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D-003

Antiplatelet Therapy
Treatment of Lipoprotein Disorders

Mixture of very-high-molecular-weight aliphatic primary acids isolated and purified from sugar cane wax (*Saccharum officinarum* L.) whose main component is octacosanoic acid, followed by triacontanoic, dotriacontanoic and tetratriacontanoic acids, whereas hexacosanoic, heptacosanoic, nonacosanoic, hentriacontanoic, tritriacontanoic, pentatriacontanoic, tetracosanoic, pentacosanoic and hexatriacontanoic acids are minor components

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Abstract

D-003 is a mixture of very-high-molecular-weight aliphatic acids purified from sugar cane (*Saccharum officinarum* L.) wax with cholesterol-lowering and pleiotropic effects potentially beneficial for preventing atherosclerosis and its complications. D-003 inhibits cholesterol biosynthesis through the regulation of HMG-CoA reductase activity. Following oral administration to rabbits and dogs, it dose-dependently reduced serum levels of total (TC) and low-density lipoprotein cholesterol (LDL-C), whereas it increased high-density lipoprotein cholesterol (HDL-C) and had no effect on triglycerides (TG); these effects persisted after long-term treatment. Further experiments in hypercholesterolemic rabbits showed that D-003 prevented the increase in serum LDL and inhibited *de novo* synthesis of cholesterol, increasing the binding of LDL to liver homogenates and the removal of serum LDL. D-003 also has antioxidant and antiplatelet effects. D-003 consistently prevented experimental arterial thrombosis and ischemia, and inhibited the development of foam cells and cuff-induced carotid neointimal proliferation in rabbits. Orally administered D-003 also exerted extravascular effects, the most relevant being prevention of bone loss induced by ovariectomy in rats. Results from experimental toxicology studies have not shown any D-003-related toxicity. In healthy subjects, D-003 (5-50 mg/day) over the short term lowered serum LDL-C and TC, increased HDL-C and had no effect on TG. Bleeding time was increased on the highest dose, but individual values were normal. D-003 (10 and 20 mg/day) for 10-14 days significantly inhibited platelet aggregation induced by arachidonic acid and collagen, but not ADP, had no effect on coagulation time, lowered serum TxB_2 and increased PGI_2 levels. D-003 (5 and 10 mg/day) prevented LDL from undergoing lipid peroxidation. In a placebo-controlled dose-effect study in patients with type II hypercholesterolemia, D-003 (5-40 mg/day for 8 weeks) significantly and dose-dependently lowered serum LDL-C and TC, and increased HDL-C. Another study in older hypercholesterolemic patients showed that D-003 (5 and 10 mg/day) reduced LDL-C and TC, raised HDL-C, and at the higher dose moderately reduced TG. Antioxidant effects were also observed and the treatment was well tolerated.

Introduction

Coronary heart disease (CHD), cerebrovascular and peripheral artery disease are major causes of mortality and morbidity in the adult population (1-3). In particular, coronary mortality is the leading cause of death worldwide. Hence, coronary prevention is a major health concern, the basic strategy being to control modifiable coronary risk factors (4-6).

Atherosclerosis is the major pathological process involved in occlusive arterial diseases (7, 8). In the arterial circulation, atherosclerosis is the most important cause of superimposed thrombosis (7). Arterial thrombi commonly develop on ruptured atherosclerotic plaques or areas under turbulent blood flow, and comprise mostly platelet aggregates held by fibrin. Several factors, such as hypercholesterolemia, hypertension and blood turbulence, induce changes on endothelial cells, triggering lipid and monocyte accumulation (7-9). Further damage to the intima surface with denuding endothelium induces platelet adhesion, releasing growth factors that stimulate smooth muscle cell (SMC) proliferation. This process renders the lesions unstable and prone to rupture, which leads to thrombus formation (9, 10).

Elevated levels of total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C), together with hypertension and smoking, are considered the major risk factors for the development of atherosclerosis (11-14). In addition, the impact of lowering LDL-C and TC on clinical outcomes has been convincingly proven in landmark secondary and primary prevention studies conducted with inhibitors of HMG-CoA reductase, also known as statins (15-21). For example, major secondary prevention studies (4S, CARE, LIPID and HPS) demonstrated that simvastatin and pravastatin prevented recurrent coronary events and all-cause mortality (15-17, 20). Also, risk reduction for coronary morbidity and mortality was demonstrated in two primary and primary/secondary prevention trials (WOSCOPS and AFACPS/TexCAPS) (18, 19). PROSPER, the first published study specifically

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examining the cardiovascular benefits of a statin in older subjects, corroborated such benefits (21).

The decrease in the incidence of clinical outcomes observed in such studies was relatively rapid. The clinical benefits were evident as soon as several months after randomization (15-21), earlier than expected from regression studies. This finding led to the hypothesis that the clinical benefits observed in these studies can not be exclusively due to the reduction in plasma LDL-C levels (22). This possibility is consistent with results indicating that, beyond their cholesterol-lowering effects, statins interfere with other major events involved in endothelial wall function (23-27). Such non-lipid-lowering actions are known as pleiotropic effects of cholesterol-lowering drugs (28) and should serve to identify different targets for intervention in preventing atherosclerosis and its complications.

Despite the strong clinical benefits on risk reduction, cholesterol-lowering drugs are associated with adverse events, those related to muscle and the gastrointestinal system, including the liver, being the most relevant. Caution is therefore advised for using these drugs in population subsets sensitive to drug-related adverse events, such as patients with hepatic disease. Statins and fibrates can induce myopathies, including rare but severe rhabdomyolysis, as well as gastrointestinal disturbances, increases in serum transaminases and creatine phosphokinase (CPK), headache, cholelithiasis, impairment of fertility and diminished libido (29-33).

Considering that atherosclerosis is the common process in occlusive arterial vascular diseases and that platelet aggregation is key in the onset of thrombotic complications of atherosclerotic plaques (7), the relevance of pharmacological inhibition of platelet aggregation is evident. Hence, the preventive use of antiplatelet drugs has been clinically evaluated and evidence indicates that they protect a wide range of subjects at high atherosclerotic risk from CHD and stroke, the risk reductions for major coronary and cerebrovascular events being approximately 20-29% (34-43).

Evidence supporting the use of antiplatelet drugs for the primary prevention of individuals at low vascular risk, however, is not conclusive. Nevertheless, although a variety of antiplatelet agents are used for managing atherothrombotic diseases, no specific guidelines establish the extent of inhibition needed for obtaining a specific impact on clinical outcomes (44).

Different antiplatelet drugs are available, aspirin being the gold standard due to its efficacy, safety and low cost (6, 35-40). In a broad range of secondary prevention patients, aspirin reduces the cardiovascular risk by approximately 25%. Nonetheless, its effects on platelet aggregation are relatively moderate, as they are mediated only by inhibition of the synthesis of thromboxane A_2 (TxA_2), a powerful proaggregatory and vasoconstrictor substance released from both platelets and endothelial wall. Aspirin, however, leaves unaffected other pathways of platelet recruitment (37). Consistently, 10-20% of aspirin-treated patients with arterial thrombosis present recurrent vascular events during long-term therapy (35-40).

