by stabilizing the mast cell membrane, however, did not inhibit the histamine activity (Alves et al. 2017). Milonine also inhibited paw edema and nociception induced by phlogistic agents and these effects were related to PGE2, bradykinin, TNF-α, and IL-1β inhibition (Silva et al. 2017).

Since milonine is involved in cellular and vascular events in the inflammatory process and the lack of specific therapies in ALI/ARDS, we hypothesize that the alkaloid could be a potential candidate to become a phytomedicine to treat lung inflammatory processes. To do so, we used the LPS-induced acute lung injury experimental model to demonstrate its anti-inflammatory activity, and understand its mechanism of action by looking at the extra- (TLR4/MD2) and intracellular (Akt/ NF-κB) signaling pathways.

MATERIALS AND METHODS

Animals

Male BALB/c mice (6 - 8 weeks old, weighing between 20 and 30 g) were purchased from the Animal Production Unit of the Institute for Research on Drugs and Medicines (IPeFarM) of the Federal University of Paraíba, João Pessoa, PB, Brazil under the protocol 7316150420. The animals were kept in polypropylene cages at a temperature of 25 ± 2 ºC, with regular ventilation, water and sufficient food throughout the experimentation period.

Milonine

Leaves of *C. sympodialis* were collected from the botanical garden of the Federal University of Paraiba, Paraiba, Brazil. A voucher specimen was deposited in the "Herbário Lauro Pires Xavier" Herbarium, No. 1456. The alkaloid, milonine, was isolated from the leaf hydroalcoholic extract and chromatographed under a neutral alumina column. The fractions were obtained with preparative thin-layer chromatography and the milonine identification was realized through the analysis of ^{1}H and ^{13}C NMR spectral data compared with those published in the literature (de Freitas et al. 1995b). Therefore, 1 mg of milonine was dissolved in 50 µL of 1N of HCl and 800 µL of NaCl solution. The pH 7.0 was adjusted with 1M of NaOH and completed to 1000 µL with NaCl solution.

Animal groups

The animals (n = 6/group) were treated, orally (p.o), with milonine at 2 mg/kg, one hour after the phlogistic agent challenge (LPS+MIL2), aiming to evaluate the treatment of the pathology, mimicking as close as possible the reality that occurs with humans. The animals of the saline group (healthy animals) were treated and challenged with saline, and the animals of the lipopolysaccharide (LPS) group (sick animals) were LPS-challenged and treated with saline. The chosen dose of milonine was defined previously in a previous study by Alves et al. (2017), in which a lower effective dose of 2.0 mg/kg was observed in a model of acute inflammation (paw edema) when induced by phlogistic agents.

The acute lung injury experimental model

The acute lung injury was developed as follows: animals were previously anesthetized with 50 µL of xylazine (1.91 mg/mL) and ketamine (29 mg/mL) and, by nasal instillation, received 2.5 mg/kg of lipopolysaccharide (LPS - *E. coli* 0111: B4-Sigma-Aldrich®) diluted in 40 µL of sterile saline. The saline group received only 40 µL of the sterile vehicle. The animals were euthanized 24h after the LPS-challenge by anesthetic overdose (300 µL) to obtain the following biological material: the bronchoalveolar lavage fluid (BALF), peripheral blood from the brachial plexus, and lungs (Xavier et al. 2019).

Inflammatory cell count and protein content

The total inflammatory cell count was realized from the BALF pellet using a neubauer chamber and the differential cells of the BALF were analyzed from cyto-centrifuged slides stained with the rapid panotic kit (Hematoxylin & Eosin) and on an optical microscope (100x objective). The protein content was measure from the BALF supernatant using the SENSIPROT kit (LabTest, Minas Gerais, MG, Brazil) and the test was carried out in accordance with the manufacturer's specifications.

Cytokine measurement

The cytokines, IL-1β, TNF-α and IL-6, were measured in the BALF and in the peripheral blood by the immunoenzymatic assay method

(ELISA) and according to the manufacturer's specifications (eBioscience).

