Trabajo Original | Toxicología Experimental

# *In Vitro* Genotoxic Potential of D-003 on the Blood Lymphocytes Chromosomal Aberration Test.

**Rafael Gámez Menéndez, Ariadne Gutiérrez Martínez, Rosa Mas Ferreiro, Balia Pardo Guerra, Haydee García Cambian, Isury Bucarano Lliteras**

Centro de Productos Naturales. Centro Nacional de Investigaciones Científicas.

Autor para correspondencia: Dr.C. Rafael Gámez Menéndez

Dirección: Calle 198 e/ 19 y 21 Atabey, Playa, C. Habana.

Teléfono: 2714225, 2714200, 2714212

Correo electrónico: rafael.gamez@cnic.edu.cu

## Abstract

**Background:** D-003, a mixture of very high molecular weight aliphatic acids purified from sugarcane wax, has been shown antioxidant, lipid-modifying and antiosteoporotic properties in preclinical and clinical studies.

**Objective:** This study investigated the *in vitro* genotoxic potential of D-003 to produce chromosomal aberrations (CA) in peripheral blood lymphocytes.

**Methods:** Fresh suspensions of D-003 were added (1 000, 2 500 and 5 000 μg/mL) to cultures with (microsomal liver fraction S9 mix) or without metabolic activation. Concurrent negative (Tween/water vehicle) and positive controls (cyclophosphamide 15 μg/mL and mitomicyn-C 0.3μg/mL in the tests with and without metabolic activation, respectively) were included. Two hundred (200) metaphases by group were examined and the numbers and frequencies of cells with aberrations and the mitotic index were quantified.

**Results:** D-003 added up to 5 000μg/mL of culture did not induce chromosomal aberrations in presence or not of S9 mix compared with negative controls and no trends with the doses were observed. Positive controls evidenced increases of the numbers and frequencies of CA. Mitotic indexes were unchanged with the treatment, which indicates that D-003 does not affect cell proliferation.

**Conclusions:** D-003 added *in vitro* (1 000-5 000 μg/mL) to peripheral blood lymphocyte cultures did not show evidences of cytotoxic or genotoxic potential in the CA test.


**Keywords:** D-003, sugar cane wax acids, antioxidants, antiosteoporotic substances, lipid-modifying substances, chromosomal aberrations test, lymphocyte cultures.

## Resumen

Potencial genotóxico del D-003 en el ensayo de Aberraciones cromosómicas *in vitro* en linfocitos.

**Introducción:** El D-003, mezcla de ácidos alifáticos de alto peso molecular purificado de la cera de la caña de azúcar, presenta actividad antioxidante, hipolipemiante y antiosteoporótica en modelos experimentales y en ensayos clínicos.

**Objetivo:** Investigar el potencial genotóxico *in vitro* del D-003 en el ensayo de aberraciones cromosómicas en cultivo de linfocitos de sangre periférica.

**Métodos:** Se adicionó a cultivos de linfocitos suspensions de D-003 (1 000, 2 500 and 5 000 μg/ml) con y sin la presencia de activación metabólica (mezcla de fracción microsomal hepática). Se empleó como control negativo el tween 65/en agua y como controles positive la ciclofosfamida (15 μg/ml) y la mitomicina-C (0.3μg/ml). Se analizaron 200 metafases por grupo de tratamiento, cuantificando la el número de células con aberraciones y el índice mitótico.

**Resultados:** El D-003 adicionado hasta dosis de 5 000μg/mL cultivo de linfocitos no indujo aberraciones cromosómicas en presencia o no de mezcla S9, resultados comparables con el control negativo, no se evidencio tendencias al incremento de las aberraciones cromosómicas con la dosis. Los controles positivos empleados incrementaron la frecuencia de aberraciones cromosómicas. El índice mitótico no cambió con el tratamiento con D-003, lo cual indica que no afecta la proliferación celular.

