Effects of D-002 on xylene-induced oedema in ear of mice

Yazmín Ravelo-Calzado, Vivian Molina-Cuevas, Sonia Jiménez-Despaine, Yohani Pérez-Guerra, Ambar Oyarzábal-Yera, Daisy Carbajal-Quintana y Rosa Mas-Ferreiro.

Pharmacology Department, Centre of Natural Products, National Centre for Scientific Research, Avenida 25 y Calle 158, Playa, Apartado Postal 6414, La Habana, Cuba.

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RESUMEN. La inflamación constituye una respuesta esencial para el mantenimiento de la salud frente a diversas enfermedades, pero una repuesta inflamatoria exacerbada se asocia a muchos trastornos patológicos. Los anti-inflamatorios no esteroidales (AINEs), usados en el tratamiento de la inflamación y el dolor, se encuentran dentro de los fármacos más comúnmente prescritos en el mundo para tratar desórdenes agudos y crónicos. No obstante, los efectos adversos asociados a su uso, principalmente trastornos gastrointestinales han limitado su utilización, hecho que justifica la búsqueda de nuevos anti-inflamatorios. El tratamiento oral a dosis única de D-002, una mezcla de seis alcoholes alifáticos obtenidos de la cera de abejas, ha mostrado poseer efectos anti-inflamatorios en el modelo de pleuresía por carragenina en ratas y en el edema inducido por xileno en oreja de ratón así como efectos gastroprotectores. Aún cuando se conoce que el D-002 presenta una acción gastroprotectora, sus efectos anti-inflamatorios han sido poco estudiados. Por tal motivo, nuevas investigaciones deberán ser realizadas. Este trabajo investigó los efectos del tratamiento oral de dosis repetidas de D-002 en el modelo de edema de la oreja de ratón inducida por xileno. Para ello, los animales fueron distribuidos aleatoriamente en seis grupos: un control negativo no tratado y cinco a los que se les aplicó el xileno: un control positivo, tres tratados con D-002 (25,50 y 200 mg/kg) y un control de referencia con indometacina (1 mg/kg). Se cuantificó la formación de edema y la actividad de la mieloperoxidasa (MPO). El tratamiento oral con D-002 (25,50 y 200 mg/kg) redujo de manera marcada, significativa y dependiente de la dosis la formación de edema en un 44,7; 60,8 y 76,4 % respectivamente mientras la indometacina (1 mg/kg) redujo este indicador en un 59,9 %. El D-002 (25,50 y 200 mg/kg) inhibió, además, la actividad de la MPO en un 38,0; 47,0 y 57,0 % respectivamente, siendo el efecto de la dosis mayor ensayada (200 mg/kg) comparable al de la indometacina (57,5 %) mientras que 25 y 50 mg/kg de D-002 no alcanzaron efectos similares. En conclusión, este estudio demostró que el tratamiento oral de dosis repetidas de D-002 posee una actividad anti-inflamatoria marcada en el modelo agudo de edema de la oreja de ratón inducido con xileno.

ABSTRACT. A well-integrated host inflammatory response is essential in maintaining health and fighting against diseases, but pathological inflammation is linked to many ailments. Non steroidal anti-inflammatory drugs (NSAIDs), used to treat inflammation and pain, are among the most commonly prescribed drugs world over to treat both acute and chronic conditions, but its use is hampered by NSAIDs-related adverse effects, mainly gastrointestinal, a fact that justifies the search for new anti-inflammatory substances. Single oral doses of D-002, a mixture of six alcohols purified from beeswax, have shown anti-inflammatory effects in the rat carrageenaan-induced pleurisy and in the xylene-induced ear mice acute inflammation model, and gastroprotective effects as well. Despite D-002-induced gastroprotection has been well documented, its anti-inflammatory effects have received less attention, and merit to conduct new research. This work investigated the effects of repeated doses of D-002 on xylene-induced oedema formation in mice ear. Mice were randomized into six groups: one negative vehicle control and five xylene-treated groups: one positive control, three treated with D-002 (25.50 and 200 mg/kg) and a reference indomethacin (1 mg/kg) group. Effects on oedema formation and myeloperoxidase (MPO) activity were assessed. Significant right ear oedema was seen in the positive control group as compared to the negative control. Repeated doses of D-002 (25.50 and 200 mg/kg), significantly, markedly and dose-dependently decreased oedema formation by 44.7; 60.8 and 76.4 %, respectively, while indomethacin 1 mg/kg decreased significantly this parameter by 59.9 %. Also, while MPO activity significantly increased in the positive control group as compared to the negative control one, oral treatment with repeated doses of D-002 (25.50 and 200 mg/kg) significantly reduced MPO activity by 38.0; 47.0 and 57.0 %, respectively. The significant reduction of MPO activity achieved with indomethacin 1 mg/kg (57.5 %) and with D-002 200 mg/kg (57.0 %) were comparable, but the effects of the other doses of D-002 (25 and 50 mg/kg) were lower than those of indomethacin. In conclusion, this study demonstrates that repeated doses of D-002 exhibit a remarkable anti-inflammatory activity in the xylene-induced mice ear oedema, a model of acute inflammation.

