



## Effect of D-002 on Models of Acute Inflammation

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### ABSTRACT

Oral treatment with D-002, a mixture of 6 high molecular weight aliphatic primary alcohols purified from beeswax, has been shown to produce anti-inflammatory effects in the carrageenan-induced pleurisy and in the cotton pellet granuloma models, but its effects on other models of acute inflammation have not been investigated. This study was aimed to investigate the anti-inflammatory effects of single oral doses of D-002 on dextran-, histamine- and serotonin-induced rat paw oedema models, and on the xylene-induced ear oedema in mice. Oral pre-treatment with D-002 (200, 400 and 800 mg/kg) significantly inhibited dextran-induced paw oedema ( $p < 0.001$ ) up to 71.4%. D-002 (800 mg/kg), not 200 or 400 mg/kg, inhibited significantly histamine - and serotonin-induced hind paw oedemas in rats by 57 ( $p < 0.01$ ) and 36% ( $p < 0.001$ ), respectively, while diphenhydramine (60 mg/kg) and serotonin (10 mg/kg) produced significant inhibitions of 60% ( $p < 0.05$ ) and 79% ( $p < 0.01$ ), respectively. Also, D-002 (200 and 400 mg/kg), not 50 mg/kg, significantly and dose-dependently ( $r = 0.951$ ,  $p < 0.05$ ) reduced by 50 % a xylene-induced oedema formation in mice ear. Thus, this study reveals that oral treatment with single doses of D-002 exhibited significant anti-inflammatory activity in experimental models of acute inflammation.

**Keywords:** Anti-inflammatory, dextran, D-002, histamine, serotonin, xylene.

### INTRODUCTION

Inflammation, a normal defensive mechanism of living tissues against noxious stimuli-induced aggression, involves several inflammatory reactions, like the synthesis and release of inflammatory mediators, activation and migration of leukocytes, increased vascular permeability and hemodynamic changes that lead to oedema formation, leukocytes infiltration and granuloma formation, all contributing to the resulting tissue damage.<sup>1-5</sup>

Although the specific chemical mediators involved in the inflammatory response vary according to the specific inflammatory process, amines [histamine (Hi), serotonin (5-HT)], lipids (prostaglandins –PG- and leukotrienes –LT-) and small peptides (kinins) are recognized as typical inflammatory mediators.<sup>4,5</sup>

Pathological inflammation is linked with many diseases, like cardiovascular diseases, asthma, chronic obstructive pulmonary disease, osteoarthritis, rheumatoid arthritis, benign prostate hyperplasia and cancer, among others.<sup>6,7</sup> Consequently, non-steroidal anti-inflammatory drugs (NSAIDs), used to treat inflammation and pain, are among the most commonly prescribed drugs world over, being prescribed in both acute and chronic conditions. Since arthritis, a major cause of adult disability, increases with age, many older subjects consume aspirin for vascular risk reduction and also some other new potential indications (chemoprevention of colorectal cancer, treatment of Alzheimer disease) have emerged, NSAID-use has been increasing and should increase greatly as a result of the aging of society.<sup>8,9</sup>

Despite their benefits, NSAIDs use is limited due to gastrointestinal side effects, ranging from dyspepsia to life-threatening bleeding from ulceration<sup>10,11</sup> associated with the NSAIDs-induced inhibition of PG synthesis and increased oxidative stress in the gastric mucosa.<sup>12-14</sup>

Also, although less gastrototoxic than traditional NSAIDs, the introduction of the selective inhibitors of cyclooxygenase (COX)-2 (coxibs) has been linked to an increase of cardiovascular risk.<sup>15</sup>

D-002 is a mixture of six higher aliphatic alcohols (C<sub>24</sub>, C<sub>26</sub>, C<sub>28</sub>, C<sub>30</sub>, C<sub>32</sub> and C<sub>34</sub> alcohols) purified from beeswax wherein triacontanol is the most abundant component.<sup>16</sup> Oral treatment with D-002 has been shown to produce anti-inflammatory effects in the carrageenan-induced pleurisy and in the cotton pellet granuloma models, lowering the pleural concentrations of LTB<sub>4</sub> in the carrageenan-pleurisy.<sup>17</sup>

