Effects of D-002, a mixture of beeswax alcohols, on the acetic acid-induced writhing test in mice: a comparison with naproxen, aspirin, and paracetamol

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Palabras clave: D-002, naproxeno, aspirina, paracetamol, contorsiones por ácido acético.

ABSTRACT. D-002, a mixture of six higher aliphatic alcohols purified from beeswax, has been shown to produce anti-inflammatory effects in experimental models, devoid of gastrotoxic or hepatotoxic effects. Some studies have demonstrated that D-002 exerts analgesic effects, but just related to its anti-inflammatory action. The objective of this study was to compare the effects of D-002, naproxen, aspirin and paracetamol on the acetic acid-induced writhing test in mice. Mice were allocated into 13 groups : a vehicle control group, four D-002 (25, 50, 200 and 400 mg/kg, respectively)-, three naproxen (10, 20 and 50 mg/kg)-, three aspirin (50,100 and 300 mg/kg) and two paracetamol (100 and 400 mg/kg)-treated groups by gastric gavage. One hour after treatment, mice were injected with 1 % acetic acid and the numbers of writhing movements were counted for 15 min. Oral treatment with D-002 (50, 200 and 400 mg/kg) significantly inhibited acetic acid-induced abdominal writhing (36, 43 and 40 % respectively). The number of wrights decreased significantly with aspirin 100 and 300 mg/kg (47 and 83 %); naproxen 20 and 50 mg/kg (37 % with both doses) and paracetamol 400 mg/kg (24 %). The lowest doses of each treatment failed to inhibit significantly the acetic acid-induced abdominal writhing. The effect of D-002 (400 mg/kg) was comparable to that of naproxen (20 and 50 mg/kg). Maximal effective doses of D-002 (200 mg/kg) and naproxen (20 mg/kg) were less effective than aspirin 300 mg/kg for inhibiting the acetic acid-induced abdominal writhing, meanwhile paracetamol 400 mg/kg was the least effective treatment**.**

RESUMEN. D-002 es una mezcla de seis alcoholes alifáticos superiores purificados de la cera de abejas con efectos antiinflamatorios demostrados en modelos experimentales sin efectos gastrotóxicos o hepatotóxicos. Algunos estudios han demostrado el efecto analgésico del D-002 relacionado con su acción antiinflamatoria. El objetivo de este trabajo consistió en comparar el efecto del D-002, naproxeno, aspirina y paracetamol sobre el modelo de contorsiones inducidas por ácido acético en ratones . Los ratones fueron distribuidos en 13 grupos (10 animales/grupo):un control con vehículo, cuatro con D-002 (25, 50, 200 y 400 mg/kg), tres con naproxeno (10, 20 y 50 mg/kg) tres con aspirina (50, 100 y 300 mg/kg) y dos con paracetamol (100 y 400 mg/kg) por vía oral. Una hora después del tratamiento, los ratones fueron inyectados con ácido acético (1,0 %) y el número de contorsiones durante 15 min registrado. El tratamiento oral con D-002 (50, 200 y 400 mg/kg) inhibió significativamente las contorsiones abdominales (36, 43 y 40 %). El número de contorsiones disminuyó significativamente con 100 y 300 mg/kg de aspirina (47 y 83 %); naproxeno 20 y 50 mg/kg (37 % con ambas dosis) y paracetamol 400 mg/kg (24 %). La menor dosis de cada tratamiento no mostró efecto analgésico. El efecto del D-002 (400 mg/kg) fue comparable al naproxeno (20 and 50 mg/kg). La máxima dosis efectiva de D-002 (200 mg/kg) y naproxeno (20 mg/kg) fueron menos efectivas que la aspirina (300 mg/kg) en inhibir las contracciones por ácido acético, mientras que el paracetamol (400 mg/kg) resultó el tratamiento menos efectivo.

