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D-003 does not possess oestrogenic potential in-vivo: findings of the uterotrophic assay

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Abstract

D-003 is a mixture of long-chain fatty acids purified from sugarcane wax that inhibits both cholesterol synthesis prior to mevalonate formation, and lipid peroxidation. D-003 has been shown to prevent bone loss and bone resorption in ovariectomized rats, and significantly improves bone resorption markers in postmenopausal women with reduced bone mineral density. As hormone-replacement therapy, D-003 displays cholesterol-lowering and anti-resorptive effects. We have studied its potential oestrogenic activity in-vivo using the uterotrophic assay. Rats were randomly distributed into five groups: a sham-operated group and four groups of ovariectomized rats, one treated with vehicle, one with D-003 (50 mg kg^{-1}), one with oestradiol benzoate (30 μg kg^{-1}) and one with D-003 (50 mg kg^{-1}) plus oestradiol benzoate (30 μg kg^{-1}). Treatments were administered for 14 days. Ovariectomy decreased the values of relative uterus weight, epithelium cell height and endometrial thickness compared with sham-operated rats, and these effects were all significantly reduced with oestradiol benzoate, but not with D-003. Concurrent administration of D-003 and oestradiol benzoate had statistically similar effects on all variables as oestradiol benzoate alone. In conclusions, D-003 orally given at 50 mg kg^{-1}, a dose that prevents bone loss and bone resorption in ovariectomized rats, did not display oestrogenic/anti-oestrogenic activity in-vivo, as assessed in the uterotrophic assay.

Introduction

Osteoporosis is a systemic disease in which reduced bone mass and impairment of bone microarchitecture lead to bone fragility and fracture risk (Cuddihy et al 2002; Bahl et al 2003) as age increases. Osteorporosis occurs in both sexes, but the risk is higher in oestrogen-deficient postmenopausal women compared with men of the same age (Looker et al 1995; Watts 1997).

Osteoporosis occurs because of an imbalance of bone remodelling, in which bone resorption exceeds bone formation. Pharmacological management of osteoporosis includes anti-resorptive and anabolic drugs (Seeman & Delmas 2006). Anti-resorptive drugs, the most widely used drugs for the management of osteoporosis, prevent bone loss mainly by reducing excessive bone resorption by lowering the number, activity and life span of the osteoclasts, and in addition bring about moderate improvements in bone formation. They can therefore preserve bone mass and reduce fracture rates. The bisphosphonates, mainly aminobisphosphonates, currently represent the leading anti-resorptive drugs used in the treatment of osteoporosis. The use of oestrogen-replacement therapy, although previously popular, has been decreasing because of undesirable side-effects such as an increased risk of uterine and breast cancer (Toniolo et al 1995).

D0003 is a mixture of long-chain aliphatic primary acids purified from sugarcane wax. Octacosanoic acid (C_{28}) is the most abundant component, but the mixture also contains tetracosanoic (C_{24}), pentacosanoic (C_{25}), hexacosanoic (C_{26}), heptacosanoic (C_{27}), nonacosanoic (C_{29}), triacontanoic (C_{30}), hentriacontanoic (C_{31}), dotriacontanoic (C_{32}), tritiacontanoic (C_{33}), tetratriacontanoic (C_{34}), pentatriacontanoic (C_{35}) and hexacotriacontanoic (C_{36}) acids, in relative concentrations that are reproducible from batch to batch (Más 2004).

D-003 has been shown to inhibit cholesterol biosynthesis prior to mevalonate formation by regulating activity of hydroxymethylglutaryl coenzyme (HMG-CoA) reductase (Menéndez

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et al 2001, 2003), consequently lowering cholesterol in experimental and clinical studies (Gámez et al 2000; Menéndez et al 2001; Castaño et al 2002, 2003a, b; Mendoza et al 2003). In addition, orally administered D-003 has been shown to inhibit lipid peroxidation of plasma lipoproteins (Menéndez et al 2002; Castaño et al 2003b). Oral treatment with D-003 (5–200 mg kg^{-1}) prevented bone loss and bone resorption in ovariectomized (ovx) rats (Noa et al 2004a; Mendoza et al 2005a, b) and in rats with prednisolone-induced osteoporosis (Noa et al 2004b). Administration of 10 mg per day for 6 months to postmenopausal women with reduced bone mineral density significantly reduced urinary deoxypyridinoline (DPD)/creatinine ratio, a marker of bone resorption, but had no effect on serum concentrations of bone specific alkaline phosphatase, a marker of bone formation (Ceballos et al 2005). These effects of D-003 are consistent with its inhibitory effects on the mevalonate-to-cholesterol pathway, which could affect production of the isoprenoids required for protein prenylation, a process essential for the osteoclast activity, thereby leading to osteoclast apoptosis and reducing osteoclastic bone resorption (Weinstein & Manolagas 2000; Manolagas 2000), as occurs with bisphosphonates (Luckman et al 1998) and statins (Staal et al 2003).

