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D-002

Antiulcer
Antioxidant
Antiinflammatory

Besswax Alcohols
BWA
Abexol®

Mixture of aliphatic alcohols isolated and purified from Beeswax (*Apis mellifera*) composed of triacontanol (25-35%), hexacosanol (7-20%), octacosanol (12-20%), tetracosanol (8-15%), dotriacontanol (18-25%) and tetratriacontanol (≤ 7.5%)

EN: 305330

Introduction

Peptic ulcer is caused by an imbalance between defensive and aggressive factors. Although the impairment of the gastric mucosal barrier plays a crucial role in gastric ulcer as compared with its contribution to duodenal ulcer, both intragastric pH and peptic activity are determinant in the development of gastric and duodenal ulcer disease (1, 2). In particular, duodenal ulcer is a chronic relapsing disease caused by an imbalance between the strength of duodenal mucosal resistance and the erosive action of acid and pepsin secretion.

Although the role of acid secretion in duodenal ulcer disease has not been fully elucidated, clinical evidences indicates that acid reduction achieved by proton pump inhibitors and histamine H_2 antagonists effectively accelerates ulcer healing (3-5). In addition, current evidence suggests that the integrity of the duodenal mucosa is of key importance in the mechanism of ulceration (6, 7).

Cytoprotective agents can exert beneficial effects on ulcer healing by sustaining the physicochemical properties of the mucosal barrier, scavenging the cytotoxic free radicals that mediate mucosal damage (8, 9).

There are different environmental factors that negatively influence the duodenal mucosa, not only predisposing to ulcer occurrence, but also increasing the period of time required for ulcer healing. Stress, alcohol, nonsteroidal antiinflammatory drugs (NSAIDs) and, above all, smoking are environmental risk factors for ulcer development (10). Thus, the first step in ulcer management is the control of these risk factors, achieved by the adherence to a healthy lifestyle based on smoking cessation and stopping or reducing the regular use of NSAIDs, alcohol and coffee consumption, especially when the stomach is empty. Nevertheless, these measures alone usually are not enough for the healing of preexisting ulcers, and pharmacological therapy is commonly needed.

Antiulcer pharmacotherapy includes a variety of effective and safe drugs, the proton pump inhibitors and H_2 antagonists (3, 4, 10-12) being the most effective for duodenal ulcer, while cytoprotective agents such as sucralfate are more effective in the prophylaxis of ulcer disease, mainly gastric ulcer (13).

Nevertheless, even though these drugs are safe, all of them show some drug-related adverse effects (3, 4, 11-15). Gastrointestinal disturbances (diarrhea, flatulence, abdominal pain and constipation) are the most common adverse effects reported for the proton pump inhibitors and H_2 receptor antagonists, and headache and dizziness/vertigo are the next most frequently reported. In addition, skin reactions, gynecomastia, impotence, weight gain and hemolytic anemia have also been documented (10-16).

The search for new agents for use in healing, relapse and preventive management of ulcer disease is thus a matter of clinical interest.

Beeswax

Beeswax is a widely used natural wax obtained from the cellular fabrications of worker bees (honeycombs) (17), which consists of a complex mixture of hydrocarbons (14%), monoesters (35%), diesters (14%), triesters (3%), hydroxymonoesters (4%), hydroxypolyesters (8%), free fatty acids (12%), acid monoesters (1%), acid polyesters (2%) and other materials (7%), and has been used in the pharmaceutical and cosmetic industry. Most data for beeswax refer to the wax obtained from the European bee *Apis mellifera*.

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Table I: Composition of beeswax alcohols.

Individual alcohols	Percent in BWA (limits)
1-triacontanol	25-35%
1-hexacosanol	7-20%
1-octacosanol	12-20%
1-tetracosanol	8-15%
1-dotriacontanol	18-25%
1-tetratriacontanol	≤ 7.5%
Total	85-92%

Impurities: diols, fatty acid salts and hydrocarbons (paraffins)

Description

D-002, recently designated as beeswax alcohols (BWA), is a mixture of higher aliphatic alcohols isolated and purified from beeswax (*Apis mellifera*) through a procedure consisting first of saponification followed by extraction steps performed with organic solvents, such as acetone and *n*-hexane (18). It is an off-white to light cream crystalline powder, which is highly insoluble in water, insoluble in hexane, methanol and ethanol, and very slightly soluble in chloroform and 1,2-dicloroethane. Melting temperature ranges from 72 to 82 °C.

The composition of D-002 is shown in Table I. Thus, the high-molecular-weight alcohols composing the mixture are: triacontanol (molecular weight, MW: 438.5 μm, $C_{30}H_{61}OH$), followed by hexacosanol (MW 382.4 μm, $C_{26}H_{53}OH$), octacosanol (MW: 410.5 μm, $C_{28}H_{57}OH$), dotriacontanol (MW: 466.5 μm, $C_{30}H_{65}OH$), tetracosanol (MW 354.7 μm, $C_{24}H_{49}OH$) and tetratriacontanol (MW: 494.7 μm, $C_{32}H_{69}OH$). It has been shown that the relative proportion of each aliphatic alcohol within the mixture is within a reproducible range and stable on storage conditions. The total content of alcohols must be 85-92% to reach quality specifications. The high-molecular-weight alcohols present in this mixture do not contain asymmetric carbon atoms in their structure. Other compounds, such as natural diols, paraffins and salts of fatty acids, are also present in the raw material as impurities.

A quality control (QC) system is applied to the manufacturing process starting from the beeswax up to the finished form, which includes organoleptic and microbiological control, as well as identification and determination of the content of higher aliphatic alcohols by a gas chromatography (GC) method performed on both the raw material and finished form. For QC of the finished form, determination of content uniformity and disintegration assays are also performed.

