J. Pharm. Pharmacol. 1996, 48: 858–860
Received December 18, 1995
Accepted March 22, 1996

Possible Cytoprotective Mechanism in Rats of D-002, an Anti-ulcerogenic Product Isolated from Beeswax

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Abstract

D-002 is an anti-ulcerogenic product, isolated from beeswax, which consists of a well-defined mixture of higher primary aliphatic alcohols. It is highly effective against ethanol-induced ulcers. This study was designed to determine if D-002 shows cytoprotective properties on gastric mucosa in ethanol-induced ulcers. The involvement of endogenous prostaglandins in the protective effect of D-002 was also investigated.

When a subulcerogenic dose of indomethacin (10 mg kg^{-1}) was injected simultaneously with oral administration of ethanol, oral pre-treatment with D-002 (5–100 mg kg^{-1}) partially inhibited the gastric protection. D-002 (5 and 25 mg kg^{-1}) administered to normal rats significantly increased the soluble mucus content and also prevented its reduction in rats with ethanol-induced ulcers. In addition, D-002 administered at 5 and 25 mg kg^{-1} prevented the increase of vascular permeability induced by ethanol (60%) and reduced the concentration of thromboxane B_2 (TXB_2) in gastric mucosa of rats with ethanol-induced ulcers.

These results support the hypothesis that the anti-ulcerogenic properties of D-002 could be related to a cytoprotective mechanism.

D-002, a natural mixture of higher primary alcohols isolated and purified from beeswax, contains triacontanol as its main component, followed by octacosanol, dotriacontanol, hexacosanol and tetracosanol. Tetracontanol is also present as a minor component.

D-002 shows mild anti-inflammatory activity in cotton pellet granuloma and carrageenan pleurisy associated with a reduction on leukotriene B4 levels in pleural exudate (Carbajal et al 1996).

Remarkable anti-ulcer effects of D-002 have also been demonstrated in different experimental models. Thus D-002 at 5–50 mg kg^{-1} prevents ulcers induced by ethanol (60%) and HCl (0·6 M); at 30 mg kg^{-1} it prevents indomethacin-induced ulcers and inhibits ulcers induced in pylorus ligated rats. These doses did not affect the volume and pH of acid secretion (Carbajal et al 1995).

Because the term 'cytoprotection' means protection against gastric mucosal injury by a mechanism different from inhibition or neutralization of gastric acid (Robert et al 1977) it is plausible to consider D-002 as a putative cytoprotective agent. In this regard several mechanisms have been associated with cytoprotection, e.g. increased mucus, bicarbonate secretion, blood flow in gastric mucosa, formation of prostaglandins, reduced gastric motility and release of leukotrienes, among others (D'Souza & Dhume 1991).

This study was designed to determine if D-002 shows the pharmacological characteristic of cytoprotective drugs in the prevention of ethanol-induced ulcers.

Materials and Methods

Animals

Female Sprague–Dawley rats, 180–200 g, from the Centro Nacional para la Produccion de Animales de Laboratorio (Cenpalab, Cuba) were housed in environmentally controlled rooms (25 ± 2°C, 12 h light/dark cycles) with free access to standard chow (Cenpalab) and tap water. The animals were randomly distributed in different experimental groups.

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Administration and dosage.

D-002 was supplied by Laboratorios Dalmer (Havana City, Cuba) The batch composition was: triacontanol (26·63%), octacosanol (17·49%), dotriacontanol (16·95), hexacosanol (15·34%), tetracosanol (13·24%) and tetratriacontanol (2·23%). It was suspended in a 1% aqueous solution of acacia gum; in all experiments D-002 was administered orally by gastric gavage (1 mL/200 g). Control groups received a similar volume of the vehicle only. The ethanol (60%) used for ulcer induction was administered by the same route.

Measurement of gastric mucus

The animals were fasted for 24 h before the experiments but had free access to water. The rats were randomly distributed in five experimental groups: sham, control (vehicle), and three groups treated with D-002 at 1, 5 and 25 mg kg^{-1}, respectively. Three hours after treatment the rats were killed by cervical dislocation and the stomachs were removed immediately, opened along the greater curvature and rinsed in saline. They were weighed and incubated for 2 h at room temperature in a solution of alcian blue (0·1%; 20 mL) in 0·16 M sucrose buffered with 0·05 M sodium acetate (pH 5·8). After this period, the stomachs were washed twice with sucrose solution (0·25 M; 10 mL) for 15 and 45 min. The dye-mucus complex was extracted for 2 h with $MgCl_2$ solution (0·5 M; 10 mL). The optical density was read at 605 nm. Results are expressed as μg alcian blue (g tissue)$^{-1}$, as described by Frechilla et al (1991).

Mucus quantification in ethanol-induced ulcers

Rats were pre-treated with D-002 and, 1 h later, ethanol (60%; 1 mL/200 g) was administered. The animals were killed 1 h later and mucus determination was performed as described above.