Correspondencia:
Lic. Yazmin Ravelo Calzado
Departamento de Farmacología, Centro de Productos Naturales, Centro Nacional de Investigaciones Científicas, Avenida 25 y Calle 158, Playa, Apartado Postal 6414, La Habana, Cuba. Correo electrónico: yazmin.ravelo@cnic.edu.cu

INTRODUCTION

A well-integrated host inflammatory response is essential in maintaining health and fighting disease, but pathological inflammation is linked to many ailments. Inflammation, a vascular and cellular response from tissues against noxious stimuli, is characterized by vasodilatation, increased vascular permeability, migration of cells from blood vessels into the tissues with oedema formation, leukocytes infiltration and release of mediators like histamine, serotonin, thromboxane, prostaglandins (PG) and leukotrienes (LT).[1,2]

Non steroidal anti-inflammatory drugs (NSAIDs), used to treat inflammation and pain, are among the most commonly prescribed drugs the world over, being prescribed in both acute and chronic conditions,[3,4] but their use is hampered by NSAIDs-related adverse effects, mainly gastrointestinal,[5-7] a fact that justifies the search for new anti-inflammatory substances.

D-002 is a mixture of six high molecular weight aliphatic primary alcohols (C_{26}, C_{26}, C_{28}, C_{30}, C_{32} and C_{34}) purified from beeswax, wherein triacontanol (C30) is the most abundant component.[8] Oral administration of D-002 has shown anti-inflammatory effects in the rat carrageenaan-induced pleurisy[9] and in the xylene-induced acute inflammation model in ear of mice,[10] and gastroprotective effects as well.[11-19]

Despite NSAIDs induce gastric damage[5-7] and D-002 exhibits well documented gastroprotective effects,[11-19] the anti-inflammatory effects of D-002 had received little attention and merit new studies.

Xylene-induced mice ear oedema reflects the oedematization occurred during the early stages of acute inflammation, which is probably related with the release of inflammation mediators.[20]

The anti-inflammatory effects of classical NSAIDs (diclofenac, indomethacin) have been assessed in this model, in which periphery sensorial neurons release the substance P, a peptide that produces vasodilatation and plasmatic extravasation of inflammatory mediators, thus causing a neurogenic oedema.[20] Despite being a model of acute inflammation, it is interesting to know the persistence of the effects of an anti-inflammatory substance on this model.

This study, therefore, investigated the effects of repeated doses of D-002 on xylene-induced oedema formation in the ear of mice.

MATERIALS AND METHODS

Animals

The study was carried out in accordance with current Cuban guidelines for Good Laboratory Practice (GLP) and for the care of laboratory animals. The independent local ethic committee for animal use approved the protocol for the study.

Adult male mice OF-1 (20-30 g) from National Centre for Laboratory Animal Production (CENPALAB, Havana) were housed for 7 d in temperature-controlled rooms (22 - 23 °C, humidity 55 - 60 %, 12 h dark-light cycles) until use. Access to water and standard chow (rodent pellets from CENPALAB) were freely allowed.

Administration and dosage

D-002 (batch: 030040109), supplied by the Plants of Natural Products (National Centre for Scientific Research, Havana City, Cuba), was used after corroborating its quality criteria. The composition of the batch, assessed with a validated gas chromatographic method,[21] 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 six alcohols) was 88 %.

D-002 was suspended in Tween 20/water (2 %) vehicle.