The methods to determine the content of higher aliphatic alcohols have been supported by the study of linearity, accuracy and precision (repeatability and reproducibility) and properly validated. Since the major components of D-002 are six higher aliphatic primary alcohols, the quality of the extract is standardized according to the composition and total content of such alcohols. The identity of the mixture, that is the relative proportion of the constituents in each batch, is determined using a validated GC method using flame ionization as the detection system, which is able to distinguish and identify the methylsilyl (MS) derivatives of the individual alcohols present in the mixture according to their relative retention times.

Once the identity of D-002 is established, the purity is determined by the quantification of the total content of higher aliphatic primary alcohols present in this mixture, which must be ³ 85% to reach the quality specifications, and, as a whole, impurities range from 8% to 15%.

Accelerated and room temperature stability studies have been conducted on the raw material and finished form, showing that the product is very stable under storage conditions. The room stability studies were conducted under ambient conditions defined for the Republic of Cuba as Zone IV (27 ± 3 °C; 80 ± 10% of R.H.) for 36 months. These studies did not show changes in the content of the mixture of higher aliphatic primary alcohols, nor in other quality specifications, of either the raw material of the finished form, so that the product has a shelf life of 3 years (19).

D-002 is available as plain tablets containing 50 mg of BWA registered in Cuba, the source of origin, under the trademark Abexol®.

The drug development approach has supported the mixture instead of the purified triacontanol alone, not only because triacontanol is very difficult to synthesize, but also because its relative proportion within the mixture is not so different from that of hexacosanol and octacosanol to be defined as a major component; hence, purification of triacontanol would require sophisticated techniques, which are not feasible on an industrial scale. In addition, the reproducibility of the mixture is high, QC methods developed are adequate and D-002 is very stable under the proposed storage conditions.

Pharmacological Actions

Table II summarizes the main preclinical pharmacological results for D-002.

Antiulcer effects of D-002: evidence of a cytoprotective mechanism

The antiulcer effects of D-002 have been demonstrated in different experimental models (20). D-002 given orally at 25 and 50 mg/kg, but not at 5 mg/kg, significantly inhibited indomethacin-induced ulcers by 25.3% and 50.2%, respectively, compared with the control group. Cimetidine given orally at 25 mg/kg induced inhibitory effects similar to those of D-002 administered at this dose, indicating similar efficacy in this experimental model. In addition, D-002 at doses of 5 and 50 mg/kg prevented ethanol-induced ulcers in rats, achieving inhibition of 55.7% and 66.9%, respectively. Cimetidine administered at 25 mg/kg, however, was ineffective in this experimental model (20). Thus, oral administration of

Table II: Summary of main studies of precilinical pharmacology of D-002.

Ref.	Studies	Administration and species used	Doses tested (mg/kg)	Main results
Carbajal *et al.* (20)	Evaluation of antiulcer activity in different experimental models	Oral, gastric gavage Rats	5-100	D-200 at 25 mg/kg inhibited ethanol, indomethacin and pylorus ligation-induced ulcers, but 100 mg/kg was required to prevent stress ulcers. Cimetidine 100 mg/kg was similar to D-002 inhibiting indomethacin but not ethanol ulcers
Carbajal *et al.* (21)	Cytoprotective mechanism in rats of D-002	Oral, gastric gavage Rats	5-100	A subulcerogenic dose of indomethacin prevented the inhibitory effects of D-002 on ethanol ulcers, indicating prostaglandin dependence. D-002 increased gastric mucus production, reduced stomach vascular permeability and TxB_2 content
Carbajal *et al.* (22)	Effect of D-002 on gastric mucus composition in ethanol-induced ulcer	Oral, gastric gavage Rats	5 and 25	D-002 5 and 25 mg/kg significantly increased the amount and protein content of gastric mucus in ethanol-induced ulcers. Also, it increased neutral glycoproteins and sulfated macromolecules
Valdés *et al.* (23)	Comparative study of the antiulcer effects of D-002, sucralfate and omperazole	Oral, gastric gavage Rats	D-002 5-100 Sucralfate 25-100 Omperazole 0.1-10	D-002 and sulcralfate were similarly and more effective than omeprazole in ethanol-induced ulcers, but in stress-induced ulcers omeprazole was more effective than D-002 and sucralfate
Carbajal *et al.* (24)	Antiinflammatory activity of D-002	Oral, gastric gavage Rats	25-200	D-002 significantly reduced the weight of cotton pellet granuloma, the exudate volume and LTB_4 levels in the exudate in carragenan-induced pleurisy
Menéndez *et al.* (34)	Inhibition of rat microsomal lipid peroxidation by D-002	Oral, gastric gavage Rats	5-100	D-002 partially inhibited *in vitro* enzymatic and nonenzymatic brain and liver lipid peroxidation, as well as *in vivo* CCL_4 and toluene-induced lipid peroxidation
Molina *et al.* (42)	Antioxidant effects of D-002 on gastric mucosa of animals with experimentally induced injury	Oral, gastric gavage Rats	5-200	Single and repeated doses of D-200 25 and 100 mg/kg, but not 5 mg/kg, reduced both ulcer length and extent of lipid perodixation occurring in gastric mucosa of rats with indomethacin-induced ulcer. D-002 100 and 200 mg/kg, but not 25 mg/kg, significantly reduced gastric erosion index and extent of lipid peroxidation in gastric mucosa in rats with injury induced by ischemia-reperfusion
Noa *et al.* (52)	Effect of D-002 on preulcerative phase of carrageenan-induced colonic ulceration in guinea pig	Oral, gastric gavage Guinea pigs	25 and 50	D-002 and 50 mg/kg significantly reduced wet weight, wall thickness, histological indices and counts of infiltrating PMN neutrophils and macrophages
Noa *et al.* (53)	Effect of D-002 on acetic acid-induced colitis in rats	Oral, gastric gavage Rats	25 and 50	Single and repeated doses of D-002 administered before or after the induction of colitis with acetic acid reduced wet weight, macroscopic injury, PMN infiltration and wall thickness of colonic mucosa
Noa *et al.* (54)	Comparative study of of D-002 *versus* sufasalazine on acetic-induced colitis in rats	Oral, gastric gavage Rats	D-002 1-100 Sulfasalazine 1-100	Significant reductions of all indicators of colonic ulceration were observed in colonic mucosa of animals treated with D-002 and sulfasalazine from 5 to 100 mg/kg, being both similarly effective