Therefore, antiplatelet agents acting through other mechanisms have been investigated. Extensive studies have been conducted with thienopyridines, such as ticlopidine and clopidogrel, which act by inhibiting the binding of ADP to its platelet receptors (37, 41-43). No antiplatelet drug is, however, actiive against all aggregating agents. Also, although antiplatelet therapy is generally safe and well tolerated, drug-related adverse events have been documented for all drugs of this therapeutic class (35-43). Hence, the search for new antiplatelet agents continues.

According to the above, a substance showing both cholesterol-lowering and antiplatelet effects would be a potential candidate for treating and/or preventing atherothrombotic disease.

Description

D-003 (sugar cane wax acids) is a mixture of very-high-molecular-weight aliphatic acids purified from sugar cane (*Saccharum officinarum L.*) wax by a manufacturing process including initial saponification followed by extraction with organic solvents. D-003 is a cream-colored crystalline powder, highly insoluble in water. Melting temperature ranges from 72 to 86 °C (45).

The purity of D-003 is expressed as the total content of high-molecular-weight aliphatic acids and is defined according to the relative composition of each acid within the mixture. The long-chain acids present in D-003 are 1-octacosanoic acid (C28), 1-triacontanoic acid (C30), 1-dotriacontanoic acid (C32) and 1-tetratriacontanoic acid (C 34), followed by 1-hexacosanoic acid (C26), 1-heptacosanoic acid (C27), 1-nonacosanoic acid (C29), 1-hentriacontanoic acid (C31), 1-tritriacontanoic acid (C33) and 1-hexatriacontanoic acid (C36). 1-Tetracosanoic acid (C24), 1-pentacosanoic acid (C25) and 1-pentatriacontanoic acid (C35) are also present as minor components. The relative proportion of each aliphatic acid is reproducible within the referred ranges and stable under storage conditions. The high-molecular-weight acids present in this mixture do not contain asymmetric carbon atoms in their structure. The identity and purity of D-003 are assessed using a validated gas chromatography (GC) method utilizing flame ionization as the detection system. The method is able to distinguish and quantify the derivatives of the individual acids present in the mixture according to their retention times relative to that of 1-nonadecanoic acid, used as internal standard (46, 47). Other compounds, such as α,β-unsaturated aldehydes, long-chain alcohols and paraffins are also present in the raw material as impurities. D-003 is formulated as film-coated tablets containing 5 mg of the long-chain acids comprising D-003.

Quality control has been applied to both active ingredient and the finished dosage form, which includes determination of the identity and content of higher aliphatic acids in both the raw material and the film-coated tablets.

In addition to routine microbiological specifications for oral solid forms, determination of uniformity of content and disintegration time are also assessed for the finished form.

Accelerated and long-term stability studies at room temperature have been conducted on both active ingredient and finished form, demonstrating high stability under storage conditions. The long-term stability studies have been conducted under climatic conditions defined for the Republic of Cuba as Zone IV (27 ± 3 °C; 80 ± 10% of RH). The stability of the active ingredient has been evaluated over 60 months, while that of the finished form has been tested for 48 months. The results have not revealed changes in the proposed specifications of either raw material or finished form.

Although octacosanoic acid is the most abundant component of D-003 and shares some pharmacological effects of D-003, for different reasons, drug development has supported the mixture instead of the purified octacosanoic acid alone. First, the synthesis or purification of octacosanoic acid is difficult, requiring laborious techniques not feasible on an industrial scale. Second, the pharmacological activity of D-003 is superior to that of octacosanoic acid in those models in which both substances have been tested (unpublished results). Third, batch-to-batch reproducibility for D-003 is high, the quality-control methods have been validated and the substance is very stable under the proposed storage conditions. Thus, being a mixture does not represent a relevant handicap.

Pharmacological Actions

Effects on serum lipid profile

D-003 has shown cholesterol-lowering effects in experimental models, particularly in normocholesterolemic rabbits. In all these studies, body weight gain, food consumption and overall animal behavior were unaffected by D-003 (48-50). Thus, D-003 (5-200 mg/kg) dose dependently reduced TC (31.7-42.7%) and LDL-C (69.4-85.2%) in normocholesterolemic rabbits, while it markedly and significantly increased HDL-C (36.8-55.3%), these effects being reversible after washout. Triglycerides, however, were unaffected by D-003 (48).

A further study (49) compared the effects of both D-003 and policosanol, a mixture of higher aliphatic alcohols obtained from the same source (50), administered at a low dose (5 mg/kg) for 30 days in the same experimental model. After 15 days on therapy, only D-003 significantly lowered LDL-C and TC values with respect to the control group, the reductions in LDL-C on D-003 being greater (45.3%) than in the policosanol group (38.1%). No other significant change in lipid profiles was observed at interim control. After 30 days, the changes in LDL-C and TC were enhanced in both groups, the reductions in LDL-C on D-003 (81.6%) being greater than on policosanol (68.7%), while decreases in TC (approximately 41%) were similar in both groups. Although both D-003 and policosanol significantly increased HDL-C, D-003 was more effective (78.8%) than policosanol (47.1%). Serum TG remained unchanged in both groups (49).

Based on these promising results, two other studies compared the effects of D-003 with those of the statins lovastatin and fluvastatin (51, 52). One study compared the cholesterol-lowering effects induced by D-003 5 mg/kg and lovastatin 10 mg/kg administered orally for 30 days. At study completion, D-003 and lovastatin significantly and similarly lowered serum LDL-C and TC. D-003, but not lovastatin, significantly increased HDL-C, whereas only lovastatin decreased TG (51). The other study compared the cholesterol-lowering effects of D-003 (5 mg/kg), fluvastatin (5 mg/kg) and their combination (52). Treatments were administered for 30 days. D-003, fluvastatin and combination therapy significantly lowered LDL-C by 81.5%, 61.4% and 75.9%, respectively. Similar effects were observed on TC levels, such that D-003, fluvastatin and combination therapy significantly lowered TC by 48.4%, 39.7% and 45.3%, respectively. D-003 and the combination, but not fluvastatin, increased HDL-C (21.5% and 19.0%, respectively), these changes being significant compared to controls. Fluvastatin and combination therapy, but not D-003, lowered TG (13.6% and 13.0%, respectively). The effects of combined therapy on HDL-C and TG were similar to those of D-003 and fluvastatin alone, respectively. The only advantage of such therapy therefore appears to be superior effects on HDL-C and TG compared to the respective monotherapies.

The long-term cholesterol-lowering effects of D-003 were explored in beagle dogs. D-003 orally administered at 200 and 400 mg/kg for 9 months significantly reduced TC levels in a dose-dependent manner, and the effects persisted throughout the study (53).

Effects on cholesterol biosynthesis

D-003 lowers cholesterol through inhibition of cholesterol biosynthesis between acetate consumption and mevalonate production, through the indirect regulation of HMG-CoA reductase activity by depressing *de novo* synthesis and/or stimulating enzyme degradation (54). When fibroblasts were cultured long term in lipoprotein-free medium, enzyme activity significantly increased. The exposure of cultured fibroblasts to lipid-depleted medium and D-003 led to a concentration-dependent decrease in cholesterol biosynthesis from [^{14}C]-acetate and tritiated water, but not from [^{14}C]-mevalonate. D-003 added to the incubation mixture did not affect HMG-CoA reductase activity, but in the presence of D-003 enzyme upregulation was suppressed, indicating that D-003 modulates rather than directly inhibiting HMG-CoA reductase enzyme activity.