Evaluation of endogenous prostaglandin in ethanol-induced ulcers

Rats were distributed randomly in seven experimental groups: control (indomethacin), control (ethanol), control (vehicle + ethanol + indomethacin), and four groups treated with ethanol + indomethacin + D-002 at 5, 25, 50 and 100 mg kg^{-1}, respectively. Immediately after administration of D-002 a subulcerogenic dose of indomethacin (10 mg kg^{-1}) was injected subcutaneously. After 1 h the animals received ethanol (60%; 1 mL/200 g); they were killed 1 h later. The inhibition of gastric mucosa damage was quantified. Lesion scores were defined as the total sum of the lengths (mm) of the gastric lesions. Observation and measurement of the length of a lesion were performed by two independent observers with no knowledge of the treatment received by the rats (Ohara et al 1992).

Evaluation of vascular permeability with Evan's blue

The rats were distributed randomly in three experimental groups: control (vehicle) and two groups treated with D-002 at 5 and 25 mg kg^{-1}, respectively. Ethanol (60%) was administered 1 h later. Permeability of Evan's blue was evaluated according to the method of Szabo et al (1985). In all groups Evan's blue (10 mg kg^{-1}) was injected intravenously under light anaesthesia 45 min after ethanol administration. Fifteen minutes later the animals were killed. Their stomachs and duodena were removed and cut along the greater curvature. The gastric wall was weighed and mixed with saline (1 mL) and the mix was homogenized with a tissue homogenizer (Ultra-Turrax 25). HCl (32%; 2·5 mL) was then added, the mixture was shaken and left in the room for 2 h, and chloroform (2·5 mL) was added. The mixture was centrifuged at 3000 rev min^{-1} for 15 min. Evan's blue was extracted with chloroform and its concentration was measured spectrophotometrically at 610 nm.

Quantification of thromboxane B_2 (TXB_2) levels in gastric mucosa

D-002 (1, 5 or 25 mg kg^{-1}) was administered to rats 1 h before administration of ethanol (60%). One hour later the rats were killed and the mucosal layers of their stomachs were separated from the muscle layers by gentle scraping. The tissue was weighed. Tris-HCl buffer (50 μM, pH 8·4; 1 mL) was added; the samples were centrifuged at 9000 *g* for 10 s, resuspended in buffer (1 mL), vortex-mixed and then centrifuged at 9000 *g* for 15 s and indomethacin (1 μg mL^{-1}; 10 mL) was added. Determination of TXB_2 was performed by radioimmunoassay using Amersham specific kits.

Statistical analysis

Comparisons between groups were performed using the Mann Whitney *U* test. An 'a priori' level of $\alpha = 0{\cdot}05$ was established for statistical significance.

Results

Table 1 summarizes the effects of D-002 on the production of gastric mucus in rats with and without ethanol-induced ulcers. It is apparent that the amounts of gastric mucus in groups treated with D-002 at 5 and 25 mg kg^{-1} were significantly higher than those in the positive control and statistically similar to those of the sham-operated groups. D-002 administered at 1 mg kg^{-1} caused no change in the production of gastric mucus. Similar effects of D-002 were observed on the production of gastric mucus of rats showing normal gastric mucosa.

Subcutaneous injection of indomethacin (10 mg kg^{-1}) to animals pre-treated with D-002 (5–100 mg kg^{-1}) inhibited the gastric protection in ethanol-induced damage compared with control (ethanol + indomethacin). At the dose tested indomethacin alone caused no gastric damage but did increase the ethanol-induced damage in rats receiving ethanol for ulcer induction (Table 2). Table 3 shows that administration of D-002 resulted in a significant reduction of vascular permeability in gastric mucosa. The doses of D-002 (5 and 25 mg kg^{-1}) effective at increasing gastric mucus production were selected for tests to determine whether the treatment changed the levels

Table 1. Effects of D-002 on gastric mucus production in rats.

Treatment	Dose (mm kg^{-1})	n	Amount of mucus (μg (g tissue)$^{-1}$)
With ethanol-induced gastric ulcer			
Sham	–	10	45·4 ± 13·7*
Positive control	–	10	33·5 ± 3·46
D-002	1	10	37·6 ± 6·38
D-002	5	10	44·3 ± 7·60**
D-002	25	10	45·5 ± 5·15**
Without ethanol-induced gastric ulcer			
Control	–	10	31·46 ± 6·70
D-002	1	10	35·39 ± 6·74
D-002	5	10	38·78 ± 4·36*
D-002	25	10	41·52 ± 5·50**

*$P < 0{\cdot}05$; **$P < 0{\cdot}01$ (Mann Whitney *U* test). For first set of results D-002 was administered previously to rats in both groups 1 h before administration of ethanol. Three hours later rats were killed and their stomachs were weighed and incubated in alcian blue (0·1%; 20 mL) for 2 h. The dye-mucus complex was extracted with $MgCl_2$ (2 h) and monitored at 605 nm. Values are means ± s.e.

Table 2. Effects of D-002 on endogenous prostaglandin levels in ulcers induced by ethanol (60%) and a subulcerogenic dose of indomethacin (10 mg kg^{-1}).

