Milonine Protects Against Acute Lung Injury By Modulating The Akt And NF- κ B Signaling Pathway

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Research Article

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Abstract

Acute lung injury (ALI) is an inflammation that triggers acute respiratory distress syndrome (ARDS) with perialveolar neutrophil infiltration, alveolar-capillary barrier damage, and lung edema. Activation of the toll-like receptor 4 complex and its downstream signaling pathways are responsible for the cytokine storm and cause alveolar damage on ARDS. Due to the complexity of inflammatory events on ALI, a defined pharmacotherapy has not been established. Thus, this study aimed to evaluate the anti-inflammatory potential of milonine, an alkaloid of *Cissampelos sympodialis* Eichl, in an ALI experimental model. BALB/c mice were lipopolysaccharide (LPS)-challenged and treated with milonine at 2.0 mg/kg. Twenty-four hours later, the bronchoalveolar lavage fluid (BALF), peripheral blood, and lungs were collected for cellular and molecular analysis. The milonine treatment decreased the inflammatory cell migration (principally neutrophils) to the alveolar cavity, the protein exudate, the pulmonary edema, and the level of pro-inflammatory cytokines (IL-1 β , IL-6, TNF- α) into the BALF. The systemic level of IL-6 level was also reduced. In the lung tissue, milonine reduced the bronchoalveolar damage. The milonine docking analyzes demonstrated that the molecule formed hydrophobic interactions with the amino-acids Ile124 and Phe126 of the TLR4/MD2 groove. Indeed, the anti-inflammatory effect of milonine was due to the negative regulation of cytoplasmic kinase-Akt and NF- κ B by interacting with the TLR4/MD2 complex. Therefore, milonine is an effective inflammatory modulator by blocking the interaction of the LPS-TLR4/MD2 complex and downregulating the intracellular inflammatory pathway axis being a potential molecule for the treatment of ALI.

Introduction

The acute lung injury (ALI) and its most severe form, acute respiratory distress syndrome (ARDS), are inflammatory lung diseases that cause respiratory failure. Their development may be due to a primary infection, which is the case of pneumonia that can be bacterial, viral or fungal. Indirect lung lesions affect the lung in a secondary way, being more common in severe sepsis. The pathological characteristic of ALI/ARDS 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 [1, 2].

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; Mazgaen and Gurung 2020). Thus, the TLR4/MD2 activation occurs when the LPS is transferred to a hydrophobic groove of the MD2, which leads to a homodimerization of TLR4 [5] leading to a pro-inflammatory signal intracellular chain and activation of phosphatidylinositol-3-kinase (PI3K) and protein kinase B (Akt or PKB) [6]. Akt has been reported to act on the nuclear factor- κ B (NF- κ B) 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-1 β [7–9], promoting the progression of ALI. Thus, the blockage of the TLR4/MD2-mediated NF- κ B signaling pathway can attenuate the LPS-induced ALI.

Although it has been advancing in the study of the pathophysiology of ALI/ARDS, the effectiveness of therapeutic approaches is still limited, the mortality rate of patients with ALI remains high (around 40–60%), and a definitive pharmacological treatment has not yet been established [10]. In this scenario, new treatments must be developed to improve clinical results and reduce the mortality rate of patients.

Milonine, an 8,14 dihydromorfinandeinonic alkaloid (Fig. 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 [11, 12]. In cytotoxic studies, using hepatocytes, fibroblasts, and Vero lineages, the IC₅₀ varied between 100–400Mm [13, 14]. Besides, *in 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 reduce blood pressure in normotensive rats [15]. 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 [16]. Milonine also inhibited paw edema and nociception induced by phlogistic agents and these effects were related to PGE₂, bradykinin, TNF- α , and IL-1 β inhibition [17].

In this form, considering the lack of specific therapies and high mortality in ALI/ARDS, and the fact that milonine is involved in cellular and vascular events in the inflammatory process, we hypothesize that the alkaloid could be a potential candidate to become a medicine to treat 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- and intracellular signaling pathways and its TLR4/MD2 interaction.

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 $\pm$ 2 °C, with regular ventilation, as well as drinking water and sufficient food throughout the experimentation period.