**Conclusiones:** El tratamiento con D-003 (1 000 - 5 000 μg/mL) *in vitro* a linfocitos de sangre periférica no mostró evidencias de potencial citotóxico o genotóxico en el ensayo de aberraciones cromosómicas.


**Palabras claves:** D-003, ácidos de la cera de la caña de azúcar, antioxidante, antiosteoporótico, hipolipemiante, aberraciones cromosómicas, cultivo de linfocitos.

## Introduction

D-003, a mixture of high molecular weight aliphatic acids purified from sugarcane (*Saccharum officinarum, L*) wax, contains octacosanoic acid as the most abundant component, and tetracosanoic, pentacosanoic, hexacosanoic, heptacosanoic, nonacosanoic, triacontanoic, hentriacontanoic, dotriacontanoic, tritiacontanoic, tetratriacontanoic, pentatriacontanoic and hexacotriacontanoic acids at lower concentrations.[1]

Oral treatment with D-003 has been shown to produce lipid-modifying and various pleiotropic effects (antioxidant, antiosteoporotic) effects in experimental and clinical studies.[1] The lipid-modifying effects of D-003 have been associated to the inhibition of cholesterol biosynthesis by regulating the enzymatic activity of hydroxymethylglutaryl Coenzyme (HMG-CoA) reductase.[1-4] Also, D-003 has proven to reduce lipid peroxidation in experimental and clinical studies.[5,6] Consequently, oral treatment with D-003 has demonstrated to prevent bone loss and bone resorption in experimental models of ovariectomy- and corticoids-induced osteoporosis in rats,[7-9] consistent with its inhibitory effects on both the mevalonate to cholesterol pathway and lipid peroxidation, as well.[2]

Previous experimental toxicological studies have failed to find D-003-related toxicity, so that single oral dose toxicity in rodents was practically absent[10] and the same happened in the oral sub-chronic (90 days) study in rats, wherein a dose of 1 250 mg/kg, the highest tested, did not produce observable toxic effects.[10] Also, lack of chronic toxicity was found in studies conducted in rats (6 months) and dogs (9 months), which confirmed the pharmacological effects of D-003, thus discarding that the lack of toxicity was not attributable to inadequate drug exposure.[11,12] On the other hand, previous *in vitro* and *in vivo* studies had shown that D-003 is devoid of mutagenic or clastogenic effects,[13,14] and that administered orally to 1 000 mg/kg did not impair fertility or reproduction.[15,16] Nevertheless, the *in vitro* genotoxic potential of D-003 on chromatid or chromosome aberrations (CA), a cause of many human genetic diseases, had not been

explored. In fact, there is substantial evidence that chromosomal damage and related events causing alterations in oncogenes and tumour suppressor genes of somatic cells are involved in cancer induction in humans and experimental animals.[17]

The *in vitro* mammalian CA is a sensitive, fast and reproducible assay widely used to assess chromosomal damage, in which cultures of established cell lines, cell strains or primary cell cultures can be used.[17-19]

In light of these issues, this study was undertaken to investigate the *in vitro* genotoxic potential of D-003 to produce CA using the mammalian test in human peripheral blood lymphocytes.

## Material and Methods

### Primary cell cultures

This study was conducted in human peripheral blood lymphocytes, a recommended model to assess the induction of CA *in vitro*,[17-19] from healthy volunteer donors.

### Cellular culture conditions

Lymphocyte cultures contained (per 10 mL): 10% foetal bovine serum; 10% whole blood from a healthy young donor; 2% phytohemagglutinin in RPMI 1 640 and incubated at 37°C in wet atmosphere. After being incubated for 48 hours, cells were exposed to the vehicle, D-003 or the positive control in conditions with or without metabolic activation (S9 mix).