Indomethacin (QUIMEFA) (Batch: 100907007) was dissolved in sodium bicarbonate (5 %).

Mice were randomized into six groups (10 mice per group): one negative control group and five xylene-treated groups: one positive control (treated with the vehicle), three treated with D-002 (25.50 and 200 mg/kg) and a reference indomethacin 1 mg/kg group. Treatments (vehicle, D-002, indomethacin) were administered by gastric gavage for 15 d .

Induction of mice ear oedema

After the last day of treatment, acute inflammation was induced. In brief, 30 μL of pure xylene was topically applied on the dorsal surface of right ear of mice. Two hours later mice, under ether anesthesia, were sacrificed by cervical dislocation.

Both ears were cut and immediately weighed in an analytic balance. The formation of oedema was calculated by the difference between the right (with oedema) and left ear (without oedema) weights (ΔO).

Myeloperoxidase

Myeloperoxidase (MPO) activity was measured according to Worthington enzyme manual.[22] Whole ear tissue was homogenized in phosphate buffer (50 mmol/L) at pH = 6 containing 0.5 % hexadecyl trimethylammonium bromide (HTAB) (100 mg tissue/mL buffer) using a homogenizer (Ultra-turax T-25). The samples were sonicated for 10 s, freezing and thawing at 20 to 30 °C three times. After that, the samples were centrifuged at 12 000 r/min for 25 min at 4 °C and supernatant was used for MPO determination. In brief, 250 μL of sample was mixed with 625 μL of phosphate buffer (50 mmol/L, pH = 6) containing 0.167 mg/mL O-dianisidine dihydrochloride and 125 μL hydrogen peroxide (0.000 5 %). The changes in absorbance at 460 nm were measured with spectrophotometer for two minutes. MPO activity was expressed as units/g of tissue (U/g tissue), one unit being defined as the degradation of 1 μmoL of peroxide per minute at 25 °C, being quantified by the following formula:

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

where:
ΔA min absorbancy variation.
Vol. cuvete cuvete final volume.
Vol. sample volume of sample aggregated (μL).

Statistical analyses

Comparisons between treated and control groups were performed with the non-parametric Mann-Whitney U test. Statistical significance was chosen for $\alpha = 0.05$. Data were processed with the Statistics Software for Windows (Release 4.2). Relation doses/effect was performed with lineal regression and correlation test using a Primer of Biostatistics program (Stanton A, Glantz Version 3.01).

RESULTS

As expected, significant right ear oedema were seen in the positive control mice as compared to the negative

control ones. Repeated doses of D-002 (25.50 and 200 mg/kg), significantly, markedly and dose-dependently decreased oedema formation by 44.7; 60.8 and 76.4 % respectively, while indomethacin 1 mg/kg decreased significantly this parameter by 59.9 % (Table 1).

Also, while MPO activity significantly increased in the positive control group as compared to the negative control one, oral treatment with repeated doses of D-002 (25.50 and 200 mg/kg) significantly reduced MPO activity by 38.0; 47.0 and 57.0 %, respectively (Table 2). The significant reduction of MPO activity achieved with indomethacin 1 mg/kg (57.5 %) and with D-002 200 mg /kg (57.0 %) were comparable, but the effects of the other doses of D-002 (25 and 50 mg/kg) were lower than those of indomethacin.

DISCUSSION

The present study demonstrates that repeated (15 d) oral doses of D-002 produce significant anti-inflammatory activity in the xylene-induced mice ear oedema, a model of acute inflammation.

The topical application of single doses of xylene on mice ear produced a marked oedema formation and increased MPO enzymatic activity that were significantly reduced by indomethacin, the reference drug used in the study, which agrees with reports of other authors,[23] and confers validity to our experimental conditions.