Histopathological analysis of the lung tissue and morphometric studies

The lungs are removed, fixed (10% formaldehyde), dehydrated, diaphanized and paraffinized. The paraffin layer was submerged in microtome sections, 4 µm thick, to obtain sheets for staining with hematoxylin and eosin. For morphometric analysis, twenty random images from slides of lung tissue were used. Under an Axiolab light microscope (Zeiss) with 440× resolution, twenty images were relayed to an image analysis system (Kontron Elektronik image analyser; Carl Zeiss, Germany—KS300 software).

Lung wet/dry weight ratio

The lungs were removed and immediately weighed on a precision analytical balance to obtain the wet weight and subsequently, the lungs were stored in a drying oven for 48 hours at a temperature of 60 °C, and then weighed to obtain the dry weight. Thus, the pulmonary edema index was obtained from the ratio between the wet weight and dry weight.

Molecules of the intracellular signaling pathway

The flow cytometry methodology was used to determine the intracellular protein Akt and the p65NF-κB expression of cells from BALF. The $5x10^5$ cells/mL were incubated with specific anti-mouse Akt PE conjugated or p65NFκB PE conjugated antibodies. The flow cytometer (Becton - Dickinson FACS Canto II) analyzed the expression of specific antibodies with BALF cell fluorescent labeling. The specific protocol for each marking was carried out in accordance with the manufacturer's specifications (BIOSCIENCE, Inc. Science Center Drive, San Diego, CA-USA).

Molecular docking analysis

The TLR4/MD2 receptor selected for molecular coupling was obtained from the protein database - PDB (https://www.rcsb.org/pdb/home/home.do), under the code PDB ID 3FXI (Park et al. 2009). For coupling, milonine was inserted in the SDF format and all water molecules and cofactors were removed. The Molegro Virtual Docker v.6.0.1 (MVD) program was used to calculate the binding energy. For the coupling procedure (receptor - ligand), a GRID with a radius of 15 Å and a resolution of 0.30 was used covering the location of the connection site, defined by using a known ligand. The model was designed to perform the adjustment with the characteristics expected between the ligand and the enzyme, using the heuristic search algorithm that combines the differential evolution and the crystallographic ligand as a model. The cavity prediction algorithm (Moldock) and the Moldock score function were selected to obtain the results (Thomsen & Christensen 2006).

Statistical analysis

The data were analyzed using the GraphPad Prism version 7.0 (GraphPad Software, San Diego, CA, USA) and the values are considered significant for $p < 0.05$. The FlowJo program, version 10, will be used to analyze the Flow cytometer data. The results are expressed by mean ± standard error of the mean (SEM) and analyzed statistically using the one-way ANOVA followed by the Tukey post-test.

RESULTS

Milonine attenuates the lung damage in LPS-induced acute lung injury in mice

The sick animals (LPS group) presented into the BALF a significant increase (p <0.0001) of the total leukocytes dependent on neutrophils, macrophages, and lymphocytes when compared

to healthy animals (saline group). The oral treatment with milonine showed a significant reduction (p <0.0001) of these cell populations independently of macrophages (Figure 2a-d). The histological analyzes of the lung tissues of the sick animals showed alveoli filled with inflammatory cells (*) with bronchoalveolar architecture disruption as compared to the lung tissues of the healthy animals (saline group). The lung tissues of milonine treated animals showed minimal destruction caused by the LPS-challenged and reduction of inflammatory cells in the alveolar spaces (Figure 2e). The morphometric data confirm the reduction of the inflammatory process (Figure 2f).

Milonine inhibits the microvascular protein permeability and pulmonary edema in LPS-induced acute lung injury in mice

The protein exudation into the bronchoalveolar cavity of the animals of the LPS group was increased when compared to the animals of