### Administration and dosage

D-003, obtained from the Plants of Natural Products (National Centre for Scientific Research, Havana, Cuba) by a process that includes sugar cane wax basic hydrolisis and further extraction and purification in organic solvents, was used after control its quality specifications. The composition of D-003, assessed through a validated gas chromatography method,[20] was as follows: tetracosanoic (0.9%), pentacosanoic (0.5%),

hexacosanoic (1.0%), heptacosanoic (3.0%), octacosanoic (47.0%), nonacosanoic (3.0%), triacontanoic (21.0%), hentriacontanoic (1.0%), dotriacontanoic (12.0%), tritriacontanoic (2.0%), tetratriacontanoic (12.0%), pentatriacontanoic (0.5%), hexatriacontanoic (6.0%). D-003 was suspended in a 0.4% Tween 20/water vehicle and further diluted in Roswell Park Memorial Institute (RPMI) 1640 medium. Fresh suspensions were added to the culture (100 µL/culture), reaching the following final concentrations: 1 000, 2 500 and5 000 µg/mL of culture, the highest dose being the upper limit dose for this assay (5 000 µg/mL).[17-19]

**Cytogenetic study**

Lymphocyte cultures contained (per 10 mL): 10% foetal bovine serum; 10% whole blood from a healthy young donor; 2% phytohemagglutinin in RPMI-1640.

After being incubated for 48 hours, cells were exposed to the treatments in presence or absence of metabolic activation. Fresh suspensions of D-003 were added to reach 1 000, 2 500 and 5 000 µg/mL of culture. Concurrent negative (vehicle-treated) and positive controls were included in both experiments. Cyclophosphamide (CP) (15 µg/mL) and mitomicyn C (MMC) (0.3 µg/mL) were used as positive controls in the tests with (liver microsomal S9 mix) and without metabolic activation, respectively.

**Experimental series without S9 mix.**

After being incubated for 48 hours, cells were exposed, without metabolic activation, to the vehicle, D-003 or mytomicin-C (MMC) (0.3 µg/mL) for 17-20 hours, a time enough to complete a cell cycle (13-15 hours). In all cases, colchicine (0.4 µg/mL) was added and incubation ran for the last 3 hours. Then, cells were collected, treated with KCl (75mM), fixed with methanol: acetic acid (3:1) and dropped onto slides, being subsequently stained with 5% Giemsa in Sörensen buffer pH 6.8, in order to perform the cytogenetic study.

**Experimental series with S9 mix.**

After the 48 hours incubation, cells were exposed for 2-3 hours to D-003, vehicle or cyclophosphamide (CP) in presence of metabolic activation. Once concluded, cells were washed three times with fresh RPMI-1640, and then placed on a culture medium supplemented with foetal bovine serum and phytohemagglutinin and incubated at 37°C for the next 17-20 hours, time enough for completing a cell cycle (13-15 hours).

Cytogenetic analyses, conducted in 200 metaphases by group, were conducted under bright field microscopy and CA classified conveniently, following the standard protocol. The number and frequency of cells with CA (excluding the gaps) and the mitotic index were quantified.[17-19]

**Microsomal fraction ($S_9$)**

The S9 microsomal fraction was obtained from the liver of male Sprague Dawley rats treated with sodium phenobarbital (30 and 60 mg/kg) and benzoflavone (80mg/kg), as recommended.[21]

## Statistical analysis

The numbers of cells with CA and the types of CA were compared among groups with the non-parametric $\chi^2$ test. The dose-response relationship was explored using a lineal regression analysis for proportions. For each case, the results of two different cultures were analysed.[17,22] Statistical analyses were performed with the software Statistics for Windows. A value of $\alpha = 0.05$ was a priori established for the statistical significance.

## Results

Table 1 summarises the results of the study. Negative controls exhibited almost no aberrations/200 cells, and low values of mitotic index, gaps and aberrant cells, all of which increased significantly in the positive control groups treated with MMC (test without metabolic activation, -S9 mix) or CP (test with metabolic activation, +S9 mix). Thus, (16.5% of the cells treated with MMC and 23.5% of the cells treated with CP showed structural CA (56 and 65 CA, respectively).