Since D-003 displays both cholesterol-lowering and antiresorptive effects, in common with hormone-replacement therapy (HRT) (Josse 1996), we wanted to investigate its potential oestrogenic activity in-vivo using the uterotrophic assay in mature ovx rats. The rodent uterotrophic bioassay has been standardized and validated by the Organization for Economic Cooperation and Development (OECD) as a screening method for detecting the oestrogenic properties of chemicals able to prevent changes in the rat uterus due to the decrease in oestrogens induced through surgical removal of the ovaries, the primary source of oestrogen biosynthesis. The induced oestrogen deficiency results in reduction of uterus weight and morphological changes of the endothelial and stromal uterine tissues, such as reductions in epithelial cell height and stromal thickness (Kanno et al 2001). This assay has became the model of choice for evaluating the oestrogenic activity of drugs, which is manifest as restoration of changes in uterine growth that result from a cascade of events starting with the binding of such agonists with the oestrogen α-receptor, inducing uterine cellular division and uterine growth (Tinwell et al 2000; Kanno et al 2003).

Materials and Methods

Animals

Three-month-old female Sprague-Dawley rats (225 ± 20 g) were obtained from the National Centre for Laboratory Animals Production (CENPALAB, Havana, Cuba) and adapted to laboratory conditions (temperature 21°C, humidity 55%, 12 h light–dark cycle) for 2 weeks, with free access to food (rodent chow from CENPALAB) and water.

Animal handling was conducted in agreement with the Cuban guidelines for the use of laboratory animals. An independent institutional board approved the study protocol and the use of the animals in the experiment.

Drug administration and dosage

D-003 was obtained from the Chemistry Department of the Centre of Natural Products (Havana City, Cuba). Composition of the batch used (D30020504), assessed using a validated gas chromatography method (Méndez et al, 2003, 2004) was: 1-tetracosanoic (0.5%), 1-pentacosanoic (0.6%), 1-hexacosanoic (2.8%), 1-heptacosanoic (2.6%), 1-octacosanoic (35.4%), 1-nonacosanoic (1.6%), 1-triacontanoic (17.2%), 1-hentriacontanoic (1.0%), 1-dotriacontanoic (7.9%), 1-tritriacontanoic (1.0%), 1-tetratriacontanoic (8.8%), 1-pentatriacontanoic (0.4%) and 1-hexatriacontanoic (2.8%) acids.

D-003 was administered suspended in a 2% Tween/water vehicle. Drug suspensions were prepared weekly, and after checking their stability, were administered orally by gastric gavage (5–10 mL kg^{-1}).

Oestradiol benzoate (IMEFA, Havana City, Cuba) was dissolved in sunflower oil and administered by subcutaneous injection in a volume of 0.1 mL per 100 g bodyweight.

Rats were randomly distributed into five groups of eight rats. One group underwent a sham operation. The four ovx groups were treated with the vehicle (positive control), D-003 (50 mg kg^{-1}), oestradiol benzoate (30 μg kg^{-1}) or D-003 (50 mg kg^{-1}) plus oestradiol benzoate (30 μg kg^{-1}). The last group was included to assess whether D-003 could modify the response of ovx rats to oestradiol benzoate. Oestradiol benzoate was injected subcutaneously and D-003 and the vehicle were administered orally, once a day for 14 days, starting 2 weeks after ovariectomy.

The dose of D-003 used here (50 mg kg^{-1}) has been shown to almost completely prevent bone loss and bone resorption in ovx rats (Noa et al 2004a, b; Mendoza et al 2005a, b), to lower serum cholesterol (Gámez et al 2000) and to inhibit lipid peroxidation (Menéndez et al 2002). Thus, meaningful oestrogenic activity that D-003 has in-vivo in relation to its antiosteoporotic and hypocholesterolaemic effects should be evident at such a dose.

