Research Article

Effects of D-002 (Beeswax Alcohols) on Lung Leukocyte Infiltration and Lipid Peroxidation in Rats with Carrageenan-Induced Pleurisy

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ABSTRACT

Inflammation, a physiological response to noxious stimuli, is implicated in diseases. D-002, a mixture of beeswax alcohols with anti-inflammatory and antioxidant effects, has been shown to reduce the pleural exudates in rats with carrageenan-induced pleurisy (CAP), a model characterised by lung neutrophil infiltration and subsequent lipid peroxidation. The effects of D-002 on these events, however, had not been explored yet. Here we investigated the ability of D-002 to inhibit neutrophil infiltration and lipid peroxidation in the lungs of rats with CAP. Rats were randomized into a negative vehicle control and six carrageenan-injected groups: one positive vehicle control, four treated with D-002 (50, 200, 400 and 800 mg/kg) and one with aspirin (150 mg/kg). Acute oral treatment with D-002 (50 – 800 mg/kg) decreased significantly, dose-dependently and almost totally neutrophil infiltration (27.4% to 99.9%) and malondialdehyde (MDA) (72.5% - 96.3%) levels in lung tissues compared to the positive control. Also, D-002 reduced significantly, but moderately (≅ 31%), the volume of the pleural exudates. Aspirin (150 mg/kg) reduced markedly (90.2%) neutrophil infiltration, not MDA, in lung tissues, and lowered the volume (47%) of the pleural exudates. In conclusion, this study demonstrates that acute oral D-002 treatment was effective for ameliorating neutrophil infiltration and lipid peroxidation in the lung tissues of rats with CAP and confirms its ability for lowering the formation of CAP-induced pleural exudates. Taken together, these data enhance our understanding of the anti-inflammatory effects of D-002.

Keywords: beeswax alcohols**,** carrageenan-induced pleurisy, D-002, inflammation, lipid peroxidation, lung neutrophil infiltration.

INTRODUCTION

The inflammatory response implicates a chain of events that involves the selective and transient migration of different cells to the affected site, fluid exudation, and some form of resolution, which implicates the relative concepts of acute and chronic inflammation.[1] Acute inflammation, the first-line response that protects the organism against noxious stimuli, is highly regulated by the actions of pro- and anti-inflammatory mediators that allow resolution of the inflamed tissue and repair.[1,2] Failure on the resolution of acute inflammation, however, may lead to chronic inflammation, a process characterised by persistent signs of inflammation, tissue damage and impaired function that has been associated to several chronic diseases.[3-8]

Inflammation involves several mediators that elicit vasodilatation, increased capillary permeability and neutrophils migration from the blood towards to the inflammatory site.[6] In particular, neutrophils (polymorphonuclear leukocytes –PMN-) are not only the major effectors of acute inflammation, but also contribute to chronic inflammatory conditions and adaptive immune responses, which may promote protective or pathological immune responses at different sites. Currently, a role of PMN in chronic inflammation is accepted and effects on this target might provide new approaches to prevent or treat chronic inflammatory conditions.[9-11]

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used to treat acute and chronic inflammatory conditions, and aspirin for vascular prevention. Inhibition of cycloxygenase (COX) pathway is responsible for the major anti-inflammatory effects of NSAIDs since by inhibiting prostaglandins synthesis, they lower vasodilation and pain, and by lowering blood flow to the inflamed site, they help lower the edema.[12-14]

Nevertheless, prostaglandins are relevant for the defensive and repair mechanisms of the gastrointestinal mucosa. The inhibition of prostaglandins synthesis by NSAIDs makes this mucosa prone to be damaged by internal aggressive factors like gastric acid and bile and less efficient to be repaired.[15–17] Also, selective COX_2-inhibitors are linked with an increased cardiovascular risk.[15 – 17] This background supports the search for anti-inflammatory substances safer than NSAIDs.