Hypercholesterolemia induced in rabbits by a fat-free, casein-rich diet is a model of endogenous hypercholesterolemia useful for evaluating the effects of cholesterol-lowering drugs that inhibit cholesterol biosynthesis.

Rabbits fed a diet enriched with casein develop endogenous hypercholesterolemia mainly due to decreased LDL receptor activity and increased LDL synthetic rate. The efficacy of HMG-CoA reductase inhibitors in decreasing TC and LDL-C levels increased by diet in this model has been previously demonstrated (55, 56).

The effect of D-003 was therefore investigated in a model of hypercholesterolemia induced by a wheat/casein-rich diet in rabbits (57). Animals were fed the diet for 4 weeks and administered D-003 (5, 50 and 100 mg/kg) simultaneously. As expected, nontreated rabbits became hypercholesterolemic. As early as 15 days following dosing, D-003 at 50 and 100 mg/kg significantly prevented the increase in TC and LDL-C compared with controls, effects which were maintained up to study completion, when HDL-C levels significantly increased in all treated groups. Incorporation of $^{3}H_2O$ into sterols in the liver and proximal small bowel of treated rabbits was significantly suppressed, indicating that D-003 inhibited the *de novo* synthesis of cholesterol. Also, D-003 significantly increased the removal rate of [^{125}I]-LDL from serum and significantly increased the binding of [^{125}I]-LDL to liver homogenates. This was the first *in vivo* evidence of the inhibitory effects of D-003 on cholesterol biosynthesis and serum LDL clearance.

These findings suggest that D-003 prevents endogenous hypercholesterolemia induced by a fat-free, casein-rich diet, at least partially through inhibition of hepatic cholesterol biosynthesis, decreasing the cholesterol concentration in hepatocytes and preventing the loss of hepatic LDL receptors induced by casein administration (55, 56). However, since casein-induced hypercholesterolemia is also a consequence of a stimulation of cholesterol absorption in the lumen and an increase in cholesterol output associated with LDL, the effect of D-003 on cholesterol absorption should be further investigated.

Effects of D-003 on rat lipoprotein peroxidation

Oxidative modification of LDL is considered a key step in atherosclerosis development. Aldehyde products of lipid peroxidation modify LDL, producing oxidized LDL (LDL), which is scavenged by macrophages, leading to the production of foam cells, the first recognized step of atherosclerotic lesions (8, 9, 58). In addition, oxidized LDL is toxic and antigenic to endothelial cells, inducing a series of inflammatory steps leading to atherosclerosis (8, 9).

We investigated the effects of D-003 (0.5-100 mg/kg) administered orally to rats on the susceptibility of plasma lipoproteins (LDL + VLDL) to undergo lipid peroxidation (59). The effects of D-003 on the kinetics of conjugated diene generation in a cell-free medium were first investigated. D-003 (5-100 mg/kg) inhibited lipid peroxidation of rat plasma lipoproteins, protecting their lipid domain from Cu^{2+}-induced peroxidation, increasing the time for the maximal production of conjugated dienes and lowering the maximal propagation rate of conjugated dienes compared with controls. D-003 also attenuated the reduction of free amino groups induced by Cu^{2+}, indicating that it also diminishes oxidative damage to the protein moiety of the lipoproteins.

Effects of D-003 on lipid peroxidation were also evaluated by examining the susceptibility of plasma lipoproteins to lipid peroxidation induced by 2',2'-azobis-2-amidinopropane hydrochloride (AAPH), a compound that releases peroxyl radicals at a constant rate when incubated at 37 °C. D-003 (5, 50 and 100 mg/kg) administered orally for 4 weeks inhibited both lipid peroxidation and carbonyl group production induced by AAPH in a dose-dependent manner, the dose of 50 mg/kg producing the maximal effect (59).

Effects of D-003 on *in vitro* lipid peroxidation in rat liver and brain homogenates were also investigated. D-003 (5, 50, 100 and 200 mg/kg/day p.o. for 4 weeks) inhibited the generation of thiobarbituric acid-reactive substances (TBARS) in native rat homogenates in which lipid peroxidation was induced by $FeCl_3$/ADP/NADPH (enzymatic system). D-003 inhibited TBARS generation in homogenates previously inactivated by heating (nonenzymatic system) in which lipid peroxidation was induced by $FeCl_3$/ADP/NADPH. Also, D-003 inhibited TBARS generation in active rat liver homogenates in which lipid peroxidation was evoked by the addition of CCl_4. In all cases, the effects were dose-dependent and the maximal inhibitory effect reached at 100 mg/kg was marked (> 80%) (59).

Effects of D-003 on platelet aggregation and thrombosis

As previously mentioned, increased platelet aggregation triggers thrombotic complications of atherosclerosis (7). Thrombosis is a key process triggering acute coronary syndromes and other critical vascular events, and the preventive effects of antiplatelet agents on the recurrence of coronary and cerebrovascular events has been documented (34-43).

D-003 (25-200 mg/kg) dose-dependently inhibited platelet aggregation in response to collagen and ADP in rats and collagen-induced platelet aggregation in guinea pigs (60). These effects have been demonstrated after single or repeated doses, including long-term (6 months) treatment (61). Consistent with its antiplatelet effects, D-003 increases bleeding time in rodents, an effect which is also dose-dependent (60, 61).

Considering that hemorrhage, mainly gastrointestinal bleeding, is one of the most relevant drug-related adverse events associated with antiplatelet drugs, the effects of D-003 on bleeding time were studied in experimental models and humans. Although D-003 increased bleeding time, this effect was not increased by prolonging treatment duration, was easily reversible after treatment withdrawal and individual values were within the normal range in rodents (60, 61) and beagle dogs (53). Hence, the potential risk of this effect appears not to be relevant. In addition, D-003 did not affect important markers of the

coagulation cascade (53, 61), indicating that the increase in bleeding time is related to an antiplatelet and not an anticoagulant effect.

Since pharmacodynamic drug interactions between D-003 and dicoumarol (warfarin) are probable, we investigated the putative benefits and risks of such combination therapy in two experimental models. Experimental venous thrombosis was used for assessing the benefits of concomitant use of D-003 and dicoumarol on efficacy, while bleeding time assessment was used as a marker of potential risk for bleeding episodes (62). D-003 and dicoumarol alone at a dose of 50 mg/kg prevented thrombus formation, reducing the thrombus frequency by 31.6% and 43.9%, respectively, compared with positive controls, and they reduced the average thrombus weight by 57.7% and 46.3%, respectively. Combination therapy was more effective than the respective monotherapies for preventing venous thrombosis, reducing the frequency of thrombus by 81.6% and thrombus weight by 86.3% *versus* positive controls. On the other hand, D-003, dicoumarol and their combination prolonged bleeding time by 128%, 152% and 184%, respectively, compared with controls. This interaction appears to be additive rather than synergistic, which is consistent with the different mechanism whereby D-003 and dicoumarol act as antiplatelet and thrombolytic agents, respectively. Since the antiplatelet effect of D-003 is generally marked, experimental drug interactions with anticoagulant drugs could be expected.