D-002 at 5 mg/kg significantly inhibited ethanol-induced ulcers, while 25 mg/kg was the minimal effective dose for inhibiting indomethacin-induced ulcers. This together with the fact that the percent inhibition induced by D-002 at 50 mg/kg in the model of ethanol-induced ulcers was larger than that achieved against indomethacin-induced ulcers, suggests that D-002 is more effective for preventing ethanol-induced ulcers than those induced by NSAIDs (20). In addition, D-002 inhibited ulcer occurrence in pylorus-ligated rats, increasing mucus production while the pH of acid secretion remained unchanged, indicating that its antiulcer effects are mediated by a cytoprotective mechanism (21).

Mucus secretion is a crucial factor for the protection of the gastrointestinal mucosa from the damage induced by different stimuli, so it is considered an important defensive factor in the gastric mucosal barrier (5, 6). Orally administered D-002 not only increased the content of soluble gastric mucus in nonulcerated rats, but also prevented the decrease in mucus production seen in ethanol-induced ulcers. In addition, it inhibited the increase in vascular permeability induced by ethanol, whereas it significantly decreased the concentration of thromboxane B_2 (TxB_2), a stable metabolite of thromboxane A_2 (TxA_2), in the gastric mucosa of rats with ulcers induced by ethanol (21).

Further studies revealed that oral treatment with D-002 (5-25 mg/kg) before the induction of ethanol-induced ulcers significantly increased not only the amount of gastric mucus, but also its protein content (22). These qualitative and quantitative changes on the mucus could explain, at least partially, the cytoprotective effects of D-002.

Nevertheless, the mechanisms whereby D-002 is cytoprotective are complex and include more than a single target, since they also depend on prostaglandin production. Thus, its antiulcerogenic effects in ethanol-induced ulcers could be prevented by the administration of subulcerogenic doses of indomethacin (22), a finding that could explain, at least in part, the increase in mucus production induced by D-002.

A comparison of the effects of D-002, sucralfate and omeprazole in ulcers experimentally induced by stress and ethanol, respectively (23), revealed that omeprazole, as expected for a proton pump inhibitor, was more effective than D-002 and sucralfate in preventing stress-induced ulcer, which is an acid-dependent induced ulcer. On the other hand, D-002 and sucralfate were more effective at preventing ethanol-induced ulcers than omeprazole (21), since ethanol acts as a necrotizing agent, so that this kind of ulcer is mainly related to the impairment of defensive factors in the gastric mucosa, which is why cytoprotective agents are very effective in this model.

Antiinflammatory effects

D-002 also shows moderate antiinflammatory effects associated with a significant reduction in leukotriene B_4 (LTB_4) levels in the pleural exudate of rats (24), indicating that a contribution of reduced leukotriene production to its cytoprotective effects cannot be ruled out.

Antioxidant effects

Oxygen-derived free radicals, such as hydroxyl radicals and superoxide anions, are cytotoxic agents which promote tissue damage, while their removal leads to the opposite effect (25). These radicals damage the cell membrane and induce the release of intracellular components, leading to further damage as part of a positive feedback cycle, which is of particular importance in the pathogenesis of gastric mucosal lesions. Scavenging such radicals therefore facilitates ulcer healing (26-31).

With these facts in mind, the possible contribution of an antioxidant effect of D-002 to its cytoprotective mechanism was investigated. The results showed that D-002, when orally administered to rats at doses of 5, 25 and 100 mg/kg, inhibited lipid peroxidation in rat liver microsomes, preventing the in vitro accumulation of thiobarbituric acid-reactive substances (TBARS) in hepatic microsomes in a dose-dependent manner (32).

The antioxidant action of D-002 was further demonstrated by *in vivo* experiments, which showed that oral administration for 2 weeks (5-100 mg/kg) significantly lowered the baseline levels of lipid peroxidation in liver and brain microsomes in a dose-dependent manner (32). It also inhibited CCl_4- and toluene-induced lipid peroxidation in brain microsomes. Toluene-stimulated lipid peroxidation was significantly inhibited by D-002 administration, since doses of 25 and 100 mg/kg fully inhibited TBARS accumulation. Since *in vivo* CCl_4 hepatotoxicity has been related to lipid peroxidation (33), while toluene is a neurotoxic chemical that acts by increasing the generation of reactive oxygen species in brain (34), the ability of D-002 to inhibit TBARS generation *in vivo* induced by both chemicals indicates that it not only protected microsomes against in vitro lipid peroxidation, but also against *in vivo* peroxidation, with maximal inhibition achieved at a dose of 25 mg/kg. The high degree of protection against oxidative stress in brain exerted by D-002 might be biologically relevant since the brain is highly susceptible to peroxidative damage because of its high level of unsaturated fatty acids in relation to endogenous antioxidant levels and high oxygen consumption (35, 36).

D-002 (5 and 25 mg/kg) increased superoxide dismutase (SOD) activity in both soluble and particulated fractions from brain and liver. In addition, a significant increase in glutathione peroxidase activity was also observed at both doses and was dose-dependent (32).

