3. Preparation of the gels

Sepigel 305 gels were prepared by water addition into Sepigel 305 and after mixing opalescent gels were made at room temperature. Propylene glycol (5, 10, 15%), ethanol (5, 10, 15%) and indomethacin (1%) were added to the gels and the mixtures were homogenized and were left to stand for 24 h.

4. Evaluation of indomethacin release

A series of six permeation chambers was used. In each donor chamber, 3.0 g of the studied formulation was placed and 20 ml of phosphate buffer (pH 6.6) was placed in each acceptor part. The acceptor phase was mixed with a magnetic stirrer. Indomethacin was left to permeate at 37 °C through a hydrophilic membrane into the phosphate buffer. The amounts of released drug were determined spectroscopically at 318 nm after 15, 30, 45, 60, 90, 120 and 180 min.

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Long-term effects of D-003, a mixture of high molecular weight acids from sugarcane wax, on bones of ovariectomized rats: a one year study

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This study was done to determine the long-term effect of D-003 on bones of ovariectomized (ovx) rats distributed in 4 groups: a false-operated and three groups of ovx rats: one treated with the vehicle and two with D-003 (5 and 250 mg/kg). D-003 significantly prevented, in a dose-dependent fashion, the trabecular bone volume (TBV), trabecular number (TbN) and trabecular thickness (TbTh) reduction induced in ovx rats and the increase of trabecular separation (TbSp) osteoclast number (OcN) and osteoclast surface (OcS/BS) increased in the positive controls versus the sham group. It is concluded that D-003 administered for 12 months prevented bone loss and decreased bone resorption in ovx rats, without evidences of impaired bone quality.

D-003 is a mixture of higher aliphatic primary acids isolated and purified from sugarcane wax, wherein octacosanoic, triacontanoic, dotriacontanoic, and tetratriacontanoic acids are the most abundant and other acids (C24-C27, C29, C31, C33, C35 and C36) are at minor concentrations (Más 2004). D-003 inhibits cholesterol synthesis prior to mevalonate formation by regulating HMG-CoA reductase activity (Menéndez et al. 2001), and displays cholesterol-lowering effects (Castaño et al. 2003) and also inhibits lipid peroxidation in rat plasma lipoprotein as in healthy human volunteers (Castaño et al. 2003). D-003 (5–200 mg/kg) administered for 3 months prevented bone loss and bone resorption in ovariectomized (ovx) rats, increasing osteoclast apoptosis (Noa et al. 2004; Mendoza et al. 2005a), and administered for 80 days prevented corticoid-induced osteoporosis in rats (Noa et al. 2004a). D-003 (10 mg/day) for 6 months reduced the urinary excretion of DPD/creatinine, in postmenopausal women with low bone mineral density (BMD) (Ceballos et al. 2005).

The ovariectomized (ovx) rat mimics the increased trabecular bone loss and resorption occurring in postmenopausal women (Mosekilde et al. 1993; Bagi et al. 1997; Glatt et al. 2001). The assessment of the potential effects of any treatment on post-menopausal osteoporosis should include studies in this model, which provides information of the effects on bone quality, difficult to obtain in humans, in a short time, and also the effects after repeated bone remodelling cycles in such species (Thompson et al. 1995). So,

Table 1: One year effects of D-003 on the trabecular bone of ovx rats: morphometric study (X ± DS)