The effects of D-003 on platelet aggregation and experimental thrombosis are associated with a significant reduction in TxA_2 and a concomitant increase in prostacyclin (PGI_2) levels (63). The endothelium is known to release vasoactive substances, including powerful aggregatory, vasoconstrictor and prothrombotic substances such as TxA_2, which is also released by platelets and endothelin, as well as antiplatelet, vasodilator and antithrombotic substances, such as PGI_2 and nitric oxide (NO) (8, 9).

Experimental pharmacological testing has also shown a positive interaction between the antiplatelet and antithrombotic effects of D-003 and aspirin, which can be explained by the different antiplatelet mechanisms of the drugs, as evidenced from changes in arachidonic acid metabolites. The effects of combination therapy with D-003 (50 mg/kg) + aspirin (3 mg/kg) on arachidonic acid-induced sudden death in mice and bleeding time were studied in rats. D-003 + aspirin significantly increased bleeding time in rats more than D-003 or aspirin alone. Also, combined therapy with higher doses, such as D-003 200 mg/kg + aspirin 5 mg/kg, protected against arachidonic acid-induced sudden death in mice synergistically compared with either agent alone (64).

Consistent with the effects described above, D-003 (5-200 mg/kg) also prevents arterial thrombosis in rodents, an effect manifested at doses as low as 5 mg/kg (60).

Effects of D-003 on atherosclerosis development

Evidence of the beneficial effect of D-003 on atherosclerosis development has been obtained by studying its effects on foam cells and smooth muscle cell (SMC) proliferation. Orally administered D-003 (25 and 50 mg/kg for 20 days) significantly and markedly prevented the occurrence of foam cells induced by lipofundin in carrageenan-induced granuloma, the inhibition achieved with the lower dose (25 mg/kg) being > 90% (65). D-003 (5 and 25 mg/kg) administered for 20 days to rabbits markedly inhibited the cuff-induced neointimal proliferation of carotid arteries by 97.5% compared to positive controls, indicating antiproliferative effects on SMCs (66).

Other vascular effects

The effects of single (25-400 mg/kg) and repeated (5-200 mg/kg) oral doses of D-003 on myocardial necrosis induced in rats by subcutaneous injection of isoproterenol were investigated in other experiments. In both schemes, D-003 was effective in decreasing necrotic area, percent infarct area and the presence of polymorphonuclear cells (PMNs) in myocardial tissue. As single doses, D-003 was effective only at 200 and 400 mg/kg, but all doses were effective following repeated administration for 10 days, indicating that the repeated-dose scheme was more effective for preventing isoproterenol-induced myocardial injury (67).

D-003 (25 and 200 mg/kg) for 10 days to rabbits prevented neurological and histological damage in spinal cord ischemia induced by abdominal aorta occlusion (68). D-003 protected the rabbits against neurological symptoms occurring 4 h after reperfusion, histopathological changes and 24-h mortality, indicating broad protective effects. Also, D-003 significantly raised PGI_2 levels in thoracic aorta rings compared with positive controls.

D-003 at 5 mg/kg for 30 days also significantly reduced the number of circulating plasma endothelial cells in rabbits, suggesting a beneficial effect on the endothelial wall (49). Doses of 5 and 25 mg/kg to cuffed rabbits for 20 days also lowered endothelemia compared to positive controls (66).

Extravascular effects of D-003

Acute hepatotoxicity induced by CCl_4 in rats has been related with an increased rate of lipid peroxidation in the liver and antioxidant compounds have proven effective in this model. The effects of D-003 on acute hepatotoxicity induced by CCl_4 in rats were therefore studied (69). D-003 at 25 and 100 mg/kg markedly, significantly and dose-dependently decreased the percent of ballooned cells and hepatocytes with lipid inclusions, and increased the percent of normal hepatocytes compared to positive controls. The percent of swollen hepatocytes was also reduced *versus* positive controls, but not in a dose-

dependent manner. Necrotic areas and inflammatory infiltrate were observed in the liver of 87.5% of positive controls, while D-003 markedly reduced both necrotic areas and inflammatory infiltrates, which were present in only 12.5% and 0% of animals treated with 25 and 100 mg/kg, respectively. D-003 protected against histological changes characteristic of CCl_4-induced hepatic injury in rats.

Subsequently, the effects of D-003 were investigated on acute hepatotoxicity induced by paracetamol in rats, a model of hepatoxicity also dependent on lipid peroxidation (70). D-003 at doses of 5 and 25 mg/kg significantly and dose-dependently decreased the percent of swollen cells and hepatocytes with necrosis and increased the percent of normal hepatocytes compared with positive controls. Necrotic areas and inflammatory infiltrate were observed in the liver of 90% of positive controls compared to only 10% of D-003-treated animals. No histological alterations in liver sections of negative controls were found. The relationship between this protective action of D-003 and its antioxidant effects, however, deserves further investigation.

Mevalonate is a precursor of the lipoids required for osteoclast activity, and inhibition of its synthesis affects bone metabolism. Inhibitors of HMG-CoA reductase might increase new bone formation through a mevalonate-dependent effect. In turn, bisphosphonates inhibit bone resorption by mechanisms involving the metabolic pathway from mevalonate to cholesterol (71, 72). Thus, considering the action of D-003 on HMG-CoA reductase activity, we investigated its effects (50 and 200 mg/kg orally for 3 months) on bone loss induced by ovariectomy (OVX) in rats (73). As expected, OVX diminished trabecular number and thickness, increased trabecular gap, osteoclast number and surface. D-003 prevented the decrease in trabecular number and thickness, as well as the increases in trabecular separation, osteoclast number and surface compared to OVX controls, preventing bone loss and decreasing bone resorption induced by OVX, but it failed to increase osteoblast surface compared to OVX controls. The effects of D-003 were comparable to those induced by alendronate 3 mg/kg.

Experimental drug interactions

As stated above, D-003 shows pharmacological interactions with dicoumarol and aspirin in experimental models (62, 64). Compared with D-003 and dicoumarol alone, concomitant therapy with D-003 + dicoumarol increased both antithrombotic efficacy and bleeding time values, an interaction which appeared to be additive (62). Combination therapy with D-003 + aspirin enhanced antiplatelet and antithrombotic effects in a synergistic manner, although the increase in bleeding time induced by D-003 + aspirin appeared to be additive (64).

Pharmacodynamic drug interactions between D-003 and fluvastatin were also investigated. D-003 + fluvastatin administered orally at 5 mg/kg for 30 days to rabbits induced similar effects on LDL-C and TC as D-003 alone. The only advantage of combined therapy was that it had better effects on HDL-C and TG than those achieved with fluvastatin and D-003 alone, respectively (52).

The effects of D-003 on *in vivo* hepatic drug-metabolizing enzymes have also been studied. In one series, rats were randomly distributed to a control group and 2 groups treated with D-003 at 1000 and 2000 mg/kg for 14 days. In a second series, the animals were distributed to a control group and 3 groups treated with D-003 250, 500 or 1000 mg/kg for 6 months. The content of microsomal P-450, cytochrome b5, total sulfhydryl groups (T-SH), nonprotein sulfhydryl groups (NP-SH), protein-bound sulfhydryl groups (PB-SH), NADPH cytochrome *c* reductase, aminopyrine demethylase, dimethylnitrosamine *N*-demethylase, 7-ethoxyresorufin *O*-deethylase (EROD), 7-pentoxyresorufin *O*-depentylase (PROD) and cytosolic glutathione *S*-transferase (GST) was assessed. D-003 at 2000 or 1000 mg/kg for 14 days or 6 months, respectively, did not affect the activities of hepatic drug-metabolizing enzymes (74). Thus, the potential risk for drug interactions between D-003 and concomitant drugs appears to be low.