These results suggest that the inhibition of lipid peroxidation induced by oral administration of D-002 may be a consequence, at least in part, of its action on antioxidant enzymes. The most important *in vitro* source of O_2^- is the electron transport chain of the mitochondria. Significant elevations in mitochondrial SOD activity by D-002 could indicate that it increases the dismutation of

superoxide radical once generated from O_2, and hence can reduce the generation of much more deleterious oxygen species and the associated oxidative damage (37, 38). Even when enhanced dismutation of superoxide radical results in the generation of H_2O_2, it is known that glutathione peroxidase can detoxify H_2O_2 (39). Therefore, elevated levels of glutathione peroxidase induced by D-002 can detoxify enhanced levels of H_2O_2. It should also be considered that glutathione peroxidase could inhibit lipid peroxidation by directly catalyzing the conversion of peroxidated lipids to alcohols.

It is known that Se-dependent glutathione peroxidase catalyzes the breakdown of inorganic hydroperoxides, whereas Se-independent peroxidase catalyzes the breakdown of organic peroxides (39). Glutathione peroxidase activity was measured using a method which determines the total peroxidase activity, indicating that the significant stimulation of glutathione peroxidase activity induced by D-002 may stem not only from an enhancement of H_2O_2 detoxification, but also from lipid peroxides produced by oxidative stress.

Nevertheless, although all this evidence supports a remarkable *in vivo* antioxidant effect for D-002 in liver and brain tissues, it does not entirely explain the potential contribution of the antioxidant effects of D-002 to its cytoprotective action on gastroduodenal mucosa. For this reason, further experiments evaluated the effects of D-002 on the lipid peroxidation occurring in gastric mucosa of rats with ulcers induced by indomethacin (40). Single and repeated oral doses of 25 and 100 mg/kg, but not 5 mg/kg, significantly reduced both the ulcer length and the extent of lipid peroxidation occurring in gastric mucosa, as assessed using TBARS and MDA (malondialdehyde) levels, in experimental gastric ulcers induced by indomethacin in rats. Similar results were obtained when its effects were assessed by levels of malondialdehyde (MDA)/mg tissue. Since the reduction in MDA levels on the 5 mg/kg dose tended towards significance ($p = 0.05$), it cannot be ruled out that longer treatment at this dose could be effective (40).

The effects of D-002 on acute gastric mucosal injury induced by ischemia-reperfusion (I-R) in rats were also investigated. The results showed that single oral doses of D-002 were not effective in reducing the erosion index and lipid peroxidation in this experimental model, even at the highest dose tested (200 mg/kg), while repeated doses of 100 and 200 mg/kg, but not 25 mg/kg, significantly reduced both the gastric erosion index and the extent of lipid peroxidation induced by I-R (40).

The rationale for including these experimental models in the pharmacological study of D-002 was based on the fact that the generation of oxygen-derived free radicals is greatly increased under pathological conditions and is implicated in tissue injury. The cytotoxic effect of oxygen free radicals is the result of their ability to react with unsaturated lipids and to initiate lipid peroxidation reactions in target cell membranes, leading to cell injury (41-44). The major sources of oxygen-reactive species in the gastric mucosa are considered to be polymorphonuclear leukocytes (PMNs) and the xanthine-xanthine oxidase system (43).

Experimental evidence supports a role for activated PMNs and the formation of oxygen free radicals in indomethacin-induced gastric injury (45, 46), and the protective effects of certain drugs on these lesions in rats is mediated through the reduction of circulating activated PMNs, followed by reduced lipid peroxidation (47, 48). Thus, there is evidence of elevated production of LTB_4, a potent chemotactic agent for neutrophils, after NSAID administration, and therefore a reduction in LTB_4 levels could produce an inhibitory effect on PMN adherence to the vascular endothelium (49-51). In this regard, the efficacy of D-002 in reducing both ulcer size and lipid peroxidation (nmol MDA/mg tissue) in the gastric mucosa in the indomethacin-induced ulcer model can also be explained by a contribution of this mechanism. Since previous work demonstrated a reduction in LTB_4 levels in the pleural exudate of rats treated with D-002 (23), its effects on lipid peroxidation could be mediated by a reduction in PMN migration to the damaged site.

On the other hand, the gastric erosions induced by I-R are due to increased tissue lipid peroxidation, caused by reactive oxygen species derived from the xanthine-xanthine oxidase system (43). Our results suggest that D-002 can exert some effect on this enzymatic system, although this remains to be further elucidated.

Effects of D-002 on experimentally induced colonic ulcerative disease

Evidence for a protective effect of D-002 on colonic ulceration was obtained investigating its effects on the preulcerative phase of carrageenan-induced colonic ulceration in the guinea pig (52). D-002 (25 and 50 mg/kg i.p. for 3 days) was administered to the guinea pigs, all of which received degraded carrageenan aqueous solution (3%) as drinking fluid for 3 days. The results showed that D-002 significantly reduced wet weight, wall thickness, counts of infiltrating PMNs and macrophages, as well as the histological index in the colonic mucosa of treated animals as compared with controls (52).

Later on, the effects of D-002 administered as single or repeated doses (25 and 50 mg/kg) on acetic-induced colitis in rats were investigated (53). Significant reductions in all parameters associated with colonic ulceration were observed in animals treated with D-002 in both protective and therapeutic regimens when compared with controls (53).

A comparative study of D-002 and sulfasalazine was performed using the same experimental model, comparing the effects of oral doses (1, 5, 25 and 100 mg/kg) on acetic acid-induced colitis (54). Significant reductions in wet weight, macroscopic injury, PMN infiltration and wall thickness were observed in the colonic mucosa of D-002- and sulfasalazine-treated animals as compared with untreated controls, the effects of both drugs being similar in this experimental model (54).

Comparison of D-002 with other mixtures of higher aliphatic primary alcohols

Since D-002 is a mixture of higher aliphatic primary alcohols, we compared its pharmacological actions not only with the more abundant individual alcohols within its composition, but also with those induced by another mixture of higher aliphatic alcohols developed by us, named policosanol (55-60).