Group	Doses (mg/kg)	TbN (#/mm)	TbTh (μm)	Tb Sp (μm)
Femoral neck				
Sham	0	7.04 ± 0.33*	84.04 ± 1.28*	191.75 ± 0.94*
Positive control (ovx)	0	4.54 ± 0.17	56.75 ± 1.59	350.96 ± 6.92
D-003	5	6.29 ± 0.28*	82.50 ± 0.98*	205.96 ± 3.31*
D-003	250	6.88 ± 0.31*, a	84.33 ± 0.5*, a	191.04 ± 2.03*, b
Distal femur				
Sham	0	1.46 ± 0.17*	92.46 ± 0.94*	213.04 ± 2.66*
Positive control (ovx)	0	0.75 ± 0.15	72.67 ± 0.87	355.46 ± 5.98
D-003	5	1.42 ± 0.15*	89.67 ± 2.30*	236.75 ± 4.10*
D-003	250	1.78 ± 0.28*	92.08 ± 0.85*, a	215.58 ± 2.64*, b
Fifth vertebrae				
Sham	0	3.33 ± 0.25*	84.04 ± 0.90*	216.42 ± 1.27*
Positive control (ovx)	0	1.88 ± 0.35	72.71 ± 0.38	259.38 ± 1.69
D-003	5	3.33 ± 0.25*	82.25 ± 1.28*	215.71 ± 0.77*
D-003	250	3.42 ± 0.24*	84.17 ± 0.67*, a	216.42 ± 1.04*

TbN, trabecular number, TbTh trabecular thickness, Tb Sp trabecular separation
* $p < 0.001$ Comparisons with control ovx, [a] $p < 0.05$, [b] $p < 0.01$, Comparisons with D003 5 mg/kg (Mann Whitney U Test)

this study investigated the long-term (12 months) effect of D-003 on bones of ovx rats.

Ovariectomy decreased the values of trabecular volume (TBV), which were 61.6%, 52.5% and 56.7% of sham values in the femoral neck, distal femur and fifth vertebrae, respectively. D-003 significantly and markedly prevented, in a dose-dependent fashion, TBV reduction induced by ovariectomy in the rats. Table 1 summarizes the effects on the main histomorphometric variables. Ovx rats showed significantly lower trabecular numbers (TbN) and trabecular thickness (TbTh), and significantly higher trabecular separation (TbSp) than those of the sham group. D-003 significantly and dose-dependently prevented the increase in TbSp and the reduction of TbN and TbTh compared with the positive controls. Table 2 lists the effects on bone resorption indicators. Both osteoclast number (OcN) and surface (OcS/BS) increased in the positive controls *versus* the sham group, an effect prevented significantly with D-003.

Table 2: One year effects of D-003 on bone resorption parameters of ovx rats

Groups	Doses (mg/kg)	OcN (#/mm)	OcS/BS (%)
Femoral neck			
Sham	0	0.45 ± 0.02*	5.71 ± 0.26*
Positive control (ovx)	0	0.74 ± 0.04	8.95 ± 0.40
D-003	5	0.51 ± 0.02*	6.13 ± 0.22*
D-003	250	0.46 ± 0.02*, b	5.88 ± 0.17*, a
Distal Femur			
Sham	0	0.94 ± 0.02*	4.54 ± 1.*
Positive control (ovx)	0	1.90 ± 0.02	6.51 ± 0.79
D-003	5	1.08 ± 0.03*	5.01 ± 0.26*
D-003	250	1.00 ± 0.04*, a	4.78 ± 0.21*, a
Fifth vertebrae			
Sham	0	0.27 ± 0.02*	1.22 ± 0.1*
Positive control (ovx)	0	0.47 ± 0.02	1.60 ± 0.04
D-003	5	0.25 ± 0.02*	1.21 ± 0.06*
D-003	250	0.27 ± 0.02*	1.22 ± 0.03*

OcN (osteoclast number), OcS/BS (osteoclast surface)
* $p < 0.001$ Comparisons with control ovx, [a] $p < 0.05$, [b] $p < 0.01$, Comparisons with D003 5 mg/kg (Mann Whitney U Test)

This study demonstrates that D-003 administered for 12 months to ovx rats prevented the typical changes of trabecular bone induced by ovariectomy, like the decrease in TBV, TbN, TbTh and the increase of TbS. In our study, the TBV values of positive control rats were similar to those of another one year study in ovx rats (Glatt et al. 2001), providing validity to this model under our experimental conditions. Also, D-003 prevented the augmented bone resorption in the ovx rat, in a dose-dependent manner. Previous data showed that D-003 prevents bone loss and resorption in the ovx rat through the increase of osteoclast apoptosis (Noa et al. 2004; Mendoza et al. 2005a, 2005b), in line with the present results.