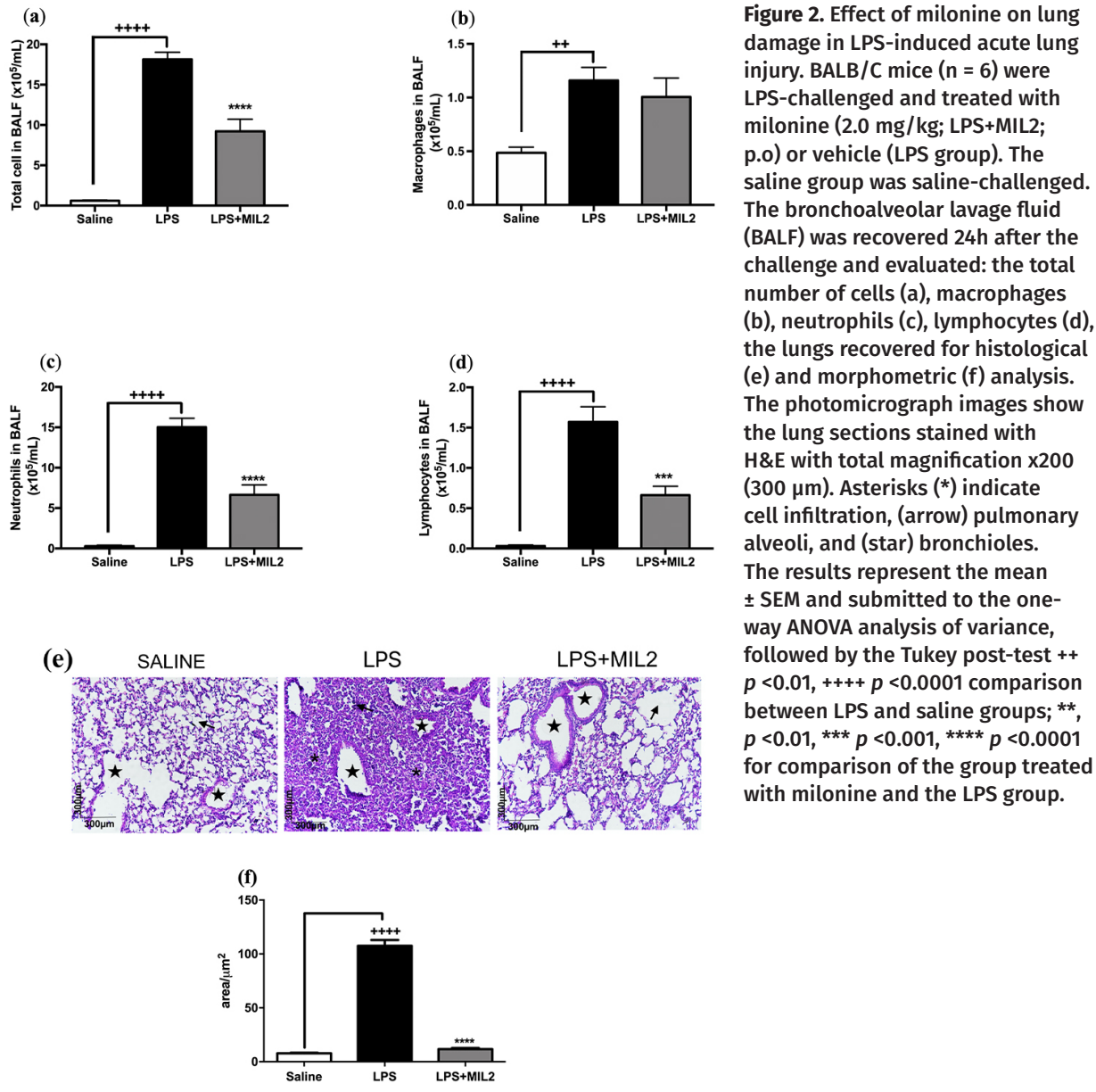

Figure 2. Effect of milonine on lung damage in LPS-induced acute lung injury. BALB/C mice (n = 6) were LPS-challenged and treated with milonine (2.0 mg/kg; LPS+MIL2; p.o) or vehicle (LPS group). The saline group was saline-challenged. The bronchoalveolar lavage fluid (BALF) was recovered 24h after the challenge and evaluated: the total number of cells (a), macrophages (b), neutrophils (c), lymphocytes (d), the lungs recovered for histological (e) and morphometric (f) analysis. The photomicrograph images show the lung sections stained with H&E with total magnification x200 (300 μm). Asterisks (*) indicate cell infiltration, (arrow) pulmonary alveoli, and (star) bronchioles. The results represent the mean ± SEM and submitted to the one-way ANOVA analysis of variance, followed by the Tukey post-test ++ p <0.01, ++++ p <0.0001 comparison between LPS and saline groups; **, p <0.01, *** p <0.001, **** p <0.0001 for comparison of the group treated with milonine and the LPS group.

the saline group. On the other hand, the LPS-challenged animals treated with milonine showed a reduction in the protein content in the bronchoalveolar fluid (Figure 3a). Related to the protein content is the pulmonary edema that was measured by the lung wet/dry ratio (w/d). The LPS group presented an increase in the lung w/d ratio as compared to the saline group. Meantime, the milonine group showed significant inhibition (p <0.05) of the lung w/d ratio as compared to the LPS group, being close to the baseline level of the saline group (Figure 3b).