The composition of the mixture of alcohols obtained from beeswax is qualitatively and quantitatively different from that obtained from sugar cane wax. Thus, octacosanol is the most abundant long-chain alcohol present in the mixture of alcohols obtained from sugar cane wax, accounting for a relative proportion of approximately less than or equal to 60% in the mixture; in beeswax, triacontanol, and not octacosanol, is the most abundant component, but in a relative proportion that represents approximately 25-35% of the mixture of high-molecular-weight alcohols obtained from this source (18, 61).

Although the similarity of the structures involved in both products suggests that similar pharmacological effects can be expected, it was demonstrated that, although some pharmacological actions are common to both products, the main effects are different (Table III). While policosanol inhibits cholesterol biosynthesis and lowers plasma cholesterol levels (54-60), D-002 did not exert a lipid-lowering effect (62). In addition, a comparison of the effects on cholesterol biosynthesis in human fibroblast cultures revealed that while policosanol and octacosanol are able to inhibit the incorporation of [^{14}C]-acetate into cholesterol by up to approximately 70% as compared to untreated controls, uptake was not affected in D-002- and triacontanol-treated cultures (55-57).

Policosanol (55, 63-67), but not D-002 (62), showed antiplatelet properties, while the opposite was true for antiinflammatory effects. Thus, policosanol was devoid of any antiinflammatory action even at doses as high as 400 mg/kg, whereas D-002 has shown moderate antiinflammatory effects in different experimental models, which are associated with a reduction in LTB_4 (24).

Finally, although some effects are elicited by both products, differences in the pharmacological potency and the extent of the effects have also been found. Although policosanol is able to inhibit ulcers induced by ethanol and aspirin, like D-002, the minimal doses required for such effects (50 mg/kg) are 10 times higher than those of D-002. Moreover, the maximal inhibition on D-002 is approximately 80%, while policosanol failed to produce more than 55-60% inhibition. Also, D-002, but not policosanol, was able to effectively inhibit ulcers induced by stress and pylorus constriction.

Although both drugs inhibited LDL lipid peroxidation, the effects of policosanol were not only greater than those elicited by D-002, but also required lower doses. Thus, policosanol doses of 5 and 10 mg/day administered for 4-12 weeks to humans increased the lag time and decreased the diene propagation rate of LDL lipid peroxidation (68, 69), whereas effects for D-002 on fractionated plasma have only been demonstrated at a dose of 50 mg/day (70, 71), as will be analyzed in the clinical studies section. On the other hand, the effects of D-002 on oxidation processes occurring in different tissues are particularly pronounced compared with those of policosanol (72, 73). This difference could be a consequence of a different bioavailability of the drugs at different targets, although the scarce pharmacokinetic information available does not allow the confirmation of such a supposition.

Nevertheless, such differences are not completely surprising, and are to be expected given the data about the difference in the biological effects induced by individual higher aliphatic alcohols previously published. Triacontanol, considered to possess plant growth-regulating properties (74), increased CO_2 fixation in the green alga *Chlamydomona*s, while octacosanol alone has no effect on photosynthetic CO_2 assimilation, but is able to inhibit the effect induced by triacontanol (75). Similar results were obtained for stimulation of ATPase activity in barley (*Hordeum vulgare*) root plasma membrane treated with triacontanol and octacosanol (76).

In line with the differences in the effects of individual alcohols is the fact that hexacosanol, but not other naturally occurring long-chain fatty alcohols, exhibited neurotrophic effects on cultured central nervous system neurons (77). This effect was further experimentally corroborated, as *n*-hexacosanol has been shown to enhance neurite outgrowth and in vivo neuronal differentiation, to promote the survival of septal neurons after axotomy (78), to reduce neuronal damage induced by the neurotoxin kainic acid (79), and to enhance mouse sciatic nerve regeneration (80).

These findings are consistent with our results and suggest that high-molecular-weight aliphatic alcohols appear to be associated with protective functions in the vegetal and animal kingdom. It is thus logical that they may provide not only new drugs and novel foods, but also serve as templates to model new structures supporting compounds with protective functions to be exerted in different target organs.

Toxicology

Experimental studies conducted with any new compound intended to be used by humans are undertaken to describe its toxicity after administration to animal species for finite periods. To increase the ability of such studies to detect any potential product-related hazard, commonly doses many times higher than that recommended for clinical use are investigated. The first rational approach is to consider any toxicity expected from the chemical structures or compounds present in the substance to be investigated. In this regard, the main components of D-002 are higher aliphatic primary alcohols, molecules documented as safe. In the case of policosanol, a cholesterol-lowering drug acting via inhibition of cholesterol biosynthesis and an increase in LDL receptor-dependent processing, no

Table III: Summary of main studies of preclinical pharmacology of D-002.

Ref.	Studies	Administration and species used	Doses tested (mg/kg)	Main results
General toxicology				
Rodeiro *et al.* (88)	Acute oral toxicity of D-002 in Sprague Dawley rats	Oral, gastric gavage Rats	500-5000	No mortality was observed in any group. Overall, D-002 did not induce any toxic symptoms, indicating that acute oral toxicity of D-002 is negligible and LD_{50} was > 5000 mg/kg
Rodeiro *et al.* (89)	Oral subacute (14 days), subchronic (90 days) and chronic (1 year) toxicity	Oral, gastric gavage Mice and rats	Subacute: 2000, 3000, 5000 Chronic: 250, 500 and 1000	No treatment-related toxicity was demonstrated in these studies. Thus, effects on body weight, food consumption, clinical observations, blood parameters, organ weight ratios and histopathological findings were similar in control and treated groups, supporting a wide safety margin for the product
Rodeiro *et al.* (90)	Oral acute and subchronic (90 days) toxicity	Oral, gastric gavage Mice	Acute: 500, 1500, 2000 and 5000 Subchronic: 24, 125 and 625	No mortality, toxic symptoms, significant changes in body weight, organ weight and histopathological findings were documented The effects of subchronic administration on mouse spermatogenesis were also evaluated and no significant differences among groups nor tendency with the doses were observed. Thus, no evidence of treatment-related toxicity was shown
Alemán *et al.* (91)	Chronic study	Oral, gastric gavage Beagle dogs	50 and 250	No mortality, toxic symptoms, significant changes in body weight, blood indicators, organ weight and histopathological findings were documented. Thus, no evidence of treatment-related toxicity was shown
Special toxicology				
Gámez *et al.* (92)	Ames test	Addition on cultures	5000 μg/ml *Salmonella typhimurum*	No evidence of mutagenic potential was found
Gámez *et al.* (93)	Micronucleus test	Oral, gastric gavage Mice	2000	No evidence of mutagenic potential was found
Rodeiro *et al.* (94)	*In vivo* genotoxic study	Oral, gastric gavage Mice	Dominant lethal assay: 25-625	No significant differences among treated and control animals were found. Thus, no evidence of any genetoxic potential was found
Rodríguez *et al.* (95)	Developmental toxicity	Oral, gastric gavage Rats and rabbits	100, 320 and 1000	There was no evidence of embryototoxicity or teratogenicity in any of the treated groups