Rodent models of inflammation have been useful for the study of inflammation and for the evaluation of substances with anti-inflammatory potential.[2] Carrageenan-induced pleurisy is a model of acute inflammation in which the pleural inflammatory response induced by carrageenan triggers the release of histamine, bradykinin, substance P, and prostaglandins; followed by exudation, cytokines release, lung neutrophil infiltration and increased lipid peroxidation.[18,19]

D-002, a mixture of six higher aliphatic beeswax alcohols, has been shown to produce anti-inflammatory and antioxidant effects.[20] Oral pretreatment with D-002

reduced the volume and leukotriene B_4 (LTB_4) concentrations of pleural exudates in rats with carrageenan-induced pleurisy.[21] Despite of PMN infiltration and lipid peroxidation represent characteristic features of this model, the effects of D-002 on these targets had not been investigated before.

In light of these sounds, this study was undertaken to investigate the ability of D-002 to inhibit neutrophil infiltration and lipid peroxidation in the lungs of rats with carrageenan-induced pleurisy.

MATERIALS AND METHODS

Animals: Male Sprague Dawley rats (250-300 g) were purchased from the National Center for Laboratory Animal Production (CENPALAB, Havana, Cuba) and adapted for 7 days to the following conditions: temperature (22-23°C), relative humidity (55-60%) and 12 hours dark/light cycles. Food and water were freely supplied.

The experiments were performed after the approval of the Institutional Board for animal use and were carried out in accordance with Cuban Guidelines for the care of laboratory animals and the Cuban Code of Good Laboratory Practice (GLP).

Administration and dosage

The batch of D-002 used in the experiments was supplied by the Plants of Natural Products (National Center for Scientific Research, Havana City, Cuba) and used after corroborate its quality criteria. Batch composition, assessed with a validated gas chromatographic method,[22] was as follows: 1-tetracosanol (5 %), 1-hexacosanol (10.2 %), 1-octacosanol (14 %), 1-triacontanol (34.21 %), 1-dotriacontanol (24.24 %) and 1-tetratriacontanol (3.03 %). Purity (total content of these six alcohols) was 90.7 %.

D-002 was suspended in 2% Tween 20/water vehicle and aspirin in 10% acacia gum/water vehicle.

Rats were randomized into seven groups (10 rats per group): a negative vehicle control and six carrageenan-injected groups: one positive vehicle control, four treated with D-002 (50, 200, 400 and 800 mg/kg), one with aspirin (150 mg/kg). All treatments were administered acutely by intragastric intubation (5 mL/kg) one hour before the induction of carrageenan-induced pleurisy.

Induction of Carrageenan-induced pleurisy

In previously anesthetized rats pleurisy was induced by an intrapleural injection of 0.3 mL of 1% carrageenan saline solution (0.9 % NaCl) into the right pleural space. The rats were sacrificed with an overdose of sodium thiopental solution 5 hours after injection of carrageenan, when the pleural cavity was washed with 1mL of 3.15% sodium citrate dissolved in sterile saline solution. The pleural exudates were then collected and their volumes measured. Any exudates with blood collection were discarded.[23]

Preparation of lung samples

When pleural exudates were collected, the lungs were simultaneously dissected out and samples taken for subsequent histological and biochemical procedures. For the microscopic evaluation lung tissue samples were fixed in 10% formaldehyde, dehydrated by graded ethanol and embedded in paraffin. Sections obtained with a microtome were deparaffinized with xylene and stained with hematoxylin and eosin (H&E). In turn, for the assessment of MDA concentrations in the lung tissue aliquots (500 mg) were gently homogenized with an Ultra-Turrax homogenizer in 150 mmol/L Tris/HCl buffer (pH 7.4) place into an ice-cold bath. Samples were stored to -20 °C up to use.

Assessment of PMN infiltration

Histological counting of neutrophils in lung sections was performed in 10 randomly fields (up-down-left-right) of each animal and group in a Primo Star Carl Zeiss microscope.