Milonine decreases the pro-inflammatory cytokine level in BALF and serum of LPS-induced acute lung injury in mice

The cytokine levels of IL-1ß, TNF-α and IL-6 were evaluated in the bronchoalveolar lavage and in the serum of all animal groups and, as shown in figure 4, the animals of the LPS group presented a significant increase in these cytokines into the BALF when compared to the animals of the saline group. The LPS-challenged animals and treated with milonine presented a decrease of all these cytokines into the BALF (Figure 4a, c, e). At a systemic level, the LPS group showed significant increase of IL-1ß and IL-6 when compared to the saline group. However, only the level of IL-6 was reduced in the LPS-challenged animals and treated with milonine. Interestingly, the level of TNF-α in both compartments was not changed among the animal groups (Figure 4b, d and f).

Milonine decreases the expression of Akt and p-65 NF-κB

To investigate the mechanism by which milonine reduces lung damage, BALF cells were analyzed for the expression of the intracellular protein Akt and p65 NF-κB. For the Akt expression, it was observed that the stimulus with LPS increased its frequency (p <0.001) (Figure 5a and b) and intensity (p <0.0001) (Figure 5c) when compared to the saline group. However, the milonine treatment was able to down-modulate its frequency (p <0.0001) and the intensity (p <0.0001) (Fig. 5b and c). Akt activation leads to

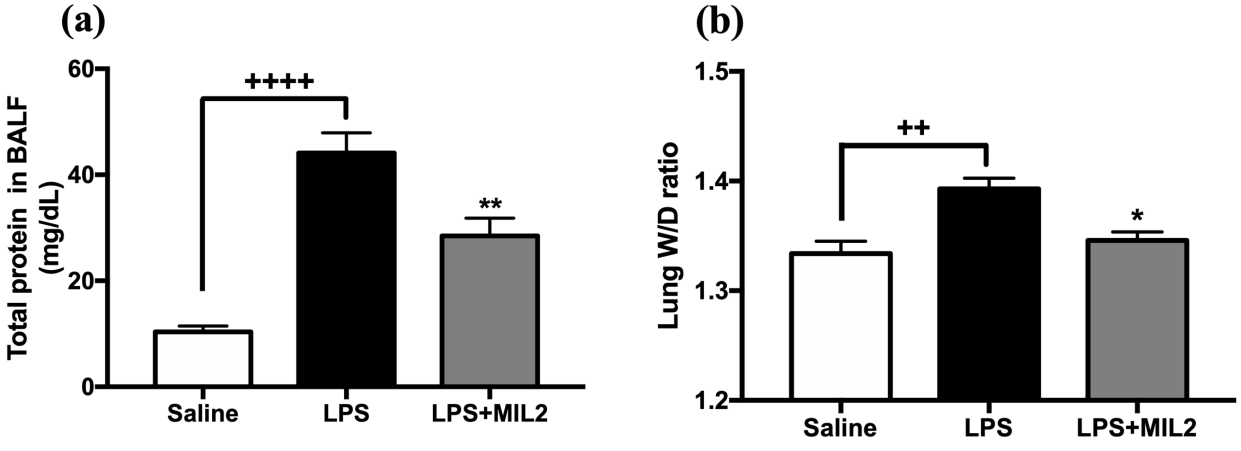

Figure 3. Effect of milonine on the microvascular protein permeability and pulmonary edema. BALB/C mice (n = 6) were LPS-challenged and treated with milonine (2.0 mg/kg; LPS+MIL2; p.o) or vehicle (LPS group). The saline group was saline-challenged. The bronchoalveolar lavage fluid (BALF) was recovered 24h after the challenge and evaluated: the total protein concentration (a) and the lung w/d ratio (b). The results represent the mean ± SEM and submitted to the one-way ANOVA analysis of variance, followed by the Turkey post-test ++ p <0.01, ++++ p <0.0001 comparison between LPS and saline group; * p <0.05 and ** p <0.01 for comparison of the group treated with milonine and the LPS group.