D-003 added (1 000-5 000 µg/mL) to lymphocyte cultures did not increase significantly the mitotic index, gaps, aberrations per cells or aberrant cells in cultures submitted or not to metabolic activation as compared to the controls. Thus, there were 7 (1.2%) and 3 (1.5%) aberrant cells in all (600) analyzed D-003-treated and control cells group, respectively, in conditions of S9 mix, meanwhile 0.7 % of cells with CA were seen in D-003-treated and 1% in control cells not submitted to metabolic activation. Overall, D-003 does not induce structural CA nor affect cellular proliferation since it unchanged the mitotic index. Likewise, no trends with the doses of D-003 were observed.

## Discussion

In accordance to the current guidelines the upper concentration of D-003 used in the experiments (5 000 µg/mL) is the maximal recommended for relatively non-cytotoxic compounds.[17-19] On the other hand, CP and MMC effects were consistent with the reported findings for this model by other authors,[23,24] these results confer validity to the present results, discarding that the negative results could be attributable to methodological reasons.

D-003 does not induce or changed mitotic index. Likewise, no trends with the doses of D-003 were observed. Therefore, our negative results demonstrates the lack of genotoxic/cytotoxic potential of D-003 in this test, consistent with previous data of the *in*

*vitro* (Ames test) and in vivo (rat and mice micronucleus bone marrow test, head-sperm morphology, comet assay) potential genotoxicity of D-003.[13,14]

High correlation had been documented between increase of CA and carcinogenicity in experimental models.[18] In such regard, the present negative results are a new evidence that supports the lacking of carcinogenicity founded in rats and mice treated with high doses of D-003.[25,26]

## Conclusions

D-003 added *in vitro* (1 000-5 000 μg/mL) to peripheral blood lymphocyte cultures did not show evidences of cytotoxic or genotoxic potential in the CA test.

**Table 1. Cytogenetic effects of D-003 (1 000 - 5 000 µg/mL) in peripheral blood lymphocytes cultures**

| Concentration | M.I. (%) | Aberrations/200 cells | | | | | Aberrant cells (%) |
|---|---|---|---|---|---|---|---|
| | | Gaps | Chromosome type | | Chromatid type | | |
| | | | Breaks | Exchanges | Breaks | Exchanges | |
| **Without metabolic activation (-S9 mix)** | | | | | | | |
| **Negative control** | 9.8 | 4 | 0 | 0 | 2 | 0 | 1.0 |
| **MMC** (0.3µg/mL) | 1.9* | 46* | 16* | 2 | 27* | 11* | 16.5* |
| **D-003** (µg/mL) | | | | | | | |
| **1 000** | 9.9 | 5 | 0 | 0 | 3 | 1 | 1.0 |
| **2 500** | 9.3 | 2 | 0 | 0 | 1 | 0 | 0.5 |
| **5 000** | 9.6 | 5 | 0 | 0 | 1 | 0 | 0.5 |
| **With metabolic activation (+S9 mix)** | | | | | | | |
| **Negative control** | 7.3 | 5 | 1 | 1 | 2 | 0 | 1.5 |
| **CP (**15µg/mL) | 2.1* | 22* | 6 | 7[a] | 39* | 13* | 23.5* |
| **D-003** (µg/mL) | | | | | | | |
| **1 000** | 7.1 | 4 | 0 | 0 | 3 | 0 | 1.5 |
| **2 500** | 7.4 | 5 | 0 | 0 | 2 | 0 | 1.0 |
| **5 000** | 7.2 | 3 | 0 | 0 | 2 | 0 | 1.0 |

Positive controls Mitomycin C (MMC) and cyclophosphamide (CP) for experiments without and with metabolic activation, respectively
*****$p<0.001$, [a] $p<0.05$ Chi-square Test. Comparison with negative control