Pharmacokinetics and Metabolism

At present, insufficient published data are available to review the pharmacokinetic profile of D-003, since most remain as data on file of the manufacturer. Due to the nature of D-003, a mixture of closely related long-chain fatty acids (C24-C36 carbon atoms), it is impractical and very difficult to investigate the pharmacokinetics of each constituent. Considering that octacosanoic acid is the most abundant component of D-003, and that the effects of D-003 in some experimental models can be attributable to octacosanoic acid, the adoption of octacosanoic acid pharmacokinetics as a surrogate for the rest of the fatty acids present in D-003 was considered an acceptable approach.

The first experimental pharmacokinetic study was performed with a GC method on a capillary wide-bore column developed to follow the time course of plasma octacosanoic acid, the presence of which in plasma was confirmed by using GC/mass spectrometry. These experiments showed that, following a single intravenous (i.v.) dose of D-003 to rabbits, plasma octacosanoic acid levels markedly decreased within the first 30 min, indicating a fast plasma elimination (75). Subsequent plasma decay was slower, so that the substance was still detected at 240 min after dosing. Octacosanoic acid showed a bicompartmental kinetic distribution, with a very fast initial distribution phase ($t_{1/2\alpha}$ = 7.82 min) and a second slower elimination phase ($t_{1/2\beta}$ = 50.16 min). Total plasma clearance was high (11.52 ml/min) and the fraction of compound remaining in plasma (0.12) was low, consistent with fast plasma elimination. These data, together with the high volume of distribution (837.81 ml), indicate that

the compound undergoes rapid distribution to extraplasmatic compartments (46).

Other pharmacokinetic studies were performed with labeled octacosanoic acid, investigating the time course of plasma total radioactivity following single oral and i.v. doses of [^{3}H]-octacosanoic acid in rats. [^{3}H]-Octacosanoic acid was labeled in no specific position, its chemical and radiological stability being checked in each experiment (76). Further studies using a molecule labeled at specific positions should provide a better description of the pharmacokinetics of octacosanoic acid and its metabolites. Nevertheless, the consistency of the results confers acceptable validity to the information obtained.

The plasma time course of total radioactivity showed fast oral absorption, with high values reached at 15 min after dosing, and peak plasma levels at 30 min. Total radioactivity values then decreased slowly, so that at 72 h appreciable levels were still detected. The elimination half-life was consistently 66 h. Biphasic distribution was observed. Five min after i.v. dosing with [^{3}H]-octacosanoic acid, very high levels of total radioactivity were observed, decaying relatively fast within the first 6 h and slowly thereafter (76).

Tissue distribution studies showed that the highest values for total radioactivity were found in the liver throughout the entire experiment. The prevalent hepatic uptake could be an advantage for a cholesterol-lowering drug, since the liver is the main organ for the synthesis and regulation of cholesterol metabolism. The distribution in other tissues was variable. Thus, within the first 2 h, the highest values were found in the liver, stomach, proximal portion of the small bowel and plasma. At 6 h, the small bowel passed to the second position, followed by plasma and the large bowel. The fast oral absorption was consistent with the high levels of total radioactivity detected in the stomach 15 min after dosing, suggesting that this could be a primary site for absorption. Total radioactivity absorption in the small bowel was to be expected considering the lipophilic nature of the compound and that the proximal portion of the small bowel is the major site for fat absorption (76). The AUC values in other tissues could be divided into two groups: tissues showing intermediate levels, such as the kidneys, heart, lungs and spleen, and tissues with low total radioactivity values, including the eyes, skeletal muscle, brain, fatty tissue, testes and adrenals (76).

Fecal excretion was the main route of elimination after oral dosing, but urinary excretion also played a role. Thus, 144 h after oral dosing, the accumulated excretion of total radioactivity in the feces (63.3%) and urine (24.7%) accounted for a total recovery of 87.9%. After i.v. dosing, the contribution of fecal and urinary excretion was similar (76). The amount absorbed, estimated using the ratio of renal excretion after oral and i.v. dosing, indicated that orally administered labeled compound was appreciably absorbed (> 50%) (76). Considering that excretion via the feces accounted for 63.3% of radioactivity, it is probable that biliary excretion is relevant, although further investigation will be necessary to confirm this.

Systemic bioavailability was limited, not due to poor absorption, but rather to extensive extraplasmatic uptake documented by the time course of plasma total radioactivity levels and tissue distribution. Presystemic accumulation of total radioactivity in the stomach and small bowel and preferential uptake and accumulation in the liver suggest a first-pass effect in the liver, which was confirmed in metabolism experiments.

To study the metabolism of the labeled compound, the distribution of total radioactivity in different lipid fractions in liver and plasma was investigated. Considering the suggested first-pass effect, the hepatic metabolism of octacosanoic acid was investigated. At 2 h after dosing, 76.1% of total radioactivity detected in the liver was associated with the lipid fraction, the highest percent (38.6%) being present in free fatty acids; 22.5%, 11.5% and 11.9% was associated with phospholipids, triglycerides (TG) and cholesterol esters, respectively. At 10 h after dosing, a different pattern was observed. Thus, total radioactivity associated with the lipid fraction and free fatty acids decreased to 57.6% and 10.2%, respectively. Total radioactivity associated with TG moderately decreased to 10.2%, while that present in phospholipids and cholesterol esters increased to 25.6% and 15.9%, respectively (76).

Hence, this study demonstrated that octacosanoic acid is metabolized in the liver and the decline of total radioactivity in the lipid fraction suggests progressive degradation of the acid. In this regard, degradation of long-chain fatty acids via hepatic peroxisomal β-oxidation has been documented. Such a process produces fatty acids with shortened aliphatic chains and acetyl-CoA remnants, accounting for the gradual reduction of total radioactivity in the overall lipid fraction and the fraction corresponding to free fatty acids extracted from the liver. In turn, acetyl-CoA remnants can be progressively incorporated in other nonlipid metabolic pathways.

The occurrence of total radioactivity in hepatic fatty acids, TG and phospholipids indicates that octacosanoic acid or shorter metabolites can be incorporated into these lipid components commonly present in cell membrane and organelles. These results are consistent with those described by Kabir and Kimura (77), who demonstrated that [^{14}C]-octacosanol is converted *in vivo* to its corresponding acid. Thereafter, the acid and/or derived metabolites can be esterified in different lipid fractions of hepatic tissue.

Results obtained for plasma samples 2 h after dosing were consistent with those found in the liver. Thus, most of the total radioactivity (88.2%) was present in the lipid fraction, distributed in TG (30.0%), free fatty acids (25.1%), phospholipids (18.5%) and cholesterol esters (11.0%). Considering that plasma lipids are mainly transported in lipoproteins, the distribution of total radioactivity in plasma lipoproteins was determined. The greatest amount of total radioactivity migrated with very-low-density lipoproteins (VLDL; 52.6%), followed by high-density

lipoprotein (HDL; 15.8%) and LDL (7.2%), such that most of the radioactivity was present in lipoproteins (75.6%) and only 24.7% in residual plasma (76).