Treatment	Dose (mm kg^{-1})	n	Ulcer length (*mm*)
Control (indomethacin)	–	10	0
Control (ethanol)	–	10	22·06 ± 0·50
Control (indomethacin + ethanol)	–	10	68·20 ± 14·5
D-002	5	10	43·80 ± 7·33
D-002	25	10	54·30 ± 9·79
D-002	50	10	49·70 ± 6·92
D-002	100	10	47·30 ± 11·24

D-002 was administered orally and indomethacin (10 mg kg^{-1}) was immediately injected subcutaneously. One hour later the animals received ethanol (60%); they were killed 1 h after ethanol administration. Inhibition of gastric mucosa damage was measured. Values are means ± s.e.

Table 3. Effect of D-002 on vascular permeability of Evan's blue in the stomachs of rats with ethanol-induced ulcers.

Treatment	Dose (mg kg^{-1})	n	Amount (mg (g tissue)$^{-1}$)	Reduction (%)
Control	–	13	0·140 ± 0·05	–
D-002	5	12	0·032 ± 0·007*	73·6
D-002	25	12	0·028 ± 0·05*	80

*$P < 0.05$ Mann Whitney U test. D-002 was administered orally and, 1 h later, ethanol (60%). All groups received an intravenous injection of Evan's blue (10 mg kg^{-1}) 45 min after ethanol administration. Fifteen minutes later the animals were killed, their stomachs were homogenized, and HCl (32%) and chloroform (2·5 mL) were added. The mixture was centrifuged and Evan's blue was extracted with chloroform and measured at 610 nm. Values are means ± s.e.

Table 4. Effects of D-002 on the TXB_2 content of the stomachs of rats with ethanol-induced ulcer.

Treatment	Dose (mg kg^{-1})	n	Amount TXB_2 (ng g^{-1} tissue)
Control	–	8	1·98 ± 0·47
D-002	1	8	1·20 ± 0·25
D-002	5	9	0·51 ± 0·13**
D-002	25	8	0·42 ± 0·07**

**$P < 0.01$ (Mann Whitney U test). D-002 was administered orally 1 h before ethanol. The rats were killed 1 h later and the mucosal layer of the stomach was separated from the muscle. Tris buffer (1 mL) was added, the mixture centrifuged and resuspended, and TXB_2 was quantified by radioimmunoassay. Values are means ± s.e.

of TXB_2 in the stomachs of rats with ethanol-induced ulcers. The results showed that D-002 significantly reduced TXB_2 levels in the stomachs of these rats (Table 4).

Discussion

This study confirms the protective effect of D-002, a natural product obtained from beeswax showing anti-ulcer effects. It is very effective against ulcers induced by ethanol (Carbajal et al 1995) and HCl, both narcotizing agents. D-002 (5 and 25 mg kg^{-1}) significantly inhibited the reduction in dye-recovery in these rats and increased mucus production in rats in which gastric ulcers had not previously been induced. Our results indicate the possible role of prostaglandins in the gastroprotective effects of D-002. Thus the lack of efficacy of D-002 against ethanol-induced ulcers when the animals were also pre-treated with subulcerogenic doses of indomethacin indicates that the anti-ulcer effectiveness of this drug depends on prostaglandin biosynthesis. Mucus secretion is a crucial factor in the protection of gastroduodenal mucosa from the gastric lesions of diverse etiology and has been regarded as an important defensive factor in the gastric mucus barrier (Matsuo et al 1986; Tanaka et al 1989; Frechilla et al 1991). In ethanol injury, on the other hand, prostaglandins appear to preserve microvascular integrity and reduce underlying vasocongestion (Motilva et al 1994) as well as modulating gastric blood flow.

Ethanol increases vascular permeability in gastric mucosa; D-002 reduced this increase. It has been demonstrated in ethanol-induced gastric mucosal injury that vascular changes seem to be the most pronounced feature and severe damage is associated with extensive lesions of mucosal capillaries, increased vascular permeability and reduction of blood flow of mucosa (Szabo et al 1982, 1985; Motilva et al 1994).

D-002 also significantly reduced TXB_2 levels in gastric mucosa in rats with ethanol-induced ulcers; TXA_2, local mediator of gastric injury (Esplugues & Whittle 1988), is considered a noxious stimulus involved in the induction of mucosal damage by ethanol (Zengil at al 1991. Because an increase in the local TXA_2 concentration causes contraction in the binary network of the gastric mucosa, leading a local ischaemia followed by tissue necrosis and ulcer formation (Rozsa et al 1989), a reduction of such levels can reach protective or therapeutic effect. The increase of gastric mucus production in the rat stomach, and the reduction both of vascular permeability and of TXB_2 levels induced by D-002 treatment are different factors that could be explained by a cytoprotective effect of D-002. It has, in addition, also been shown that this action is prostaglandin-dependent. The relative contribution of each mechanism to the whole effect as well as the cause-effect relationship must be elucidated in further experiments.

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