Nevertheless, instead of producing gastric damage D-002 cause gastroprotective effects, mainly against ethanol and NSAID-induced ulcers,<sup>18-20</sup> which have been linked with the increased secretion of gastric mucus, improvement of mucus composition<sup>21,22</sup> and reduction of lipid peroxidation in the gastric mucosa.<sup>23</sup>

Despite this background, the anti-inflammatory effects of D-002 have received less attention than its gastroprotective effects, being limited to the report of its effects on carrageenan-pleurisy and cotton pellet granuloma models.<sup>17</sup>

In light of these issues, this study investigated the effects of D-002 on acute inflammation models, like dextran-induced rat paw oedema, Hi-induced rat paw oedema, 5-



HT-induced rat paw oedema and xylene-induced mouse ear oedema.

## MATERIALS AND METHODS

### Animals

Male Sprague Dawley rats (200-250 g) and adult male mice OF-1 (20-30g) were obtained from the National Centre for laboratory Animal Production (Cenpalab) and adapted for 7 days to experimental conditions: (22-23 °C, humidity 55-60%, 12 hours dark/light cycles), with free access to standard rodent pellet diet acquired in Cenpalab and tap water. These conditions were maintained over the whole study.

Experiments were carried out in accordance with Cuban guidelines for the care of laboratory animals and the Cuban Code of Good Laboratory Practice (GLP). The independent local ethic committee for animal use approved the protocol for these experiments.

### Treatments and dosage

D-002 (batch: 030040109), supplied by the Plants of Natural Products (National Center for Scientific Research), was used after corroborate its quality criteria. The composition of the batch, assessed with a validated gas chromatographic method,<sup>24</sup> was as follows: tetracosanol (7.1%), hexacosanol (11.2%), octacosanol (13.8%), triacontanol (31.4%), dotriacontanol (22.1%) and tetratriacontanol (2.5%). Purity (total content of these 6 alcohols) was 88%. D-002 was suspended in Tween 20/water (2 %) vehicle.

Serotonin (MERCK), histamine and dextran (BDH) were dissolved in saline, indomethacin (QUIMEFA) in sodium bicarbonate (5%), while diphenhydramine and cyproheptadine (QUIMEFA) were dissolved in arabic gum /water vehicle (1%) (VEL).

### Evaluation of anti-inflammatory effects

In all experiments, treatments were given as single oral doses. All control groups received Tween 20/water (2 %) vehicle.

### Dextran induced paw oedema

Acute inflammation was produced by the subplantar injection of 0.1 ml dextran (1.0% w/v in normal saline) as the oedemogen.

Rats were randomly allocated into 5 groups (eight rats each): one vehicle control group, three treated with D-002 (200, 400 and 800 mg/kg, respectively) and other with indomethacin (10 mg/kg) as reference drug.

Before treatment (0 h) and after 1, 2 and 3 hours of dextran injection paw diameter was measured with the help of slide calliper. The anti-inflammatory activity was evaluated as the difference in the changes on paw diameters in treated and control groups.<sup>25</sup>

### Hi-induced plantar oedema

Rats were distributed into 5 groups of 10 rats each: one vehicle control group + Hi; three with D-002 (200, 400 and 800 mg/kg) + Hi; and one with diphenhydramine (60 mg/kg) + Hi (reference group).

### 5-HT-induced plantar oedema

Rats were randomized into 5 groups of 10 rats: one vehicle control group + 5-HT; three with D-002 (200, 400 and 800 mg/kg) + 5-HT; and one with cyproheptadine (10 mg/kg) + 5-HT (reference group).

Histamine (30 mg/kg) and serotonin (1 mg/kg) were injected (0.1 ml) into the rat left hind paw. One and two hours after the injections of 5-HT and Hi, respectively, the oedema was measured as mentioned earlier.<sup>25</sup>

### Xylene induced ear edema

Mice were randomized into six groups (10 mice per group): one negative control without ear oedema induction and six xylene-treated groups: one positive vehicle control, three treated with D-002 (50, 200 and 400 mg/kg), and a reference indomethacin (10mg/kg)-treated group. Treatments (vehicle, D-002, indomethacin) were administered acutely by gastric gavage (5 ml/100g).

One (1) hour after oral administration, the dorsal sides of the right ears of mice received 30 µl of xylene by topical application. Two hours later the mice were sacrificed and 9 mm punches were made in the right and the left ears by the borer. Each ear disc was weighed and the differences in weights of the right and left ear discs of mice were recorded as oedema level.<sup>26</sup>

Oedema formation was calculated by the difference between the right ear (with oedema) and left ear (without oedema) weights ( $\Delta O$ ).