The highest concentration of total radioactivity in TG and VLDL electrophoretic zones among hepatic lipids and plasma lipoproteins is to be expected. Thus, dietary TG are degraded by pancreatic lipase during fat absorption in the small bowel, and the resulting fatty acids penetrate within the enterocyte through passive diffusion or fatty acid carrier-facilitated transport. Subsequently, these fatty acids are transported in chylomicrons to the endoplasmatic reticulum and used for new synthesis of TG, chylomicrons being further degraded by lipoprotein lipase to chylomicrons + remnant chylomicrons, used for VLDL production in the liver. Thus, it is logical that relevant concentrations of total radioactivity arise from the intestinal absorption of [^{3}H]-octacosanoic acid after single doses. It therefore appears that the metabolism of octacosanoic acid does not take place exclusively in the liver, but also in the small bowel, as indicated by the high total radioactivity levels detected in this tissue. Nevertheless, the putative intestinal metabolism of octacosanoic acid requires further investigation.

Toxicity

The toxicological evaluation of D-003 has included studies of general and special toxicology aimed at determining the potential risks associated with oral use of D-003 in humans. These studies have included acute toxicity and repeated-dose studies (subchronic and chronic) performed in rodents (61, 78) and other animal species (51, 79). Also, studies investigating cytotoxicity, mutagenic potential and reproductive toxicity of D-003 have been concluded (80-84), and long-term carcinogenicity studies in rodents are ongoing (85).

The species selected are those commonly used in toxicological studies of new compounds. In addition, we selected species in which pharmacological effects of D-003 have been documented, such as rats, rabbits and beagle dogs. For example, inhibitory effects of D-003 on platelet aggregation and bleeding time, experimental thrombosis and lipoprotein oxidation, have been demonstrated in rats, indicating that this species is adequately sensitive to assess the putative toxicity of D-003. Likewise, cholesterol-lowering effects of D-003 have been demonstrated in rabbits and beagle dogs.

The dose levels selected for rodent studies were multiples of the effective antiplatelet and/or antioxidant doses in these species, while the doses used in other species were multiples of doses effective for lowering cholesterol. The doses investigated in humans have ranged from 5 to 50 mg/day. Toxicological studies have generally included the highest recommended doses for each type of study, 1000 mg/kg being used for most repeated-dose studies. The highest dose tested in humans has been 50 mg/day, which corresponds to 0.7 mg/kg on the assumption of a mean body weight of 70 kg. Hence, experiments investigating the toxicity of D-003 administered up to 1000 mg/kg have included doses 1,429 times higher than the maximal dose investigated in humans.

Results of preclinical toxicology studies have shown no D-003-related toxicity, indicating that the drug is safe and that the potential risk for humans is low.

The acute oral toxicity of D-003 was investigated in rodents (rats and mice) and rabbits. All studies recorded mortality, daily clinical observations, food consumption and body weight gain during 14 days after dosing. Also, in some studies blood analyses, necropsy and histopathological examinations were performed at study completion on the survivors. These studies were conducted in animals of both sexes randomly distributed to experimental groups. We conducted acute oral toxicity studies in rats following the Acute Toxicity Class method (86), while in the others we used the classic LD_{50} method. The oral LD_{50} in rats, mice and rabbits was > 5000 mg/kg. Clinical observations, body weight gain, as well as blood determinations in survivors at the end of these studies (14 days), did not show differences between treated and control groups. Moreover, organ weight analysis and anatomopathological examination did not show any differences between groups. Overall, it appears that the acute oral toxicity of D-003 is practically negligible (78, 79).

The major study of the subchronic oral toxicity of D-003 was conducted in Sprague-Dawley (SD) rats randomly distributed to 4 experimental groups: a control group treated with the vehicle and 3 groups treated orally with D-003 at 50, 500 and 1250 mg/kg for 90 days (78). The lowest dose was a multiple of the minimal effective antithrombotic and antioxidant dose in this species (5 mg/kg), and the higher doses of 500 and 1250 mg/kg were 10 and 25 times larger than the lowest dose, respectively. In this study, inhibition of platelet aggregation was corroborated in satellite groups. No differences in mortality, weight gain, clinical observations, biochemical and hematological parameters, organ weight and histopathological findings were observed between treated and control groups. Thus, the highest dose used (1250 mg/kg) did not cause drug-related toxicity, and was considered the no-observed-effect level (NOEL).

Another study investigating the toxicity of repeated oral doses of D-003 was conducted in young adult OF-1 mice. Animals were randomly distributed to 4 experimental groups: a control and 3 groups treated orally with different doses of D-003 (5, 50 and 500 mg/kg) for 60 days (78). The analysis of mortality, weight gain, clinical observations, behavioral tests, necropsy, organ weight analysis and histopathology performed at study completion did not show differences between control and treated animals, even at the highest dose tested.

A similar study was also conducted in SD rats distributed to 4 experimental groups (20 animals/sex/group) treated orally for 6 months with D-003 at 250, 500 and 1000 mg/kg, or included as controls (61). Effects on platelet aggregation and bleeding time were corroborated, but no evidence of drug-related toxicity was found. Thus, no significant differences between control and

treated animals were found regarding mortality, clinical symptoms, body weight gain, food consumption, hematology, blood biochemistry, organ weight data and histopathological findings. A dose of 1000 mg/kg was considered the NOEL in this study. Considering the lack of drug-related toxicity found and that this dose is more than 1,408 times larger than the maximal dose investigated in humans (50 mg/day), D-003 can be considered safe after long-term administration.

A study was conducted in beagle dogs of both sexes randomly distributed to 3 experimental groups treated orally for 9 months with D-003 at 200 and 400 mg/kg, or used as controls (53). As expected, D-003 lowered serum TC, inhibited platelet aggregation and increased bleeding time, without inducing toxic signs or affecting safety parameters. No significant differences between control and treated animals were found. Hence, oral administration of D-003 up to 400 mg/kg, a dose 570 times larger than the maximum dose studied in humans, did not induce drug-related toxicity, indicating that it is safe after long-term oral administration to nonrodents.

The increases in bleeding time seen in toxicological studies after repeated oral doses were persistent but reversible after treatment withdrawal and similar after short- or long-term administration; individual values remained within normal ranges for each species.

The potential cytotoxicity of D-003 was investigated using three assays: neutral red uptake, the kenacid blue test and the MTT test. Results did not show cytotoxic potential for D-003 (80).

The mutagenic potential of D-003 was assessed using both *in vitro* and *in vivo* assays (80, 81). Overall, assessment of potential genotoxicity indicated that D-003 does not cause any genetic changes in prokaryotes or eukaryotes.

The Ames test to detect gene mutations was performed in different strains of *Salmonella typhimurium* (TA 1535, TA 1537, TA 1538, TA 98 and TA 100) with and without metabolic activation. D-003 was suspended in Tween 20/water vehicle and added to cultures at 5, 50, 500, 2000 and 5000 μg/plate (80). The maximal concentration used is the highest recommended for this test. Negative and positive control cultures treated with *N*-acetylaminofluorene and aflatoxin B_1 were used. Increases in the number of revertant colonies were observed in the positive control group, thus validating the system used to detect gene mutations. D-003, however, did not increase the number of revertant colonies Thus, no evidence of mutagenic potential of D-003 was detected in this study.