MDA concentrations in the lung tissue

MDA levels in the lung tissue were determined as an indicator of lipid peroxidation. in terms of thiobarbituric acid (TBA) reagent substances (TBARS) according to Ohkawa.[24] For that, a 100 μL aliquot of the lung homogenates was added to a reaction mixture containing 200 μL of 8.1% (w/v) sodium dodecyl sulfate (SDS), 1.5 mL of 20% (v/v) acetic acid (pH 3.5), 1.5 mL of 0.8% (w/v) TBA acid and 700 μL distilled water. Samples were then boiled for 1 hour at 95°C and centrifuged at 3,000 ×g for 10 min. To prevent the production of TBA reactants 50 µL of 1 mmol/L butylated hydroxytoluene was added to the mixture. After cooling, 5 mL of n-butanol:piridine (15:1 v/v) mixture were added, stirring vigorously with vortex, and centrifuged at 4000 rpm for 20 min. The absorbance of the supernatant was measured using spectrophotometry (Genesys 10 UV) at 534 nm. TBARS concentrations were determined from a standard curve of MDA bis-(dimethyl acetal) and reported as nmol MDA/mg protein.

Protein concentration was measured by using a modification of the Lowry method.[25]

Assessment of oedema inhibition

Pleural exudates homogenized in 1 mL sodium citrate (3.15 %) were collected and measured. The percentage of oedema inhibition was calculated as follows:

$$Inhibition = 100 - \left(\frac{\Delta VEt \times 100}{\Delta VEc}\right)$$

Where

ΔVE_t: difference between the average volume of exudate in each treated group and that of the negative control

ΔVE_c: difference between the average volume of exudate in the positive control and that of the negative control

Statistical analyses

Comparisons among groups were done with the Kruskal Wallis test, paired comparisons between each treated and control groups with the Mann-Whitney U test. Statistical significance was chosen for $\alpha = 0.05$. Data were processed with the Statistics Software for Windows (Release 6.1 Stat Soft Inc, Tulsa OK, USA). Relation doses/effect was performed with lineal regression and correlation test using a Primer of Biostatistics program (Stanton A, Glantz; Copyright (c) 1992, McGraw-Hill, Inc Version 3.01).

RESULTS

Pre-treatment of rats with single oral doses of D-002 (50-800 mg/kg) decreased significantly and markedly PMN infiltration (27.4% to 99.9%) and lung tissue concentrations of MDA (72.5% - 96.3%), in a dose-dependent manner ($p< 0.001$; $r = 0.999$ for PMN infiltration, $p< 0.05$; $r = 0.986$ for MDA concentrations) as compared to the positive control values (Table 1).

Also, D-002 reduced significantly the volume of the pleural exudates (≅ 31%). The maximal effective dose for lowering the edema was 400 mg/kg, as 800 mg/kg did not cause greater decreases (Table 2).

Aspirin (150 mg/kg) reduced markedly (90.2%) neutrophil infiltration and unchanged MDA concentrations in the lung tissues, whereas it lowered the volume (47%) of the pleural exudates (tables 1 and 2).

Table 1: Effects on the infiltration of neutrophils and malondialdehyde (MDA) levels on lung tissues in rats with carrageenan-induced pleurisy

Groups	Doses (mg/kg)	Neutrophils (numbers)	I (%)	MDA (nmol/mg protein)	I (%)
Negative control	—	24.01 ± 0.75***	—	2.86 ± 0.20***	—
Positive control	—	34.85 ± 0.77	—	3.95 ± 0.13	—
D-002	50	31.88 ± 1.03*	27.4	3.16 ± 0.29*	72.5
D-002	200	28.26 ± 0.81**	60.8	2.99 ± 0.25*	88.1
D-002	400	26.05 ± 0.94***	81.2	2.94 ± 0.22**	92.7
D-002	800	24.09 ± 0.47***	99.3	2.90 ± 0.16**	96.3
Aspirin	150	25.01 ± 0.53***	90.8	3.87 ± 0.20	7.3

I (%): inhibition percent; * p <0.05; ** p <0.01; *** p< 0.0001 Comparison with the positive control; (Mann WhitneyU test)

The negative control group was not injected with carrageenan and received orally the vehicle only; all the other groups were injected with carrageenan and received orally the respective treatments, such as vehicle (positive control), D-002 at different concentrations or aspirin

Table 2: Effects on the volume of pleural exudates in rats with carrageenan-induced pleurisy

Groups	Doses (mg/kg)	Volume (mL)	I (%)
Negative control	—	0.02 ± 0.01***	—
Positive control	—	1.52 ± 0.10	—
D-002	50	1.12 ± 0.07*	**26.7**
D-002	200	1.07 ± 0.07*	**30**
D-002	400	1.05 ± 0.05**	**31.3**
D-002	800	1.05 ± 0.03***	**31.3**
Aspirin	150	0.81 ± 0.08***	**47.3**