evidence of drug-related toxicity has been found even when administered for long periods (54-60, 81-86).

Nevertheless, the mixture of higher aliphatic alcohols present in D-002 is qualitatively and quantitatively different from that present in policosanol. In consequence, its pharmacological profile is also different, indicating the importance of a proper evaluation of the potential toxicity of D-002. The toxicity of D-002 was therefore investigated in studies of general and special toxicology, comprising acute systemic toxicity testing and repeated-dose studies in rodent and nonrodent species, and studies to evaluate mutagenic potential, as well as fetal and reproductive toxicity. These studies have shown no treatment-related toxicity (Table III), indicating that D-002 is very safe.

The species selected for the different studies were those commonly used in experimental tests for any new compound. As antiulcer and antioxidant effects of D-002 have been demonstrated in the rat, this proves that this species is sensitive and thus adequate to assess the putative toxicity of the product.

The dose levels selected for the different studies were multiples of the effective antiulcerogenic and antioxidant doses in such species and the dose recommended for humans. The standard daily dose is 50 mg/day, or approximately 0.7 mg/kg on the assumption of a mean

body weight of 70 kg. For the management of ulcer disease, this dose can be increased up to 200 mg/day if required, which represents approximately 1.5 mg/kg.

The toxicological experiments were conducted following the ethical recommendations for animal management and Good Laboratory Practices guidelines. Animals used in the different studies were purchased from the Center for Production of Laboratory Animals (CENPALAB, Havana, Cuba). Once received and quality specifications corroborated, animals were adapted to a quarantine period according to that established for each species under laboratory conditions of controlled temperature, humidity and light/darkness cycles, as regulated in the Standard Operational Procedures (SOP).

Acute oral toxicity studies

The acute oral toxicity of D-002 was investigated in rodents (rats and mice), rabbits and dogs (87, 88). All studies recorded mortality, daily clinical observations, food consumption and body weight gain during an observation period of 14 days after dosing. In addition, blood parameters, necropsy and histopathological examination were performed on the survivors after sacrifice. These studies were conducted in animals of both sexes randomly distributed in 5 experimental groups: a control group receiving only the vehicle acacia gum/water and 4 groups treated with D-002 (10 animals/sex/group) at 500, 1500, 2500 and 5000 mg/kg. The rationale for such high doses in acute studies was based on the negligible toxicity of higher aliphatic primary alcohols and the highest dose tested is that accepted as the maximal dose to be included in acute studies when the LD_{50} is not reached.

The results showed that the oral LD_{50} was > 5000 mg/kg (5 g/kg) in all cases. Mortality analysis, clinical observations, weight gain, behavioral assays and blood biochemistry and hematological determinations in surviving animals at the end of the test did not reveal differences between control and treated groups. In addition, organ weight data and histological examination of the survivors did not show any toxic effect attributable to the product. Overall, these results indicate that the acute oral toxicity of D-002 is practically negligible (87, 88).

Toxicity upon repeated administration

1) Subchronic oral toxicity in rats

A study of the subchronic oral toxicity of D-002 was conducted in Sprague-Dawley rats of both sexes randomly distributed in 5 experimental groups: a control group and 4 groups treated orally with 5, 25, 125 and 625 mg/kg D-002 for 90 days (88, 89). The rationale for such doses considered the lowest dose as a multiple of the dose effective in this species as an antioxidant and antiulcer drug; the other doses were 5, 20 and 125 times larger than the lowest dose.

The analysis of mortality, weight gain, clinical observations, blood indicators, necropsy, organ weight analysis and histopathology performed at study completion in all surviving animals did not show differences between control and treated animals. In conclusion, D-002 administered orally for 90 days to Sprague-Dawley rats did not induce any toxicity even at the highest dose administered (625 mg/kg), which is approximately 880 times the standard dose recommended in humans as an antioxidant and 220 times larger than the dose recommended for ulcer treatment.

2) Subchronic oral toxicity in mice

Another study investigating the toxicity of repeated oral doses of D-002 was conducted in young adult Swiss mice. Animals were randomly distributed in 4 experimental groups (10 animals/dose/sex): a control group and 3 groups treated orally with D-002 at 25, 125 and 625 mg/kg for 60 days (87). The analysis of mortality, weight gain, clinical observations, behavioral tests, necropsy, organ weight analysis and histopathology performed at study completion in all surviving animals did not show differences between control and treated animals; even the highest dose tested did not induce any treatment-related toxicity. In conclusion, oral administration of D-002 for 60 days to mice did not induce any toxicity even at the highest dose administered (625 mg/kg).