Bodyweight

Bodyweight was recorded a week after the surgical procedure, before the beginning of the treatment and at the end of treatment.

Uterotrophic assays

This assay was conducted as per a modification of the classic protocol (Kanno et al 2001). Rats underwent bilateral ovariectomy or a sham procedure under anaesthesia with sodium pentobarbital (50 mg kg^{-1} iv). For ovariectomy, the dorsolateral abdominal wall was opened at the midpoint between the costal inferior border and the iliac crest and a few millimetres lateral to the margin of the lumbar muscle. The ovaries were detached by incision at the junction of the oviduct and each uterine horn. The abdominal wall and skin were closed by sutures. Animals were allowed to recover for 14 days.

After drug treatment, rats were killed under anaesthesia and the uterus was carefully dissected and weighed, and the uterus weight calculated as a percentage of bodyweight (uterus:bodyweight ratio). For all protocols, blotted weight

rather than wet weight was used, since it is more sensitive to the detection of uterotrophic effects (Kanno et al 2003). Each uterine horn was then cut and the luminal fluid extracted by application of gentle pressure. Samples of each uterine horn were fixed in 10% buffered formaldehyde (BDH, Poole, Dorset, England). Tissues were dehydrated in graded alcohol, embedded in paraffin and cut in 4 μm transverse sections, which were stained with haematoxylin and eosin (Merck, Darmstadt, Germany) and examined using an Olympus BH2 microscope (Tokyo, Japan).

Morphometry

Uterine sections were evaluated microscopically to quantify changes in endometrial thickness and uterine epithelial cell height along the endometrial surface lining. Average cell height and endometrial thickness were calculated in five fields per animal. The measurements were performed with an ocular graticule at the same magnification.

Statistical analysis

Statistical comparisons of continuous data (bodyweight, uterine weight, uterus:bodyweight ratio, epithelial cell height and endometrial thickness) between control and treated groups were performed using analysis of variance. The Mann–Whitney U test was used to assess paired comparisons between the groups. An $\alpha = 0.05$ was a-priori selected for the statistical significance. Statistical analyses were performed using Statistics for Windows (Kernel release 5.1, Statsoft, Inc.1998, Tulsa, OK, USA).

Results

Bodyweight

Seven days after the surgical procedures, before starting drug treatment, bodyweight had increased significantly ($P < 0.05$) in all groups of ovx rats compared with the sham group.

As can be seen from Table 1, oestradiol benzoate administered alone for 14 days, but not D-003, prevented the bodyweight increase induced by ovariectomy. Bodyweight gain in rats treated with both oestradiol and D-003 was of similar magnitude to that with oestradiol benzoate alone.

Uterotrophic assays

Uterus:bodyweight ratio (uterus weight expressed as percentage of bodyweight) was significantly lower in the positive control group than in the sham-operated group; this effect was prevented with oestradiol benzoate, which increased the relative uterus weight compared with the positive controls. D-003 alone did not prevent the reduction of uterus weight induced by ovariectomy, and did not affect the effect of oestradiol benzoate when administered concurrently (Table 1).

Uterine morphology

Epithelial cell height and the endometrial thickness were similarly reduced in the positive-control and D-003-treated ovx rats compared with the sham group ($P < 0.001$). Epithelial cell height and endometrial thickness were significantly greater in the groups treated with oestradiol benzoate, alone or co-administered with D-003, than the positive controls (Table 2). Differences in the effects of oestradiol benzoate administered alone or together with D-003 were similar.

Discussion

This study demonstrates that D-003 given orally at 50 mg kg^{-1} to ovx rats for 14 days did not display oestrogenic effects in the uterotrophic assay. The rationale for exploring this effect was that D-003, like oestrogens, reduces serum cholesterol and prevents bone loss and bone resorption in the ovx rat, the best animal model for studying postmenopausal osteoporosis. Although HRT had been used to treat osteoporosis for years, reports from large clinical studies of negative effects on cardiovascular events, thromboembolism and breast cancer have led to reductions in its use as antiosteoporotic therapy.

Oestrogens play a pivotal role in the development, regulation and endocrine control of the female genital tract and mammary glands, while their capacity to induce cell proliferation in

Table 1 Effect of D-003 on the relative uterine weight in sham-operated and ovariectomized (ovx) rats treated with D-003 (50 mg kg^{-1}) and oestradiol benzoate (30 μg kg^{-1}) daily for 14 days. Data are mean ± s.d.