D-003 administered for a long-term period covering repeated bone turnover cycles, equivalent to 4 years of human exposure (Thompson et al. 1995) displays antiosteoporotic effects. The present results could be considered as a consequence, at least partially, of the inhibition of cholesterol synthesis before mevalonate formation produced with D-003, through a mechanism involving the regulation of HMGCoA reductase activity, since the inhibition of pathway of mevalonate to cholesterol, increase osteoclast apoptosis and impair osteoclast activity with an antiresorptive effect (Mendoza 2005a, 2005b). Nevertheless, the inhibition of lipid peroxidation produced with D-003 (Castaño et al. 2003), could also contribute to the present results, since hypercholesterolemia and lipid oxidation predisposes to osteoporosis development.

No impairment of bone quality was observed in the treated groups compared with positive controls, consistent with the negative results of toxicity studies in the rat (Gámez et al. 2002). All these results encourage to continue the clinical assessment of D-003 including the evaluation of the effects on variables like BMD and fracture rates, for establishing whether the bone protector effects of D-003 are clinically meaningful.

It is concluded that D-003 (5–250 mg/kg) orally administered for 12 months, including repeated cycles of bone turnover, prevented bone loss and bone resorption in the

ovx rat, in a dose-dependent manner, without evidences of impaired bone quality.

Experimental

Sprague-Dawley rats were obtained from the National Centre for Laboratory Animals Production (Havana, Cuba), adapted to laboratory conditions, with free access to food and water. Animals were handled according to the Cuban ethical regulations for the use of laboratory animals.

Rats were ovariectomized bilaterally or sham operated under anaesthesia. D-003 was obtained from the Chemistry Department of the Centre of Natural Products (Havana, Cuba), after corroborating its quality specifications. The identity and purity of the batch used in the study were assessed through a validated gas chromatography method (Marrero et al. 2002). D-003 was suspended in Tween/water vehicle, adjusting the concentrations according to the bodyweight gain. Treatments were administered orally by gastric gavage, once a day for 12 months. Rats were randomly distributed in 4 groups (10 rats/group): a false-operated and three groups of ovx rats: one treated orally with the vehicle and two with D-003 (5 and 250 mg/kg). The doses of D-003 here used were the lowest effective dose and a dose 5 times higher than the maximal effective dose (50 mg/kg) in this model. (Castaño et al. 2003; Noa et al. 2004).

At study completion, rats were sacrificed under ether anaesthesia. Treatment effects were assessed through microscopic and morphometric studies. The right femur and fifth lumbar vertebrae were removed for the morphological study. The right femur was cut through the intertrochanteric line as described, while the distal femur was taken at the second 0.5 cm from the distal end of the femur (Mosekilde et al. 1993). Bones were decalcified in 0.5 M disodium ethylenediaminetetraacetic acid (EDTA, pH 7.4) at 4 °C for four weeks, embedded in paraffin, sectioned and stained with haematoxylin and eosin (Mosekilde et al. 1993).

Morphometry was conducted as described (Parfitt et al. 1987). Histomorphometric changes in trabecular bone volume (TBV) and structure, such as trabecular number (Tb.N, #/mm), thickness (Tb Th, μm), and separation (Tb Sp, μm), osteoclast number (OcN) and surface (OcS/BS) were the primary efficacy variables. Values of histomorphometric variables were derived from primary measurements of areas and perimeters. The calculation related to trabecular bone volume (TBV) for estimation of bone mass was performed as described. (Parfitt et al. 1987). Histomorphometric analysis was conducted using an image analysis system.

Comparisons between groups were done using the Mann-Whitney U test. An $\alpha = 0.05$ was *a priori* selected for the statistical significance. Dose relationships were assessed by regression analysis. Statistical analyses were performed using the software Statistics for Windows.

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