INTRODUCTION

Inflammation is the response of living tissues to several noxious stimuli. Acute inflammation, the short-term response to injuries, involves vasodilation, enhanced capillary permeability and neutrophils migration to the inflammatory site. In fact, chronic inflammation is a component of most relevant long-lasting human diseases.[1] Non-steroidal anti-inflammatory drugs (NSAIDs), therefore, are widely prescribed worldwide to treat pain and inflammation and their use should raise according to the concomitant increase of life expectancy and chronic inflammatory diseases.[2,3] A limitation for NSAIDs use, however, is the adverse effects they produce, mainly gastrointestinal, renal, and cardiovascular, but also hepatic and dermatological effects, among others.[4,5]

Inflammatory process involves high levels of arachidonic acid (AA), generated by the phospholipase A2 enzyme from damaged cell membrane phospholipids, which is then metabolized through the cyclooxygenase (COX) and lipoxygenase (LOX) enzyme pathways, just to produce prostaglandins (PG), thromboxanes, prostacyclins, and highly inflammatory leukotrienes (LT).[6,7] NSAIDs cause adverse side effects due to COX-1 inhibition (gastric damage, bronchospasm) and/or to COX-2 inhibition (cardiovascular side effects), so that their use in the elderly should be monitored.[2-5] Non selective NSAIDs and COX-2 inhibitors display their anti-inflammatory effects by inhibiting the COX pathway, which suppresses the production of gastroprotective PG and displaces the AA metabolism towards the LOX pathway, thus raising the synthesis of pro-inflammatory and gastrotoxic LT.[6] It is interesting to notice that high concentrations of leukotriene B_4 (LTB_4) in the walls of NSAID-induced gastric ulcers attract leukocytes to the stomach and contribute to cause ulceration,[8] enhancing the gastrotoxicity due to PG deficit.[6] On its side, paracetamol (acetaminophen) is recommended as first-line therapy to treat mild to moderate pain in different conditions that seems to offer some advantages as compared to NSAIDs due to its gastric, cardiovascular and renal safety profile.[9] The exact mode of action of paracetamol, however, remains to be elucidated. Recent data imply that paracetamol inhibits the activity of both COX isoforms, mainly of COX-2, in peripheral tissues with low levels of peroxide by reducing the higher oxidation state of COX enzymes.[10] Nevertheless, paracetamol has little, if any, anti-inflammatory action and at high doses may induce hepatotoxicity.[11]

Therefore, the search for new and safer agents to treat inflammation and pain is justified. dual acting anti-inflammatory drugs may cut PG synthesis for ensuring an anti-inflammatory effect, but concomitantly may prevent the switch to increased LT production through the inhibition of 5-LOX, as side effect. Despite this potential advantage, few dual anti-inflammatory drugs have been introduced in clinical practice.[12,13]

D-002, a mixture of high molecular weight aliphatic alcohols (C_{24}, C_{26}, C_{28}, C_{30}, C_{32}, C_{34}) purified from beeswax, has been shown to produce anti-inflammatory effects in experimental models,[14, 15] and to inhibit COX and 5-LOX enzyme activities,[16] which supports that may produce anti-inflammatory effects without concomitant gastrotoxicity. Instead, D-002 has been shown to produce gastroprotective effects by increasing gastric mucus secretion[17,18] and reducing the oxidative stress in gastric mucosa.[19,20]

Also, D-002 has been shown to reduce the acetic acid-induced writhing response, but failed to modify the hot plate response in mice, which suggests that exert an analgesic effect, just related to its anti-inflammatory action, since it was devoid of effect on the

open field and rotarod behaviour tests in mice.[15] The magnitude of the purported analgesic effects of D-002, however, has not been established yet.
In light of these issues, this study compared the effects of D-002, naproxen, aspirin and paracetamol on the acetic acid-induced writhing test in mice.