### Myeloperoxidase (MPO) activity assay

Myeloperoxidase (MPO) activity was measured (Worthington enzyme manual, 1972)<sup>27</sup> in the model of xylene-induced ear oedema. In brief, whole ear tissue was homogenized in phosphate buffer (50 mM/l) (pH=6) containing 0.5% hexadecyl trimethylammonium bromide (HTAB) (100mg tissue/ml buffer). Samples were sonicated for 10 seconds, freezing and thawing at 20 to 30°C for three times. After that, the samples were centrifuged and the supernatant was used to assess MPO activity. The change in absorbance at 460 nm was measured in the spectrophotometer.

MPO activity was expressed as:

$$U/g \text{ tissue} = \Delta A \text{ min.} \times \text{final Vol} / 8.3 \times \text{Vol. sample} \times 10$$

( $\Delta A$  min.: absorbance variation, final vol: final volume in the cuvette, Vol. sample: volume -µl- of the added sample).

## Statistical Analysis

Statistical analyses were performed by using the Mann Whitney U Test. Data was analyzed by Statistics software for Window (Release 4.2; Stat Soft, Inc, Tulsa, USA).

## RESULTS

Oral administration of D-002 showed significant anti-inflammatory activity in all tested models.

### Effect of D-002 on dextran-induced paw oedema

Following 2 hours of dextran dosing, D-002 (200, 400 and 800 mg/kg) inhibited paw oedema by 34.7, 43.4 and 52.1%, respectively. At 3 hours the reductions achieved with D-002 did not wear off, being almost unchanged in the lower dose groups (33.3% with 200 mg/kg, 47.6% with 400 mg/kg) and even improved (71.4%) in the highest dose (800 mg/kg) group.

Indomethacin 10 mg/kg decreased the dextran-induced paw oedema at 2 and 3 hours by 43.3 and 52.3%, respectively. The effect of D-002 (800 mg/kg) was greater than that of indomethacin. No anti-inflammatory effect was observed at one hour of dextran dosing (Figure 1).

### Effect of D-002 on Hi and 5-HT-induced paw oedema

Oral treatment with D-002 (400 and 800 mg/kg) significantly inhibited Hi-induced plantar oedema (37 and 57%, respectively) and the same occurred, albeit less pronounced, on 5-HT-induced (26 and 36%, respectively) plantar oedema. In turn, diphenhydramine (60 mg/kg) and cyproheptadine (10 mg/kg) inhibited Hi- and 5-HT-induced plantar oedema by 60 and 79%, respectively (Table 1).

**Table 1:** Effect of D-002 on mediator-induced paw edema

Treatment	Doses (mg/kg)	Paw diameter (mm)	Inhibition (%)
<b>Hi-induced paw edema<sup>†</sup></b>			
Control	0	0.1 ± 0.007	-
D-002	200	0.068 ± 0.011*	32
D-002	400	0.063 ± 0.008*	37
D-002	800	0.043 ± 0.009**	57
Diphenhydramine	60	0.04 ± 0.01*	60
<b>5-HT-induced paw edema<sup>†</sup></b>			
Control	0	0.39 ± 0.02	-
D-002	200	0.33 ± 0.02	15.3
D-002	400	0.29 ± 0.01**	26.0
D-002	800	0.25 ± 0.01***	36.0
Cyproheptadine	10	0.08 ± 0.01**	79.0

<sup>†</sup>Hi: histamine (30 mg/kg), 5-HT: serotonin (1 mg/kg); \*p< 0.05 \*\* p<0.01 \*\*\* p<0.001 (Comparisons with control group) Mann Whitney U Test

**Table 2:** Effects of D-002 on edema in the xylene-induced mouse ear edema

Treatments	Doses (mg/kg)	Edema (ΔO) <sup>a</sup>	Edema inhibition (%)	MPO (U/g tissue)	MPO inhibition (%)
Negative control	0	2.67 ± 1.08	—	0.59 ± 0.11	—
Positive control	0	37.87 ± 5.34 ***	—	2.41 ± 0.26 **	—
D-002	50	32.24 ± 3.35	16.0	1.61 ± 0.12 +	43.9
D-002	200	18.67 ± 3.25 + Δ	54.5	1.42 ± 0.15 +	54.3
D-002	400	19.25 ± 3.71+ Δ	52.9	1.31 ± 0.11 +	60.4
Indomethacin	10	28.48 ± 2.74 +	26.7	1.25 ± 0.08 ++	63.7