NF-κB activation and, as expected, the milonine treatment also decreased the frequency (p <0.0001) and intensity (p <0.05) of p65 NF-κB expression in these cells when compared to the LPS group (Fig. 6d-f).

Milonine binds to the MD2 groove of the TLR-4 complex

The interaction of milonine and the extracellular TLR4/MD2 complex was verified by molecular docking analysis. The results showed that milonine had a binding energy of -43.96 kcal/mol forming hydrophobic interactions with binding affinity to the amino acids Ile124 and Phe126 of the MD2 groove (Figure 6).

DISCUSSION

Acute lung injury (ALI) is a life-threatening condition with an inflammatory origin caused by many stimuli, such as viral or bacterial pneumonia. The pulmonary inflammatory response is characterized by inflammatory cell migration, pulmonary edema, and pro-inflammatory cytokine production, which leads to respiratory failure. The disease has currently no effective treatments, therefore, is considered a serious clinical problem with high morbidity and mortality, arousing great interest in therapeutic approaches (Rezoagli et al. 2017, Matthay et al. 2012, Bellani et al. 2016). In this context, milonine, a morphinane

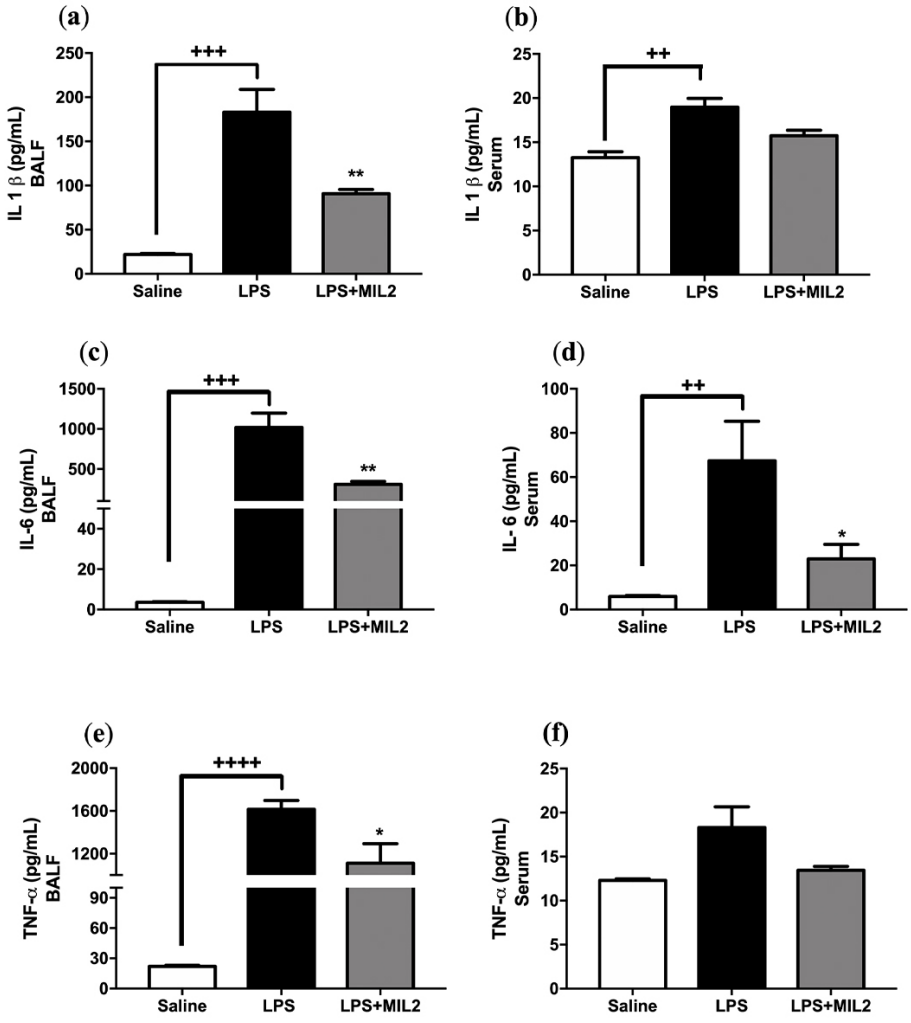

Figure 4. Effect of milonine on the level of proinflammatory cytokines in BALF and serum. BALB/C mice (n = 6) were LPS-challenged and treated with milonine (2.0 mg/kg; LPS+MIL2; p.o) or vehicle (LPS group). The saline group was saline-challenged. The bronchoalveolar lavage fluid (BALF) and