MATERIALS AND METHODS

Animals

The study was conducted according to the Cuban guidelines for Good Laboratory Practices and to the Cuban Code of Care of Laboratory Animals. An independent ethic board approved the protocol and the use of the animals for the study.
Adult male mice OF-1 (20 – 25 from the National Centre for Laboratory Animal Production (CENPALAB, Havana), were housed in wire-mesh cages and kept under conventional laboratory conditions (22-23 ^{0}C, humidity 55-60 %, 12 h dark/light cycles), for 7 d with free access to water and standard chow (rodent pellets from CENPALAB) was allowed. Prior to the test, mice were fasted overnight with water *ad libitum*.

Administration and dosage

The batch of D-002, used in experiments, was supplied by the Plants of Natural Products (National Centre for Scientific Research, Havana, Cuba, composition of the batch, assessed with a validated gas chromatographic method,[22] was as follows: tetracosanol (7.2 %), hexacosanol (11.3 %), octacosanol (13.9 %), triacontanol (32.4 %), dotriacontanol (22.9 %) and tetratriacontanol (2.5 %). Purity (total content of these six alcohols) was 90.1 %.
Aspirin, naproxen and paracetamol were supplied by the Medical Pharmaceutical Industry (QUIMEFA, Cuba). All treatments were suspended in 2 % acacia gum/water vehicle.
Mice were divided into 13 groups (10 animals/group): A control group of animals that received the vehicle only, four groups treated with D-002 (25, 50, 200 and 400 mg/kg, respectively), three with naproxen (10, 20 and 50 mg/kg), three with aspirin (50,100 and 300 mg/kg) and two with paracetamol (100 and 400 mg/kg). All treatments were performed carefully by gastric gavage.
One hour after treatment, each animal was injected (10 mL/kg) with 1.0 % acetic acid in water (v/v 0.2 mL20 g) by (ip) route.
Writhing movements were characterized by specific abdominal contractions accompanied by elongation of body with arching of back, belly touching the ground and dragging of hind limbs. The number of writhing movements were counted after acetic acid injection for 15 min and the percent of writhes inhibition in each group were calculated.

Statistical analyses

Data are presented as the mean ± SEM and analysed with the one-way ANOVA followed by Duncan's multiple comparison test. Level of significance was set at $P < 0.05$. Data were processed with the Statistics Software for Windows.

RESULTS

Table 1 shows the results of the experiment

Oral pre-treatment with D-002 (50, 200 and 400 mg/kg), not with 25 mg/kg, significantly inhibited acetic acid-induced abdominal writhing in a dose-dependent fashion (36, 43 and 40 % respectively). The dose of 200 mg/kg seems to be the maximal effective dose in this model since the last one did not produce a greater effect.

The number of wrights decreased significantly with aspirin 100 and 300 mg/kg (47 and 83 %); naproxen 20 and 50 mg/kg (37 % with both doses) and paracetamol 400 mg/kg (24 %). The lowest doses of each treatment failed to inhibit significantly the acetic acid-induced abdominal writhing. The effect of the maximal doses of D-002 (400 mg/kg) was comparable to that of naproxen (20 and 50 mg/kg).

We did not reach a maximal effect with aspirin, since a ceiling response was not achieved. Nevertheless, the effect achieved with the maximal doses effective of D-002 (200 mg/kg) and naproxen (20 mg/kg) was significantly lower than that of aspirin 300 mg/kg. In turn, the effect of paracetamol 400 mg/kg was the lowest.