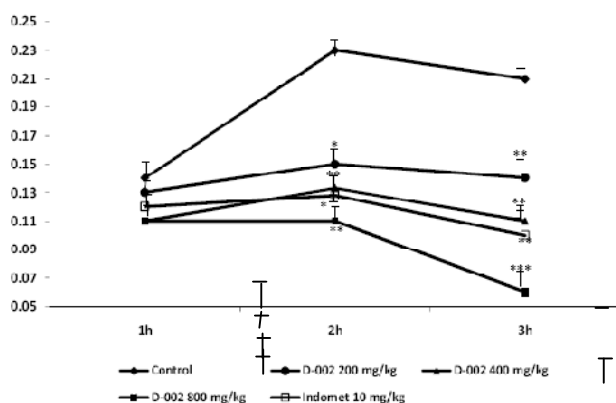
MPO: myeloperoxidase; (ΔO)<sup>a</sup> Oedema variation; \*\*\*p< 0.001 Comparison with negative control, +p< 0.05, ++p<0.01 Comparison with positive control; Δ p<0.05 Comparison with indomethacin group (Mann Whitney U test)

### Effect of D-002 on xylene-induced mouse ear oedema

Single oral doses of D-002 (200 and 400 mg/kg, not 50 mg/kg) significantly and dose-dependently ( $r = 0.951$ ,  $p < 0.05$ ) reduced xylene-induced oedema formation in mice ear by about 50%. Significant right ear oedema was seen in the positive control (Table 2). D-002 (50 – 400 mg/kg)

significantly, markedly (about 60%) and dose-dependently ( $r = 0.954$ ,  $p < 0.05$ ) decreased MPO enzymatic activity compared with the positive control. While the effects of D-002 (200 and 400 mg/kg) on oedema formation were higher than those of indomethacin 10 mg/kg, the effects of both substances on MPO activity were similar.



**Figure 1:** Effect of single doses of D-002 on dextran-induced paw edema

Values are mean  $\pm$  S.E.M; \* $p < 0.05$  \*\*  $p < 0.01$  \*\*\*  $p < 0.001$  Comparisons with control group (Mann Whitney U Test)

## DISCUSSION

This study demonstrates that oral single doses of D-002 displayed anti-inflammatory activity in classical models of acute inflammation: dextran-induced paw oedema in rats, Hi and 5-HT-induced plantar oedema in rats, and xylene-induced mouse ear oedema.

The inflammatory response consists of two main components namely the vascular and cellular cascades. Vascular events locally increase the supply of cellular and humoral effectors at the immediate site of inflammation and cellular events work to engulf, kill, and digest the stimulus so that the area can be cleared for the repair process. The vascular components consist in changes in the calibre of the blood vessels, changes in the vessel wall and in the flow of blood through the vessels. Vasodilation follows an initial but brief vasoconstriction and persists for the duration of the inflammatory response. The cause of this dilation is primarily due to some mediators, (histamine, prostaglandins and complement cascade components C3 and C5) whilst the axon reflex and autonomic system may exert additional influences.

The cellular components of the inflammatory response include the early emigration of the phagocytes (neutrophils; polymorphonucleocytes or PMN's) from the vessels. This is followed by several other species leaving the main flow, including monocytes, lymphocytes, eosinophils, basophils.<sup>28</sup> The main groups of chemical mediators responsible for chemotaxis are some components of the complement cascade, lymphokines, factors released for the PMN's and peptides released from the mast cells in the damaged tissue.<sup>29</sup>

The acute inflammation is a rapid response to an injurious agent that serves to deliver mediators of host defense-leukocytes and plasma proteins to the site of injury. It is typical of early phases of the inflammatory response and of relatively short duration, lasting for minutes, several hours, or a few days and is linked to increased vascular permeability, which causes exudation of fluid rich in plasma proteins (immunoglobulins, coagulation factors)

and cells into the injured tissues with subsequent local oedema, which is reinforced by the release of local mediators like Hi, 5-HT, bradykinin and P substance.