The *in vivo* clastogenic activity of D-003 was investigated by measuring the occurrence of micronucleated polychromatic erythrocytes (MPE) in mouse bone marrow cells (81). According to the standard protocol, mice of both sexes were randomly distributed to 3 experimental groups. Two groups were treated by gastric gavage for 5 days: a control group treated with the vehicle and the other with D-003 at 2000 mg/kg, the highest dose recommended for this test. The third group was a positive control treated with a single i.p. dose of cyclophosphamide. Cyclophosphamide, but not D-003, increased MPE compared with the negative controls.

The effects of D-003 on primary spermatocytes and spermatogenesis were evaluated in male Swiss albino mice. Animals were randomly distributed to 4 experimental groups: a control group treated with the vehicle and 3 groups treated with D-003 at 250, 500 and 1250 mg/kg for 60 days. No cytotoxic or genotoxic effect was seen for D-003, whereas a significant increase in the number of resorptions was observed in the positive controls (81).

The Comet assay provides information on DNA damage, and is used as an optional complementary tool within the battery of genotoxicity tests. In this assay, after short- and long-term treatment D-003 did not show any evidence of DNA damage in rats (82).

Assessment of the fetal and reproductive toxicity of D-003 included studies of teratogenicity in rats and rabbits, segment I and peri- and postnatal studies. D-003 administered orally at up to 1000 mg/kg during organogenesis did not produce embryotoxic or teratogenic effects in rats or rabbits (83, 84). In addition, it did not affect fertility and reproduction in rats (83).

Carcinogenicity studies are ongoing and no conclusive results have yet been obtained. The interim report of the first year of the 24-month study in rats revealed, however, that D-003 at doses of 50, 250 and 1000 mg/kg did not affect any safety parameter. Thus, to date, the highest dose appears to be the NOEL. No evidence of drug-related carcinogenicity has been obtained, and mortality and the tumor occurrence rate have been very low (85), but it is still too soon to reach conclusions, since the second year is pivotal for this type of study in rats.

In conclusion, preclinical toxicology studies have not demonstrated evidence of D-003-related toxicity even after long-term administration at 1000 mg/kg. Pharmacological actions of D-003, on the other hand, such as cholesterol reduction and antiplatelet effects, have been demonstrated at much lower doses in the same species used in toxicological studies. Also, in some toxicological studies, pharmacological effects of D-003 have been seen. The doses tested in most studies have been the highest recommended for each type of study. Thus, the lack of D-003 toxicity can not be attributable to insufficient drug exposure, but rather to a low intrinsic toxicity of the substance.

Clinical Studies

An initial single-blind, randomized, placebo-controlled, parallel-group study included 2 phases (87). The first compared the effects of single doses of D-003 with controls at 2, 4, 8, 24 and 48 h, 7 and 14 days. Thirty-two subjects were randomized to receive placebo or D-003 5, 25 or 50 mg. The second phase investigated the effects of D-003 administered for 30 days, followed by a 30-day washout period. In this step, 38 subjects were randomized to placebo or D-003 5, 25 or 50 mg/day.

Bleeding time, lipid profile, physical and blood safety parameters were measured, and adverse events were recorded. In the first part, 2 h after dosing, D-003 (25 and 50 mg) significantly increased bleeding time, but only the higher dose was different from placebo. The effect was moderately dose-dependent, but individual values remained within normal limits. The effect was readily reversible and had disappeared at 4 h. No drug-related disturbances in safety parameters were reported. Only 1 placebo-treated subject reported a mild adverse event (insomnia). In the second part, after 7 days on treatment, only the highest dose of D-003 increased bleeding time compared to baseline and placebo, and the effect persisted up to study completion, all individual values remaining within normal levels. This effect was reversible after washout. In addition, D-003 (5-50 mg/day) for 30 days significantly lowered TC (13.3-17.4%) and LDL-C (11.6-22.6%), while it raised HDL-C (14.6-29.7%). Triglyceride levels were unaffected by D-003. A significant increase in HDL-C was observed as early as 14 days. The effects on the lipid profile were reversible, although after 14 days of washout the effects on HDL-C and LDL-C partially persisted. No other drug-related changes were detected. Nine subjects (4 placebo, 5 D-003) reported adverse events, none drug-related. Only 2 D-003 subjects treated with 25 and 50 mg/day discontinued due to flu and fever occurring during the washout period and the first week of therapy, respectively.

A second double-blind, randomized, placebo-controlled study investigated the effects of D-003 on platelet aggregation induced by arachidonic acid (0.75 and 1.75 mM), collagen (1 μg/ml) and ADP (1 and 2 μM) in healthy volunteers (88). Forty-one subjects were randomized to placebo or D-003 (5, 25 or 50 mg/day) for 10 days, followed by a washout period. At baseline, after active treatment and at washout completion, platelet aggregation, lipid profile and safety parameters were assessed. D-003 significantly, reversibly and dose-dependently inhibited arachidonic acid-induced aggregation at the doses of 10 mg/day (0.75 mM: 60.4% to 21.0%; 1.5 mM: 61.5% to 26.8%) and 20 mg/day (0.75 mM: 57.2% to 11.2%; 1.5 mM: 65.1% to 21.6%), but not at the lowest dose. These doses also inhibited collagen-induced aggregation, although the effect was moderate, not dose-related and recovered after washout. Platelet aggregation induced by ADP and coagulation time were unaffected by D-003. Total cholesterol and TG levels remained unchanged after therapy, as expected for such a short administration period. Nevertheless, D-003 (10 and 20 mg/day) significantly increased HDL-C (20%). This effect was not dose-dependent and partially recovered after washout. In consequence, the final LDL-C values in the group treated with 20 mg/day were lower than on placebo. No significant changes in the lipid profile occurred on placebo. No drug-related adverse events were observed. Four subjects, 1 from each group, withdrew from the study, but none due to adverse events.

A third double-blind, randomized, placebo-controlled phase I study in healthy volunteers explored the effects of D-003 on the susceptibility of LDL to undergo lipid peroxidation induced by copper ions (89). Forty-five individuals were randomized to placebo or D-003 5 or 10 mg/day for 8 weeks. Laboratory tests and physical examination were performed at baseline and after 4 and 8 weeks on therapy, and compliance and adverse event interviews were done at weeks 4 and 8. At study completion, D-003 significantly lowered LDL-C by 20.8% (5 mg/day) and 28.8% (10 mg/day), reduced TC (12.7% and 17.5%, respectively) and increased HDL-C (8.8% and 13.1%, respectively). Triglycerides were significantly reduced (8.8% and 13.1%, respectively) compared to baseline but not compared to placebo. Responses assessed at 4 weeks showed reductions in LDL-C and TC with both doses of D-003, whereas HDL-C was significantly increased. Triglycerides, however, remained unchanged. No significant changes in the lipid profile occurred in the placebo group (90). D-003 at 5 and 10 mg/day significantly increased lag time (18.3% and 32.0%, respectively) and decreased the maximum rate of diene propagation (12.7% and 19.1%, respectively) of Cu^{2+}-induced LDL peroxidation. D-003 attenuated the reduction in the reactivity to TNBS by 19.9% and 32.0%, respectively, at doses of 5 and 10 mg/day. This study showed that D-003 protected LDL particles from lipid peroxidation. The treatment was well tolerated. Three subjects (1 from each group) discontinued the study, but only 1, treated with D-003 5 mg/day, discontinued due to an adverse event (gastritis).