Comparing with previous results,[10] the present results indicate the persistence of the antinflammatory effects of D-002 on the xylene-induced oedema. The oedema reductions shown here (44.7 % with 25 mg/kg, 60.8 % with 50 mg/kg and 76.4 % with 200 mg/kg, respectively) were better than those reached with the same doses given acutely (8.8; 16 and 54.4 % with 25.50 and 200 mg /kg, respectively, which were not significant with 25 and 50 mg/kg). Likewise, while indomethacin 10 mg/kg given as single doses reduced oedema formation by only 26.7 %, repeated doses of indomethacin 1 mg/kg produced a 59.9 % swelling inhibition. The fact that D-002 produced a rough 50 % to 70 % inhibition of oedema formation, the physiological inflammatory response to xylene induced chemical aggression, indicates a marked anti-inflammatory effect of repeated oral doses of D-002 on this model. Also, the reduction of xylene-oedema (44.7 %) with D-002 25 mg/kg was superior to those (16.0 %) of a roughly similar dose of casticin (20 mg/kg), the main alkaloid component of the ripe fruits of *Fructus viticis*, a folk medicine used to treat headaches, colds, migraine and eye pain.[24] Altogether, these data support that the anti-inflammatory effect of D-002 could be of clinical relevance.

Repeated oral doses of D-002 (25.50 and 200 mg/kg) also inhibited (38.0; 47.0 and 57.0 %, respectively) the xylene-induced increase of MPO activity, which suggests that its anti-inflammatory action could involve the inhibition of neutrophils infiltration and decrease of leukocytes migration since a direct relation between increased MPO activity and neutrophils concentration in the inflamed tissue has been documented.[25] The inhibition of neutrophils infiltration is consistent with

Table 1. Inhibitory effect of repeated doses of D-002 on xylene-induced ear oedema in mice.

Treatments	Doses (mg/kg)	Oedema (ΔO)	Swelling inhibition (%)
Negative control		3.12 ± 1.41	—
		Xylene-treated groups	
Positive control		35.04 ± 3.63 ****	—
D-002	25	20.76 ± 3.63 $^{+}$	44.7
D-002	50	15.63 ± 3.05 $^{++}$	60.8
D-002	200	10.64 ± 4.50 $^{++}$	76.4
Indomethacin	1	15.92 ± 3.71 $^{++}$	59.9

X Mean. SD Standard deviation. **** $p < 0.000\ 1$ Comparison with negative control.
$^{+}$ $p < 0.05$, $^{++}$ $p < 0.01$ Comparison with positive control (Mann Whitney U test).

Table 2. Inhibitory effect of repeated doses of D-002 on the increase of MPO activity in mice with xylene-induced ear oedema.

Treatments	Doses (mg/kg)	MPO activity (U/g tissue)	MPO activity inhibition (%)
Negative control		0.59 ± 0.08	—
		Xylene-treated groups	
Positive control		2.59 ± 0.13 ***	—
D-002	25	1.83 ± 0.21 $^{+}$	38.0
D-002	50	1.65 ± 0.23 $^{++}$	47.0
D-002	200	1.45 ± 0.19 $^{++}$	57.0
Indomethacin	1	1.44 ± 0.06 $^{+++}$	57.5

X Mean. SD Standard deviation. *** $p < 0.001$ Comparison with negative control. $^{+}$ $p < 0.05$, $^{++}$ $p < 0.01$, $^{+++}$ $p < 0.001$ Comparison with positive control (Mann Whitney U Test).

the D-002 induced reduction of pleural concentrations of leukotriene B_4 (LTB_4), a chemotactic factor for neutrophils, observed in the pleural exudates of rats with carrageenan-induced pleurisy.[9]

Nevertheless, repeated doses of D-002 (25 – 200 mg/kg) were more effective to decrease xylene-induced oedema formation (up to 76.4 %) than to inhibit xylene-induced MPO activity (inhibited up to 57.0 %), which indicates that mechanisms others than MPO inhibition contribute to the anti-inflammatory action of D-002 on this model. In such regard, since D-002 has been shown to reduce pleural concentrations of LTB_4 in another model of acute inflammation like carrageenan-induced pleurisy in rats,[9] it is plausible that it may inhibit 5-lipoxygenase (LPO) activity, a supposition that requires further research. The inhibitory effects of D-002 on lipid peroxidation markers demonstrated experimentally[14,26,27] and clinically[28-31] are in line with this last hypothesis.

This study demonstrates that repeated doses of D-002 exhibit a remarkable anti-inflammatory activity in the xylene induced mice ear oedema, a model of acute inflammation, which expands the knowledge about the anti-inflammatory effects of this substance and suggests that the anti-inflammatory effect of D-002 is potentially meaningful to treat inflammation, although such affirmation requires further research.

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