blood were recovered 24h after the challenge to measure the cytokine levels: (a, b) IL-1ß; (c, d) TNF-α; (e, f) IL-6; in BALF and serum, respectively. The results represent the mean ± SEM and submitted to the one-way ANOVA analysis of variance, followed by the Turkey post-test ++ p <0.01, +++ p <0.001, ++++ p <0.0001 comparison between LPS and saline group; * p <0.05 and ** p <0.01 for comparison of the group treated with milonine and the LPS group.

alkaloid recognized for having analgesic, antitussive, vasorelaxant, antinociceptive and anti-inflammatory activities (Cavalcante et al. 2011, Alves et al. 2017, Silva et al. 2017, Rice 2014), was tested in lipopolysaccharide (LPS)-induced ALI in mice to identify its anti-inflammatory property and define its mechanisms of action.

As a result, milonine treatment attenuated the lung edema, tissue damage and inflammatory cell migration; inhibited the production of inflammatory cytokines and the expression of Akt and NF-κB dependent on the binding to the TLR4/MD2 complex. Indeed, milonine treatment attenuated the inflammatory cell migration to the alveolar cavity, which was confirmed by the histological analysis. The reduction of this cell population to the lung was dependent on neutrophils, indicating a protective effect of milonine in ALI. In a previous study, Silva and collaborators (2017) demonstrated that milonine was able to inhibit the carrageenan-induced peritonitis by decreasing the polymorphonuclear cell migration and TNF-α and IL-1β production pointing out the