Milonine

The alkaloid - milonine was isolated from the *Cissampelos sympodialis* leaf hydroalcoholic extract and chromatographed under a neutral alumina column, and the fractions were obtained with a preparative

thin layer chromatography [11]. Professor José Maria Barbosa Filho of Federal University of Paraiba supplied the purified alkaloid. The milonine solution was obtained by dissolving 1 mg of the alkaloid in 50 μ L of 1N HCl and in 800 μ L of NaCl 0.9%. The pH was adjusted to 7 with 1M NaOH and the volume of the solution was completed to 1000 μ L with NaCl 0.9%

Animal groups

The animals (n = 6/group) were treated, orally (p.o), with milonine at 2 mg/kg (MIL 2 group), one hour after the phlogistic agent challenge. 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 [16, 17].

The acute lung injury experimental model

The acute lung injury was developed as follows: animals were previously anesthetized with 50 μ L 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 μ L of sterile saline. The saline group received only 40 μ L of the sterile vehicle. The animals were euthanized 24h after the LPS-challenge by anesthetic overdose (300 μ L) to obtain the following biological material: the bronchoalveolar lavage fluid (BALF), peripheral blood from the brachial plexus, and lungs [18].

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-1 β , TNF- α and IL-6, were measured in the BALF and in the peripheral blood (from the brachial plexus) 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 μ m 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 $\times$ 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- κ B expression of cells from BALF. The 5×10^5 cells/mL were incubated with specific anti-mouse Akt PE conjugated or p65NF κ B 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 [19]. 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 [20].

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 $\pm$ 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 (Fig. 2A-D). The histological analyzes of the lung tissues of

the sick animals showed that the alveoli were filled with inflammatory cells (*), in contrast to the lung tissues of the animals of the saline group which showed absence of inflammatory cells in the alveolar spaces with preservation of the bronchoalveolar architecture. In contrast, the animals treated with milonine showed minimal destruction of lung tissue caused by the LPS-challenged as well as a reduction in the number of inflammatory cells in the alveolar spaces (Fig. 2E). In fact, the morphometric data confirm the reduction of the inflammatory process (Fig. 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 the saline group. On the other hand, the animals challenged with LPS and treated with milonine showed reduction in the protein content into the bronchoalveolar fluid (Fig. 3A). Thus, the pulmonary edema of each animal of all groups was measured by the ratio between the wet and dry lung weights (w/d). The lung w/d ratio of the LPS group was increased as compared to the lung w/d ratio of 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 (Fig. 3B).

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

The level of cytokines such as IL-1 β , TNF- α and IL-6 was evaluated in the bronchoalveolar lavage and in the serum of all animal groups and, as shown in Fig. 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 animals challenged with LPS and treated with milonine presented a decrease of all these cytokines into the BALF (Fig. 4A, C, E). At a systemic level, the LPS group showed significant increase of IL-1 β and IL-6 when compared to the saline group. However, only the level of IL-6 was reduced in the animals challenged with LPS and treated with milonine. Interestingly, the level of TNF- α in both compartments was not changed among the animal groups (Fig. 4B, D and F).

Milonine decreases the expression of Akt and p-65 NF- κ B

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- κ B. For the analysis of Akt expression, it was observed that the stimulus with LPS increased its frequency ($p < 0.001$) (Fig. 5A and B) and intensity ($p < 0.0001$) (Fig. 5C) of expression when compared to the saline group. Also, the treatment with milonine was able to modulate the frequency ($p < 0.0001$) and the intensity ($p < 0.0001$) of Akt expression in cells (Fig. 5B and C). Akt activation leads to NF- κ B activation and, as expected, the treatment with milonine also decreased the frequency ($p < 0.0001$) and intensity ($p < 0.05$) of p65 NF- κ B expression in these cells when compared to the LPS group (Fig. 6D-F).

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

To investigate a possible mechanism of action of milonine at the extracellular TLR4/MD2 complex, the interaction of the alkaloid and this receptor complex was verified by molecular docking analysis. The results demonstrated 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 (Fig. 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, being considered a serious clinical problem with high morbidity and mortality, arousing great interest in new therapeutic approaches [1, 10, 21]. In this context, milonine, a morphinane alkaloid recognized for having analgesic, antitussive, vasorelaxant, antinociceptive and anti-inflammatory activities [15–17, 22], 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- κ B 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-1 β production pointing out the 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 [23–25].