Another double-blind, randomized, placebo-controlled phase I study investigated the effects of D-003 for 30 days on platelet aggregation induced by arachidonic acid (0.75-1.5 mM), collagen (1-2 μg/ml) and ADP (1-2 μM), as well as on plasma fibrinogen values (90). Fifty-four subjects were randomized to placebo or D-003 (5 or 10 mg/day) for 30 days. Induced platelet aggregation was assessed at baseline and at study completion. Tolerability to treatment was also explored. This study showed that D-003 administration for 30 days inhibited platelet aggregation in response to arachidonic acid and collagen, but not to ADP. The antiplatelet effect was present after longer treatment, even at 5 mg/day. Cholesterol-lowering effects of D-003 were consistent with those expected for such a short period of treatment. D-003 at 10 mg/day also lowered plasma fibrinogen. Both doses reduced TC and LDL-C, increased HDL-C, but did not change TG. The treatment was well tolerated. No disturbances in safety parameters were seen on D-003. One subject (D-003 5 mg/day) discontinued the study, and 4 (3 D-003, 1 placebo) reported adverse events such as headache (2 D-003, 1 placebo) and polyphagia (1 D-003).

A further double-blind, randomized, placebo-controlled study investigated the effects of D-003 on platelet aggregation induced by arachidonic acid (1.75 mM) and collagen (1 μg/ml) and on serum levels of the arachidonic acid metabolites TxB_2 and PGI_2 (91). Thirty-one subjects were randomized to receive placebo or D-003 20 mg/day for 14 days. Serum levels of TxB_2 and PGI_2 and arachidonic acid- and collagen-induced platelet aggrega-

tion were assessed at baseline and at treatment completion, and tolerability was also assessed. D-003 significantly reduced serum levels of TxB_2 (36.4%) and increased PGI_2 (31%) compared to baseline and placebo. As expected, D-003 inhibited arachidonic acid-induced aggregation (1.5 mM: 81.9% to 65.6%) and collagen-induced aggregation (75.3% to 62.3%). No subject withdrew from the study and no disturbances in safety parameters were observed. Four subjects, 3 treated with D-003 and 1 placebo, reported adverse events during the study: headache (2 D-003, 1 placebo) and asthenia (1 D-003).

The first phase II clinical study of the effects of D-003 on lipid profiles was conducted in patients with isolated or combined hypercholesterolemia (92). The mean percentage change in serum lipid variables represented the differences between the assessments done at the end of active therapy and the end of a baseline period of dietary control. Dietary restrictions based on adherence to a step I cholesterol-lowering diet as recommended by the NCEP continued throughout the study (4). This trial involved once-a-day administration as fixed doses. The percent change in LDL-C was the primary efficacy variable, while changes in TC, HDL-C and TG were secondary efficacy variables. After concluding a 5-week diet-only baseline period, 55 patients were randomized to placebo or D-003 (5, 10, 20 or 40 mg) once daily for 8 weeks. An interim checkup was performed at week 4. Drug tolerability was assessed throughout by the changes in physical and blood safety parameters, and adverse event interviews. After 8 weeks of therapy, D-003 significantly and dose-dependently lowered serum LDL-C, with reductions of 20.5% to 26.1% from the lowest to the highest dose investigated. At study completion, 43 of 44 (97.7 %) randomized patients treated with D-003 achieved LDL-C reductions of > 15 % compared with baseline, and 30 of 44 patients (68.2%) reached LDL-C targets. D-003 also lowered TC, TC/HDL-C and LDL-C/HDL-C significantly and dose-dependently, while it increased HDL-C (11.7-16.7%) significantly but not dose-dependently. No significant changes occurred on placebo. D-003 was well tolerated, with no changes seen in any safety parameter. All patients completed the study and only 4 reported mild adverse events: headache (1 placebo, 1 patient treated with D-003 20 mg/day), insomnia (1 patient treated with 5 mg/day) and polyuria (1 patient treated with 40 mg/day). This study thus demonstrated that D-003 was safe and effective for lowering LDL-C in patients with type II hypercholesterolemia.

Another double-blind, randomized, placebo-controlled study investigated the effects of D-003 on serum lipid peroxidation markers in older individuals with mild hypercholesterolemia (93). The effects of D-003 on LDL lipid peroxidation represented the primary efficacy variable, and effects on plasma total antioxidant status (TAS), malondialdehyde (MDA) levels and the activities of plasma antioxidant enzymes were evaluated as secondary variables. Additionally, effects on the lipid profile were assessed. Fifty-one patients were randomized to receive placebo or D-003 for 8 weeks. Laboratory tests and physical examination were performed at baseline and after 4 and 8 weeks on therapy, and compliance and adverse events were assessed at weeks 4 and 8. D-003 5 and 10 mg/day significantly increased the lag phase by 24.7% and 29.3%, respectively, while it decreased propagation rate by 22.7% and 25.8%, respectively. Also, D-003 5 and 10 mg/day increased TAS *versus* baseline by 17.7% and 23.0%, respectively, but only the changes achieved on 10 mg/day were different from placebo. At the higher dose, D-003 significantly lowered plasma TBARS levels with respect to baseline. D-003 did not modify superoxide dismutase (SOD) and GPX (glutathione peroxidase) activities. Doses of 5 and 10 mg/day significantly reduced LDL-C (13.94% and 21.8%, respectively) and TC (9.4% and 12.5%, respectively), and increased HDL-C (5.7% and 18.5%, respectively). Although the changes on both doses were significant compared to baseline, the differences compared to placebo were significant only for the higher dose. D-003 at 10 mg/day also moderately but significantly reduced TG (10.9%). No significant changes in lipid peroxidation or lipid profiles occurred in the placebo group. Treatment was well tolerated and no patient withdrew from the study. Only 3 subjects (2 placebo, 1 D-003 5 mg/day) reported adverse events: insomnia and acidity on placebo and acidity on D-003.

Data from short- and long-term clinical studies indicate that D-003 is well tolerated. To date, 8 of 347 (2.3%) patients included (6 D-003, 2 placebo) have discontinued the studies prematurely for any reason. Of these, only 3, all on D-003, discontinued due to adverse events of gastritis (1 subject) and flu (2 subjects). The most frequently reported (1% or greater) adverse event in D-003-treated patients has been headache (3.7%), but the incidence was not greater than on placebo (7.7%). The other adverse events referred by D-003 patients in these trials have shown a frequency of 1.0% or less, similar to placebo. These other adverse events include insomnia, somnolence, acidity, gastritis, gum bleeding, flu, asthenia, polyphagia and polyuria. Safety parameters controlled during the studies have not shown any D-003-related disturbances.

Conclusions

D-003 is a new drug consisting of a defined mixture of very-high-molecular-weight aliphatic acids purified from sugar cane (*Saccharum officinarum* L.) wax. Cholesterol-lowering and pleiotropic effects of D-003 beneficial for the prevention of atherosclerosis and its complications have emerged from experimental models and studies in healthy volunteers, including inhibition of platelet aggregation and the susceptibility of LDL to undergo lipid peroxidation. Overall, the results obtained in the clinical studies encourage further investigation of D-003 and 3 phase II studies are in progress.

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