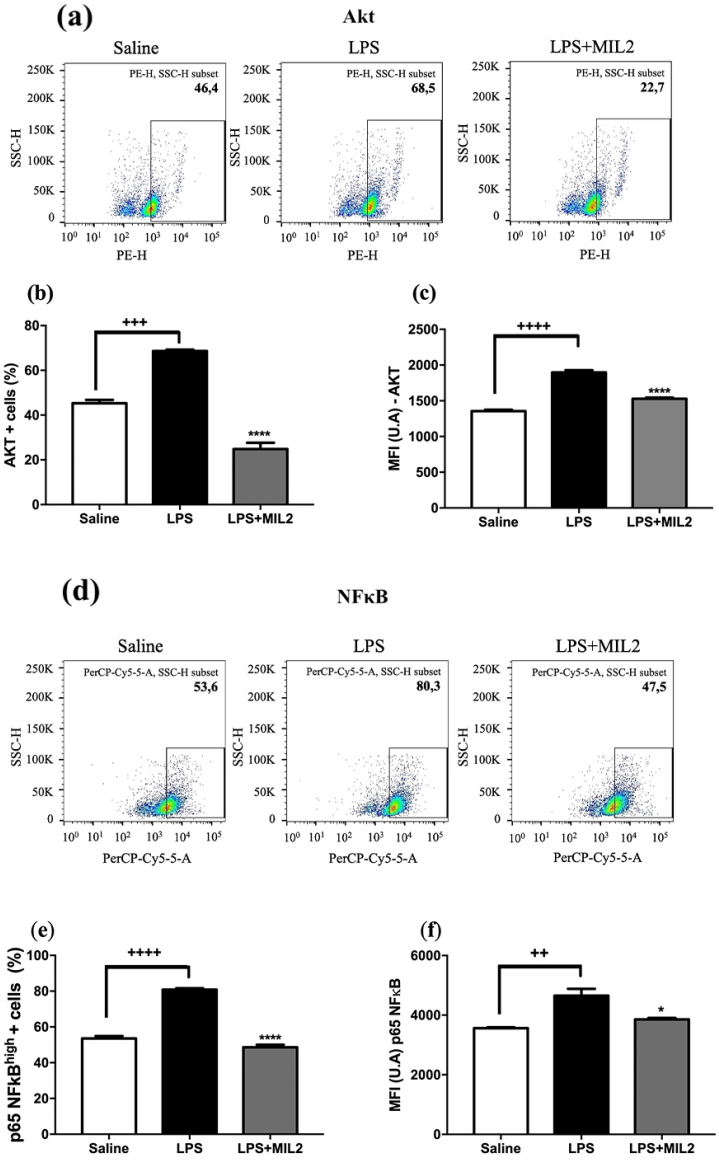

Figure 5. Effect of milonine on the expression of Akt and p65 NF-κB. BALB/C mice (n = 6) were LPS-challenged and treated with milonine (2.0 mg/kg; LPS+MIL2; p.o) or vehicle (LPS group). The saline group was saline-challenged. The BALF inflammatory cells were recovered 24 h after the challenge, and then analyzed by the flow cytometry technique: (a) cell dotplot analyses, Akt antibody label. (b) and (c) frequency of activation and fluorescence intensity (MFI) of the anti-Akt labeling, respectively. (d) cell dotplot analyzes, p65NF-κB antibody label. (e) and (f) frequency of activation and MFI of the anti-p65NF-κB label, respectively. The results were expressed as mean ± SEM and submitted to the one-way ANOVA analysis of variance, followed by the Turkey post-test +++ *p* <0.001, ++++ *p* <0.0001 in comparison between LPS and baseline groups; * *p* <0.05, ** *p* <0.0001 for comparison of the treated group with the LPS group.**

anti-inflammatory property of the alkaloid. Thus, the intense influx of neutrophils to the lung in ALI is considered the hallmark of the disease due to the degranulation process that releases toxic products as proteinases, reactive oxygen species and cationic polypeptides. The release of these mediators promotes an increase of local oxidative stress contributing to the destruction of the alveoli architecture, damage of the type I or II pneumocytes and disruption of the surfactant layer (Zemans et al. 2009, Grommes & Soehnlein 2011, Yang et al. 2021).

The milonine treatment also attenuated the lung protein exudate and the lung edema. In previous studies, we demonstrated the anti-edematogenic effect of the alkaloid in phlogistic agent-induced paw edema (Silva et al. 2017). In these experimental models, milonine reduced the paw edema induced by LPS, prostaglandin E2, and bradykinin, independently of the serotonin-induced paw edema. However, the alkaloid reduced the nociceptive behavior of paw licking induced by formalin at the inflammatory phase of the test indicating its anti-nociceptive effect. Also, the alkaloid prevented peritoneal vessel changes and edema formation induced by the acetic acid (Silva et al. 2017, Alves et al. 2017). Thereby, taken all these data together, we conclude that the alkaloid presents anti-inflammatory and anti-edematogenic effects by decreasing the inflammatory cell migration and protein exudate to the inflamed site.

The development of ALI is dependent on mediators that contribute to the increase of the vessel permeability and leukocyte recruitment. Among them are the cytokines as TNF-α, IL-1β and IL-6. The TNF-α and IL-1β induce the neutrophil accumulation at the lung and promote the release of other cytokines including the IL-6. The increase of the IL-6 level into the serum is an indicator of severe inflammatory process with multiple organ fail in ALI (Bhatia & Moochhala