3) Chronic oral toxicity in rats

This study was also conducted in Sprague-Dawley rats of both sexes distributed in 4 experimental groups (20 animals/sex/group) used as controls or treated orally for 12 months with D-002 at 250, 500 and 1000 mg/kg (89). No significant differences were found between control and treated animals regarding all parameters investigated, such as mortality, clinical symptoms, behavior, body weight gain, food consumption, hematology, blood biochemistry, necropsy, organ weight data and histopathological findings (88). These results demonstrated that oral administration of D-002 up to 1000 mg/kg, a dose more than 1,408 times larger than the recommended dose for humans, did not induce any treatment-related toxicity, which indicates that it can be considered safe even after long-term administration.

4) Chronic oral toxicity in dogs

This study was conducted in beagle dogs of both sexes randomly distributed in 3 experimental groups (4 animals/sex/group) treated orally for 12 months with D-002 at 50 and 250 mg/kg, or used as controls (90). No significant differences were found between control and treated animals regarding all parameters investigated. These results demonstrated that oral administration of

D-002 up to 250 mg/kg, a dose 352 times larger than the recommended antioxidant dose (50 mg/day) and 86 times the recommended dose for ulcer management (200 mg/day) in humans, did not induce any treatment-related toxicity, indicating its safety even after long-term administration.

Mutagenic potential

The mutagenic potential of D-002 was investigated using both *in vitro* and *in vivo* assays (90, 91).

1) In vitro *tests*

The Ames test to detect gene mutations was performed on 5 different strains of *Salmonella typhimurium* (TA1535, TA1537, TA1538, TA98 and TA100) with and without metabolic activation. D-002 was suspended in Tween 20/water vehicle and added to cultures at final concentrations of 5, 50, 500, 2000 and 5000 μg/plate (92). Negative control and positive control cultures treated with *N*-acetylaminofluorene and aflatoxin B1 were used. In all cases, schemes with and without metabolic activation induced by the metabolizing system AROCLOR-induced rat liver S9 fraction were investigated.

The results showed that D-002 did not increase the number of revertant colonies, while an increase was observed in the positive control group, thus validating the system used to detect gene mutations. No evidence of mutagenic potential of the product was detected in this study (92).

2) *In vivo* tests

The *in vivo* clastogenic activity of the product, *i.e.*, the effects on the occurrence of micronucleated polychromatic erythrocytes (MPE) in mouse bone marrow cells, was investigated (91). According to standard protocol, mice of both sexes were randomly distributed in 3 experimental groups (5 animals/group): 2 groups were treated by gastric gavage for 5 days, *i.e.*, a control group receiving only vehicle and a group treated with D-002 at 2000 mg/kg, the highest dose recommended for this test, while the third group was a positive control treated with cyclophosphamide (CP) as a single intraperitoneal dose. The results showed that oral administration of D-002 did not induce a significant increase in MPE compared with the controls, whereas an increase was observed in positive controls (93).

The effects of D-002 on both primary spermatocytes and whole spermatogenesis in male albino Swiss mice were also investigated. Animals were randomly distributed in 4 experimental groups: a control group receiving only vehicle and 3 groups treated with D-002 at 25, 125 or 625 mg/kg for 60 days. No cytotoxic or genotoxic effect of D-002 was observed in this study, whereas a significant increase in the number of resorptions was observed in the positive controls (94).

Fetal and reproductive toxicity studies

Inseminated female animals were randomly distributed in 4 experimental groups (25 animals/group): a control group and 3 groups treated orally with D-002 at 100, 320 and 1000 mg/kg from day 6 to day 15 of gestation according to established standard protocols. On day 20 of gestation, the dams were euthanized by ether inhalation and fetuses were analyzed. The results showed that D-002 did not produce teratogenic effects in Sprague-Dawley rats (95).

In summary, extensive experimental toxicology studies have been conducted with D-002 and no evidence of drug-related toxicity has been found, indicating that treatment with D-002 is very safe even at the highest dose tested (1000 mg/kg), which is approximately 1,408 times the standard dose recommended in humans. Thus, the potential risk of oral administration of D-002 to humans is practically negligible.

Pharmacokinetics and Metabolism

At present there are insufficient data to review the pharmacokinetic profile of D-002, since most of the information is derived from data on file from the supplier. D-002 contains a mixture of six closely related molecules, which is why pharmacokinetic studies for each alcohol or/and its metabolites are very complex. Thus, during pharmacokinetic studies, the major effort focused on following the blood levels of triacontanol as the most abundant component of the mixture, as well as showing some of the main pharmacological effects of the mixture (62).

Serum levels of triacontanol were followed in monkeys using GC/mass spectrometry (GC/MS) methods. The results revealed that, after oral dosing, absorption is rapid, with a time to peak levels of 1 h and a half-life of 3 h. The fast and remarkable decay in plasma suggests a rapid distribution to extraplasmatic compartments, as occurs with other long-chain alcohols (96).

Clinical Studies

Table IV shows the results of the main clinical trials conducted investigating the effects of D-002.

Antiulcer effects

A double-blind, randomized, placebo-controlled study was conducted to investigate the effects of D-002 on duodenal ulcer healing and symptoms. Ninety-two eligible patients with duodenal ulcers entered the study and were

Table IV: Summary of main clinical studies of D-002.