Table 1. Effect of D-002, naproxen, aspirin and paracetamol on acetic acid induced writhing in mice

Treatment	Doses (mg/kg)	Wright X ± ESM	Inhibition (%)
Control + acetic acid	-	40.18 ± 2.24	-
D-002 + acetic acid	25	32.54 ± 3.36	19
	50	25.54 ± 3.09**	36
	200	23.09 ± 3.54***	43
	400	24.0 ± 3.22**	40
Naproxen + acetic acid	10	33. 9 ± 2.6	16
	20	25.4 ± 1.7**	37
	50	25.12 ± 2.7**	37
Aspirin + acetic acid	50	35.49 ± 2.4	12
	100	21.30 ± 2.9***	47
	300	6.62 ± 1.6****	83
Paracetamol + acetic acid	100	37.1 ± 2.6	8
	400	30.4± 2.21*	24

Values are means ± SEM * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, comparison to the control group. All comparisons were performed using one-way analysis of variance (Duncan's test).

DISCUSSION

The present study demonstrated, for the first time, that the analgesic effect of D-002 administered orally (50, 200 and 400 mg/kg) was as effective as naproxen, more effective than paracetamol, but less effective than aspirin for inhibiting acetic-acid-induced abdominal contractions in mice.

Acetic acid-induced pain, a sensitive procedure for detecting analgesic effects of medicinal agents,[21,22] is characterized by abdominal cramps (visceromotor) and autonomic responses that involve distension, ischemia and inflammation, with the participation of local peritoneal receptors and concentrations of PGE_2 and PGF_2

produced by COX enzyme.[23,24] Then, acetic acid-induced writhing should be effectively inhibited by peripheral COX inhibitors.

Previous studies have demonstrated that D-002 exhibit anti-inflammatory effects in models of acute (carrageenan-induced pleurisy in rats, xylene-induced ear inflammation in mice)[14,15] and chronic (cotton-induced granuloma)[14] inflammation and in specific models of formaldehyde and monoiodoacetate-induced osteoarthitis in rodents.[25]

Curiously, instead of exhibit concomitant gastrotoxicity, D-002 has been shown to produce gastroprotective effects through a multifactorial mechanism that involves the increase of gastric mucus secretion[17,18] and reduction of oxidative stress in gastric mucosa.[19,20] The coexistence of anti-inflammatory and gastroprotective effects of D-002 is consistent with the D-002-induced dual inhibition of COX and 5-LOX,[16] which reduces the potential of causing NSAIDs-like adverse effects and seems to be an advantage of D-002 to treat inflammatory conditions.

D-002 has been reported to produce analgesic effects, but associated to its anti-inflammatory activity, since it did not produce behavioral changes on the hot plate and rota-rod tests, which indicates non-participation of the supraspinal components in pain modulation and no motor abnormality.[26] These effects are consistent with those recently found for octacosanol, one of the main components of D-002.

Indeed, the ability of D-002 for reducing pain, associated to inflammatory condition is a key matter for establishing its comparative value with NSAIDs, as pain is the most disturbing sensation for patients suffering condition. Consequently, comparison of the analgesic effect of D-002, NSAIDs and paracetamol was the focus of this experimental study.

Since the present results demonstrates that D-002 produces a dose-dependent analgesic effect and that the maximal dose (400 mg/kg) is as effective as the maximal dose of naproxen, and more effective than paracetamol 400 mg/kg, (both drugs widely used in clinical practice), D002 is a good predictor of its ability for ameliorating pain associated to inflammatory condition. Aspirin 300 mg/kg, however, exhibited the highest efficacy among the tested treatments.

Keeping in mind all these results, which remarks the analgesic action of D-002 as compared to drugs traditionally used to manage pain in several clinical conditions, together with the gastroprotective effects of D-002,[27-29] and its hepatoprotective effects,[30] the anti-inflammatory and analgesic effects D-002 requires clinical investigation, in order to achieve definitive conclusions of its potential to treat common and limiting inflammatory diseases, such as osteoarhtritis, which requires effective and safe treatment of long-term therapy.

CONCLUSIONS

The maximal doses effective of D-002 (200 mg/kg) and naproxen (20 mg/kg) were less effective than aspirin 300 mg/kg for inhibiting the acetic acid- induced abdominal writhing, meanwhile paracetamol 400 mg/kg was the least effective treatment.

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