Oral D-002 (200, 400 and 800 mg/kg) inhibited paw oedema induced after 2 or 3 hours, but not at 1 hour following dextran administration. The highest inhibition at 3 hours (71.4%, 800 mg/kg) was greater than that achieved at 2 hours after dextran administration (52.1%). Likewise, the same occurred with indomethacin, the standard anti-inflammatory drug used in the experiment, which after 2 and 3 hours inhibited the oedema by 43.3% and 52.3%, respectively. Although the inhibition produced with the highest dose of D-002 (800 mg/kg) was greater than with indomethacin 10 mg/kg, we cannot conclude that D-002 was more effective than indomethacin in this model because we only tested the effects of one dose of this drug. The dextran-induced oedema involves a partial degranulation of mast cells characterized by the release of different mediators that contribute to oedema formation through capillary vasodilation and increased vascular permeability, 5-HT, Hi and prostaglandins being the main mediators responsible of the effects that occur at 1 and 2 hours after dextran administration.<sup>30</sup> Our results suggest that the anti-inflammatory effects of D-002 are Hi- and PG-related rather than 5-HT-associated, since it was devoid of efficacy at 1 hour, when 5-HT plays the pivotal role in the inflammatory response that leads to paw oedema. Consistently with these data, D-002 (400 and 800 mg/kg) was more effective to inhibit Hi- than 5-HT-induced plantar oedema (57% vs 36%, respectively). Topical application of single doses of xylene on mice ear produced oedema formation and increase of MPO enzymatic activity, in accordance with other reports,<sup>31</sup> and indomethacin, a COX unspecific inhibitor used as reference drug, inhibited the ear oedema by 26.7%, consistently with the 21.5% inhibition reported by other authors.<sup>32</sup> These results confer validity to this model in our experimental conditions. Hence, the effects of D-002 here shown are treatment-related.

Xylene-induced mouse ear oedema reflects the oedematization during the early stages of acute inflammation, which is probably related with the release and inhibition of inflammation factors.<sup>33</sup> The fact that D-002 (200 and 400 mg/kg) produced a rough 50% inhibition of oedema formation, the physiological inflammatory response to the xylene-induced chemical aggression, supports its efficacy on this model. Also, D-002 markedly inhibited the xylene-induced increase of MPO activity by 60.4%, an effect similar to that produced by indomethacin 20 mg/kg, which inhibited MPO activity by 63.7%.

Although the elucidation of the mode of anti-inflammatory action of D-002 is beyond the objectives of this study, the present data support some logical approaches. First, the action of D-002 on the dextran-induced paw edema, on Hi-induced plantar oedema and on 5-HT-induced plantar oedema could involve the inhibition of the synthesis, release or action of Hi, rather



than that of 5-HT. Second, since oral treatment with D-002 markedly reduced the increase of xylene-induced increase of MPO activity, its anti-inflammatory action could involve the inhibition of neutrophils infiltration and decrease of leukocytes migration, since acute inflammation involves neutrophils infiltration from blood vessels into the interstitial spaces, and a direct relation between increased MPO activity and neutrophils concentration in the inflamed tissue.<sup>34</sup> Nevertheless, it should be noted that the reductions of MPO activity caused by D-002 and indomethacin (grossly about 60%) were greater than the decreases of ear oedemas (about 27% with indomethacin, about 53% with D-002), which suggests the contribution of other mechanisms to oedema formation in this model.

We should comment, in addition, that the present results are in line with early reports of the anti-inflammatory effects of triacontanol,<sup>35,36</sup> the most abundant component of D-002.

Bearing in mind that D-002 reduced the pleural concentrations of LTB<sub>4</sub>, a chemotactic factor for neutrophils,<sup>37</sup> in rats with carrageenan-induced pleurisy,<sup>24</sup> has been shown to produce antioxidant effects in experimental and clinical studies<sup>38-41,23</sup> and is able to produce gastroprotective effects<sup>22</sup> instead of gastric damage, the present results confirm and extend the evidences of the anti-inflammatory effects of D-002, whose mechanism merits further investigation, being relevant to assess its effects on COX and 5-lipoxygenase (LPO) enzymes.

## CONCLUSION

We can concluded that the present results demonstrate that single oral doses of D-002 produce effective anti-inflammatory activity on the early phase of inflammation in acute inflammation models.

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