I (%): inhibition percent; ** p <0.01; *** p< 0.0001 Comparison with the positive control; (Mann WhitneyU test)

DISCUSSION

Here we investigated the inhibition by single oral doses of D-002 (50-800 mg/kg) of PMN infiltration and lipid peroxidation in the lung tissues of rats with carrageenan-induced pleurisy. We found marked and almost complete inhibition of both processes in the lung tissues that had not been reported before. In addition, we confirm the anti-edema effects of D-002 on this model, which is in agreement with previous findings.[21]

Carrageenan-induced pleurisy is a classical model of acute inflammation involving various types of chemical mediators of inflammation like vasoactive amines, complement fragments, prostaglandins, and cytokines. The injection of carrageenan into the pleural space is characterized by protein-rich fluid accumulation and PMN

leukocytes infiltration.[26] In our study, the injection of carrageenan into the pleural cavity of positive control rats was characterized by the accumulation of pleural fluid and by the infiltration of PMN and subsequent lipid peroxidation (as determined by MDA levels) in lung tissues. All these variables, with the exception of MDA concentrations in lung tissues, were significantly lowered by aspirin. These features agree with those reported for this model,[27,28] making valid our experimental conditions and the D-002 effects here reported.

The fact that acute oral administration of D-002 (50-800 mg/kg) practically abolished PMN infiltration and markedly reduced the concentrations of MDA in the lung tissue (reductions greater than 95% in both cases) supports the potential interest of this substance for the management of airway inflammatory conditions. Indeed, activated neutrophils are one of the main sources of reactive oxygen species (ROS) and reactive nitrogen species, which have been demonstrate to contribute to the pathogenesis of inflammatory lung diseases.[29]

In our study, we demonstrated that D-002 treatment prevented the formation of MDA, a good marker of lipid peroxidation, thus lowering the extent of oxidative stress in the lung tissue of rats with carrageenan-induced pleurisy. This finding matches well with the antioxidant effects of D-002 seen in experimental and clinical studies, which include the reduction of lipid peroxidation and protein oxidation, as well.[30-33]

Despite this is the first evaluation of the effects of D-002 on PMN infiltration and lipid peroxidation in the lung tissue in this model, our results are in line with those found for the administration of octacosanol, one of most abundant alcohols within D-002, which significantly reduced the neutrophils influx in mice with carrageenan-induced pleurisy. In that study the authors pointed that the mechanism of the anti-inflammatory effect of octacosanol appears to be partly linked with the inhibition of α2-adrenergic transmission and of pro-inflammatory cytokines-dependent pathways.[34]

On its side, the inhibition of the inflammatory edema induced by carrageenan by D-002 here seen agrees with data of an early study, which also demonstrated a D-002-induced reduction of the concentrations of leukotriene B_4 (LTB_4) in the pleural exudates.[21] In such regard, recent studies have shown that D-002 acts like a dual inhibitor of both 5-lipoxygenase (5-LOX) and cycloxygenase (COX) enzymes, with a predominant effect on the former,[35] which reinforces the findings of the early study.[21] Since leukotrienes (LTs), particularly LTB_4, are potent mediators of inflammatory and allergic reactions produced by 5-lipoxygenase (5-LOX),[36,37] the potential relevance of the effects of D-002 on respiratory targets merits further research.

The present results demonstrate that the ability of D-002 treatment for lowering neutrophil infiltration and lipid peroxidation in the lung tissues was marked, much more pronounced than effects on the pleural oedema induced by carrageenan in rats, which encourage to develop further studies of the effects of D-002 on other experimental models of lung inflammation.

CONCLUSION

This study demonstrates that acute oral D-002 treatment was effective for ameliorating neutrophil infiltration and lipid peroxidation in the lung tissues of rats with carrageenan-induced pleurisy, and confirms its anti-oedema effects on this model. Taken together, these data enhance our understanding of the anti-inflammatory effects of D-002 and support its potential for treating lung inflammatory pathologies.

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