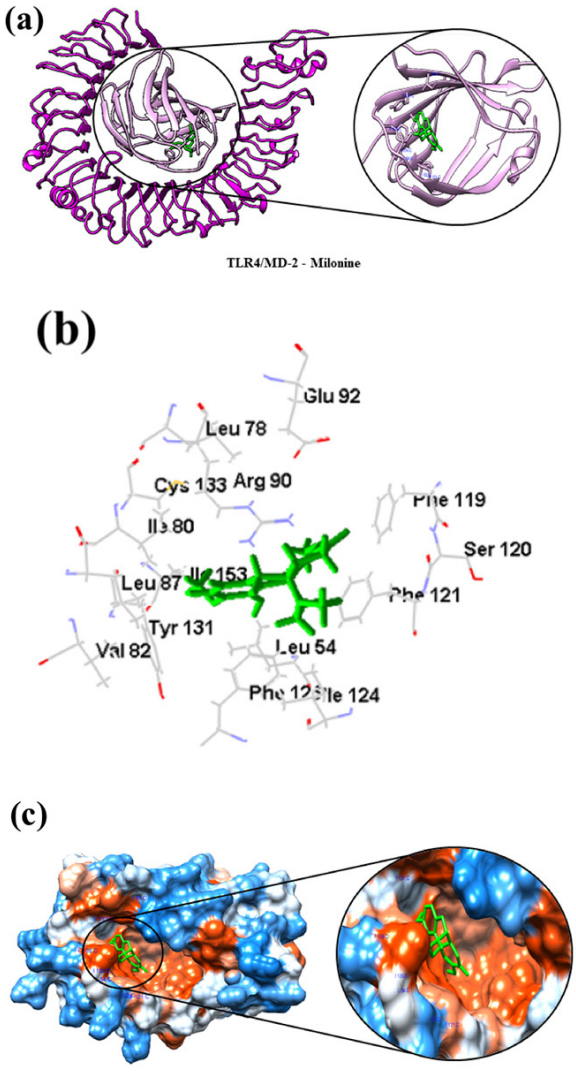

Figure 6. Structure of the TLR4/MD2 complexed with milonine. (a) General structure of the TLR4/MD2-milonine complex. The TLR4 part is colored in dark lilac, the MD2 part is colored in light lilac, and milonine in green. (b) 3D interactions of the MD2 complex and milonine. (c) Representation of the surface of the MD2. Positively and negatively charged residues are colored in blue and orange, respectively.

2004, Ciesla et al. 2005, Goodman et al. 2003, Lin et al. 2018). Therefore, downregulating these cytokines leads to improvement of the inflammatory process in this illness. Then, the results of this study showed that milonine reduced all three mentioned cytokines into the BALF and IL-6 at a systemic level, indicating the

alkaloid as a promising drug to be further tested in clinical trials (Swaroopa et al. 2016). Other studies demonstrated that the morphinane alkaloid sinomenine showed similar results in ALI experimental models with reduction of these inflammatory cytokines (Liu et al. 2018, Li et al. 2013).

To define the mechanism of action of milonine in the ALI model we looked at the signaling pathways implied at the cytokine gene activation. One of these pathways is related to the TLR4/MD2 complex that plays a crucial role in the defense of the host against pathogenic microorganisms. The LPS, the compound of gram-negative bacteria, is the agonist of the TLR4/MD2 complex by triggering a cascade of intracellular events that culminates with the NF-κB activation (Chen & Hua 2020). Several intracellular kinases are implied at this signaling pathway as Akt and p38MAPK, that control the NF-kB inhibitor (IkB) phosphorylation and nuclear translocation, and perform cytokine overproduction (Yum et al. 2001, Strassheim et al. 2004, Nie et al. 2019).

Finally, we analyzed the alkaloid effect on the Akt/NF-κB signaling pathways and, as expected, cells from the LPS group presented upregulation of both signaling routes. However, milonine treatment induced downregulated both signaling pathways. In addition, the molecular docking studies indicated that the alkaloid inhibited these intracellular events by binding to the MD2 groove at the amino acids Ile124 and Phe126 via hydrophobic interactions. Other studies demonstrated that morphinane compounds as dextromethorphan, naltrexon, sinomenine, oxycodone, inhibited the NF-κB signaling pathway in inflammation protocols (Xu et al. 2020, Chen et al. 2013, Qin et al. 2016, Li et al. 2020). It was also reported that some morphinans decrease the NFκB expression by reducing the TLR4 activation (Li et al. 2020, Xu et al. 2020, Zeng & Tong 2020). Therefore, all the data described above pointed out milonine as a potential drug to treat acute lung injury induced by LPS. However, additional studies must be carried out to clarify this hypothesis.

CONCLUSION

The present study reported, for the first time, the mechanism of action of milonine on lipopolysaccharide-induced acute lung injury. The alkaloid has an anti-inflammatory effect by downregulating intracellular Akt activation and the p65 NF-kB phosphorylation dependent on its binding affinity to the MD2 groove of the TLR4/MD2 complex. This interaction inhibits the IL-1-β, TNF-α, IL-6 production and inflammatory cell migration to the lung. Therefore, milonine is a morphinane alkaloid with anti-inflammatory properties, and its mechanism of action was described in cells from acute lung injury induced by lipopolysaccharide.

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