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 [16, 17]. 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-1 β and IL-6. The TNF- α and IL-1 β 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 [26–29]. 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 [30]. Other studies demonstrated that the morphinane alkaloid sinomenine showed similar results in ALI experimental models with reduction of these inflammatory cytokines [31, 32].

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- κ B activation [33]. Several intracellular kinases are implied at this signaling pathway as Akt and p38MAPK, that control the NF- κ B inhibitor (I κ B) phosphorylation and nuclear translocation, and perform cytokine overproduction [7–9].

Finally, we verify the alkaloid effect on the Akt/NF- κ B signaling pathways and, as expected, cells from the LPS group presented upregulation of both signaling routes. However, milonine treatment induced downregulation of both routes. In accordance with these data, morphinane compounds as dextromethorphan, naltrexon, sinomenine, oxycodone, inhibited the NF- κ B signaling pathway in inflammation protocols [34–37]. Thus, by using the molecular docking methodology, we demonstrated that the alkaloid inhibited these intracellular events by binding with the amino acids Ile124 and Phe126 amino acids of the MD2 groove via hydrophobic interactions. This binding interaction probably inhibits the LPS binding to its receptor, however, additional studies should be carried out to clarify this hypothesis.

Conclusion

The present study reported, for the first time, the mechanism of action of the alkaloid milonine on LPS-induced ALI. The alkaloid has anti-inflammatory effect by regulating intracellular kinase Akt activation as well as the p65 NF- κ B phosphorylation dependent on its binding affinity with the MD2 groove of the TLR4/MD2 complex. This effect provoked the inhibition of pro-inflammatory cytokines as IL-1- β , TNF- α , IL-6, being the last one downregulated at a systemic level. Therefore, milonine is one of the morphinane alkaloid described with anti-inflammatory properties.

Declarations

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Conflicts of interest/Competing interests

The authors declare that they have no conflict of interest.

Availability of data and material

Not applicable

Code availability

Not applicable

Authors' contributions

LRB, LKDPF, LAMPF and MRP outlined, designed the experiments; LRB, LKDPF, LAMPF, CIDV, JBO, LML performed the experimental model of ALI and ELISA protocol; LRB, LKDPF and LAMPF performed the flow cytometry protocol; AFA and RSA performed the lung histology; LRB, LAMPF, LKDPF and MRP analyzed the data; MSM and MTS performed the molecular docking simulations; JMBF was responsible for the alkaloid Milonine extraction, purification and make available for the study; LRB and MRP wrote, reviewed and edited the manuscript.

Ethics approval and Consent to participate

All experimental procedures were conducted by following the guidelines of the National Animal Experimentation Control Council (CONCEA) and adhered strictly to the guidelines of the Brazilian Law (n°. 11.794/ 2008) which establishes rules for use and care of laboratory animals. The Committee on Ethics in Uses of Animal (CEUA/UFPB) approved the experimental procedures under certificate n° 7316150420. The used animals 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.

Consent for publication

The manuscript is approved by all authors for publication.

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Figures

Figure 1

Three-dimensional structure of milonine.

Figure 2

BALB/C mice ($n = 6$) were LPS-challenged and treated with milonine (2.0 mg/kg; MIL 2; 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 μ m). Asterisks (*) indicate cell infiltration, (a) pulmonary alveoli, and (b) bronchioles. The results represent the mean $\pm$ E.P.M 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.

Figure 3

BALB/C mice ($n = 6$) were LPS-challenged and treated with milonine (2.0 mg/kg; MIL 2; 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 $\pm$ E.P.M 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.

Figure 4

BALB/C mice (n = 6) were LPS-challenged and treated with milonine (2.0 mg/kg; MIL 2; 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-1 β ; (C, D) TNF- α ; (E, F) IL-6; in BALF and serum, respectively. The results represent the mean $\pm$ E.P.M 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.

Figure 5

BALB/C mice ($n = 6$) were LPS-challenged and treated with milonine (2.0 mg/kg; MIL 2; 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- κ B antibody label. (E) and (F) frequency of activation and MFI of the anti-p65NF- κ B label, respectively. The results were expressed as mean $\pm$ E.P.M. 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.

Figure 6

The 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.

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