Ref.	Studies	Design	Dosage and treatment duration	Main results
Hano *et al.* (97)	Effects of D-002 on ulcer healing and symptom relief in patients with duodenal ulcers	Randomized, double-blind, placebo-controlled	100 and 200 mg/d, 4 weeks	D-002 significantly improved ulcer healing rate and symptoms relief compared with placebo
Terry *et al.* (98)	Effects of D-002 in patients with osteoarthritis treated with piroxicam	Randomized, double-blind, placebo-controlled, all patients treated with piroxicam	50 mg/d, 4 weeks	D-002 significantly reduced the frequency of gastrointestinal adverse events compared with placebo, without affecting the efficacy profile of piroxicam
Menéndez *et al.* (70)	Antioxidant effects of D-002 in healthy volunteers	Randomized, double-blind, placebo-controlled	50 mg/d, 8 weeks	D-002 significantly increased plasma total antioxidant status compared with baseline and placebo. Likewise, it increased lag phase and reduced diene propagation rate in copper-induced peroxidation in fractionated plasma
Menéndez *et al.* (71)	Antioxidant effects of D-002 in older subjects	Randomized, double-blind placebo-controlled	50 mg/d, 8 weeks	Compared with baseline and placebo, D-002 significantly increased plasma total antioxidant status, lag phase and reduced diene propagation rate in copper-induced peroxidation in fractionated plasma

randomized to 3 groups: placebo (n = 31), D-002 100 mg/day (n = 30) and D-002 200 mg/day (n = 30). Endoscopic examinations were performed at baseline and after 4 weeks of treatment. Safety was assessed by determining hematological and blood chemisty indicators at baseline and after 4 weeks of therapy. Adverse events were assessed by physical examination, laboratory analysis and reports by patients.

At study completion, the mean healing score of patients who received D-002 200 mg/day (1.11 ± 0.96) was significantly lower ($p < 0.05$) than in patients who received placebo (1.90 ± 1.26); the mean healing score of patients who received D-002 100 mg/day (1.32 ± 1.49) was not significantly different from that of the placebo group. Healing rates in the intent-to-treat population were significantly higher in the D-002 200 mg/day (70.9%) and 100 mg/day groups (43.3%) compared with placebo (16.1%; $p < 0.001$ and $p < 0.05$, respectively) (95).

After 4 weeks, the combined rate of partial and complete symptom relief was 87.1% (27/31) in the D-002 200 mg/day group, 76.7% (23/30) in the D-002 100 mg/day group and 25.8% (8/31) in the placebo group. The rates were significantly higher in the treatment groups compared with the placebo group ($p < 0.001$) (95).

It was concluded from this study that D-002 200 mg/day was effective in healing and relieving symptoms of duodenal ulcers, and was safe and well tolerated by study patients. Given that D-002 acts through a cytoprotective mechanism, further studies investigating the possible effects of D-002 on the healing and/or relapse of gastric ulcer are warranted.

Another clinical study was conducted to investigate whether D-002 could protect against gastrointestinal adverse effects (GAE) induced by piroxicam, a well-known NSAID, in patients with osteoarthritis. Sixty eligible patients, all receiving piroxicam (10 mg twice daily), were randomized to receive, under double-blind conditions, placebo or D-002 (100 and 200 mg/day) for 14 days. The efficacy of D-002 was assessed by the reduction or disappearance of piroxicam-induced GAE, and the influence of D-002 on piroxicam's analgesic effect was also assessed. The frequency of GAE reported by D-002-treated patients was lower than that experienced by placebo patients, but D-002 did not impair the analgesic action of piroxicam. These results suggested that D-002 could be used as an adjunctive treatment in patients with chronic arthritis under treatment with NSAIDs (97).

Antioxidant effects

The antioxidant effects of D-002 were first investigated in healthy volunteers (69). A double-blind, randomized, placebo-controlled study showed that D-002 administered at a dose of 50 mg/day during 12 weeks inhibited lipid peroxidation in these subjects. D-002 prolonged the lag time before the onset of conjugated diene generation during in vitro copper-induced peroxidation of unfractionated plasma, significantly decreased plasma MDA levels and increased plasma total antioxidant status (TAS).

The elucidation of the mechanism whereby D-002 inhibits lipid peroxidation was beyond the scope of this study. Nevertheless, some indications can be taken from the results obtained. In this study, consistent with the

decrease in TBARS levels, D-002 increased plasma TAS, which suggests that it effectively inhibited lipid peroxidation and that the observed decrease in MDA may be related to an augmentation of the antioxidant capacity of plasma upon treatment with D-002.

Moreover, treatment increased total plasma lipid oxidation resistance, suggesting a relationship between the decrease in MDA and a possible protection of plasma lipids against lipid peroxidation *in vivo*. However, the fact that oral treatment with D-002 increased not only the resistance of plasma lipids to copper-induced peroxidation, but also the free radical-trapping capacity of plasma, suggests that it may be metabolized after entering the circulation and that the metabolites may exert antioxidant effects.

On the other hand, there is an intrinsic defense system limiting free radical-mediated effects in humans comprising a variety of scavenging enzymes such as superoxide dismutases (SODs), catalases and the most important H_2O_2-removing enzymes in human cells, glutathione peroxidases (GSHPXs) (37-39). Also, radical-scavenging antioxidants in plasma have been claimed to be important *in vivo* antioxidants (36). Our results showed that oral treatment with D-002 at a dose of 50 mg/day did not modify SOD and GSHPX, which indicates that it does not act through an increase in the intrinsic defense system, at least at this dose.

Another double-blind, randomized, placebo-controlled study investigated the antioxidant effects of D-002 administered at 50 mg/day for 12 weeks in elderly patients (70). D-002 significantly decreased plasma susceptibility to copper-induced lipid peroxidation, decreased MDA levels and increased TAS, results consistent with those previously found in healthy volunteers (69).

Although the free radical theory of aging is far from conclusive, there is no question that several diseases associated with free radical overproduction are very frequent in elderly patients (98-100). Thus, it seems clear that in sick elderly protective antioxidant mechanism are reduced and supplementation with antioxidants may reduce the deleterious effects of free radical reactions on cells. Therefore, D-002 treatment could be useful to prevent or manage such pathological conditions in the elderly.

In all clinical studies, D-002 was safe and well tolerated. No treatment-related disturbances regarding any safety indicator were observed and the frequency of withdrawals was similar in the D-002 and placebo groups. Importantly, these results were obtained in populations consuming concomitant medications, such as Ca^{2+} antagonists, β-blockers, NSAIDs, diuretics, myorelaxants, nitrates, antihistaminics and anxiolytics, among others, suggesting that no clinically relevant interactions occurred.

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