Group	Bodyweight (g)	Uterus:body weight ratio (%)
Sham operated	240.69 ± 8.08**	0.23 ± 0.10*
Positive control (ovx)	264.36 ± 10.40	0.05 ± 0.009
Ovx + D-003	281.56 ± 21.78†	0.05 ± 0.008†
Ovx + oestradiol benzoate	248.88 ± 14.63*	0.24 ± 0.07**
Ovx + D-003 + oestradiol benzoate	246.96 ± 6.63**	0.23 ± 0.04**

*$P \leq 0.001$; **$P \leq 0.0001$ vs control group; $^{\dagger}P \leq 0.001$ vs sham-operated group.

Table 2 Effect on D-003 on uterine epithelial cell height and endometrial thickness of ovx rats treated with D-003 (50 mg kg^{-1}) and oestradiol benzoate (30 μg kg^{-1}) daily for 14 days. Data are mean ± s.d.

Groups	Epithelial cell height (μm)	Endometrial thickness (μm)
Sham-operated	14.60 ± 0.25*	285.43 ± 0.60
Positive control (ovx)	9.56 ± 0.08	273.38 ± 1.21
Ovx + D-003	9.58 ± 0.09†	270.66 ± 3.02†
Ovx + oestradiol benzoate	14.21 ± 0.55*	285.73 ± 1.71*
Ovx + D-003 + oestradiol benzoate	14.64 ± 0.35**	284.85 ± 1.00*

*$P \leq 0.001$; **$P \leq 0.0001$ vs control; $^{\dagger}P \leq 0.001$ vs sham-operated group.

target tissues supports their role in carcinogenesis. Then, it is important to know if substances that display oestrogen-like effects, regardless of chemical structure, have oestrogenic activity in-vivo, which could be relevant to their effects on human health. In light of these facts, it was relevant to know whether D-003, which has potential for use in the management of postmenopausal osteoporosis, can produce oestrogenic effects in-vivo.

Our results show that ovariectomy produced the characteristic reduction of uterine weight and morphological changes, and that oestradiol benzoate exhibited its typical uterotrophic response, since it prevented the bodyweight increase induced by ovariectomy and compensated for all changes induced by ovariectomy, significantly increasing uterine weight, epithelial cell height and stromal thickness compared with the positive control group. D-003 administered at 50 mg kg^{-1} did not change the ovariectomy-induced gain in bodyweight and reductions in epithelial cell height and stromal thickness, thus indicating that it does not possess oestrogenic activity at this dose.

Although only one dose of D-003 was assessed (50 mg kg^{-1} per day), this dose has, compared with positive controls, prevented both bone loss and bone resorption in different trabecular bone structures by at least 90%; furthermore this dose has been used for comparative studies versus oestradiol, alendronate and pravastatin in the ovx rat (Noa et al 2004a, b; Mendoza et al 2005a, b). Thus our negative result is enough to affirm that the anti-resorptive effects of D-003 are not mediated through oestrogenic activity.

Although previous data did not support a rational suspicion for potential anti-oestrogenic effect of D-003, we used this assay to evaluate whether D-003 could modify the oestrogenic effect of oestradiol benzoate. The results demonstrated that D-003 at 50 mg kg^{-1} did not significantly modify the response of bodyweight and uterine changes to oestradiol benzoate, indicating that, at this dose, D-003 was also devoid of anti-oestrogenic activity.

Thus, indirectly these results support that the notion that the preventive effects of D-003 on increased bone loss and bone resportion in the ovx rat may be associated with its inhibitory effect on cholesterol synthesis. In addition, since D-003 also inhibits lipid peroxidation (Menéndez et al 2002; Castaño et al 2003), a process linked to the development of osteoporosis (Xu et al 1995; Parhami et al 1997), such an effect could also contribute to the anti-osteoporotic and anti-resorptive effects of D003 previously demonstrated (Noa et al 2004a, b; Mendoza et al 2005a, b).

Conclusions

D-003 given orally at 50 mg kg^{-1}, a dose that prevents bone loss and bone resorption in ovx rats, did not display oestrogenic/anti-oestrogenic activity in-vivo, as assessed in the uterotrophic assay.

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