## References


1. Mas R. D-003: A new substance with promising lipid modifying and pleiotropic effects for atherosclerosis management. Drugs of the Future 2004; 29: 773-86.
2. Menéndez R, Mas R, Amor AM. et al. Inhibition of cholesterol biosynthesis in cultured fibroblasts by D-003, a mixture of very long chain saturated fatty acids. Pharmacol Res 2001; 44: 299-304.
3. Gámez R, Mendoza S, Más R. et al. Dose-dependent cholesterol-lowering effects of D-003 on normocholesterolemic rabbits. Curr Ther Res Clin & Exptl 2000; 61:8-16.
4. Castaño G, Mas R, Fernández L. et al. Effects of D-003 (5-40 mg/day) on lipid profile of patients with Type II hypercholesterolemia: a Phase II clinical study. Clin Drug Invest 2003; 23: 789-802.
5. Menéndez R, Mas R, Pérez Y, et al. Inhibition of rat lipoprotein lipid peroxidation by the oral administration of D-003, a mixture of very long chain saturated fatty acids. Can J Physiol Pharmacol 2002; 80:13-17.
6. Castaño G., Menéndez R., Mas R., et al. Effects of D-003 on lipid profile and lipid peroxidation in healthy volunteers. Clin Drug Invest 2003; 23: 193-203.
7. Noa M, Más R, Mendoza S, et al. Effects of D-003, a mixture of high molecular weight aliphatic acids from sugar cane wax, on bones from ovariectomized rats. Drugs Exptl Clin Res 2004; 30: 35-41.
8. Noa M, Mendoza S, Más R, et al. Effect of D-003, a mixture of very high molecular weight aliphatic acids, on prednisolone-induced osteoporosis in Sprague Dawley rats. Drugs in R&D 2004; 5: 281-290.
9. Mendoza S, Noa M, Más R, Mendoza N. Effects of D-003 (5-200 mg/kg), a mixture of high molecular weight aliphatic acids from sugarcane wax, on bones and bone cell apoptosis in ovariectomized rats. Int J Tiss React 2005; XXVII: 213-22.
10. Gámez R, Más R, Noa M. et al. Acute and subchronic oral toxicity of D-003 in rats. Toxicol Letters 2000; 118: 31-41.

**11.** Gámez R, Mas R, Noa M. et al. Six-month toxicity study of oral administration of D-003 in Sprague Dawley rats. Drugs in R&D 2002; 3: 375-86.

**12.** Gámez R, Más R, Noa M, et al. Effects of chronic administration of D-003, a mixture of sugar cane wax high molecular weight acids, in beagle dogs. Drugs Exp & Clin Res 2004; 30: 75-8

**13.** Gámez R, González JE, Rodeiro I, et al.. *In vivo* genotoxic evaluation of D-003, a mixture of very long chain aliphatic alcohols. J Med Food 2001; 4: 85-92.

**14.** Gámez R, Rodeiro I, Fernández I, Acosta P. Preliminary evaluation of the cytotoxic and genotoxic potential of D-003: A mixture of very long chain fatty acids. Teratog, Carcinog, Mutagenesis 2002; 22: 175-81.

**15.** Rodríguez MD, Gámez R., González JE. et al. Lack of developmental toxicity of D-003: a mixture of long chain fatty acids in rats. Food Chem Toxicol 2003; 41: 89-93.

**16.** Rodríguez MD., Gutiérrez A, Marrero G, García H. Perinatal/postnatal study of D-003, a mixture of long-chain fatty acids in rats. J Med Food 2006; 9: 223-30.

**17.** OECD Guideline 473. In vitro Mammalian Cytogenetic Test. Available from: http://www.oecd.org/

**18.** Toxicological principles for the Safety Assessment of Food Ingredients. Redbook 2000. November 2003. IV.C.1.b. In vitro Mammalian Chromosomal Aberration Test. U.S Food and Drugs Administration. Available from: http://www.fda.gov/

**19.** ICH. Guidelines on genotoxicity. **S2B**, 1997

**20.** Marrero D., Méndez E., González V., et al. Determination of D-003 by capillary gas chromatography. Rev CENIC Ciencias Químicas 2002; 33:99-103.

**21.** Elliot M., Combes RD, Elcombe CR. Report of UKA environmental mutagen society working party. Alternative to aroclor 254-induced S9 in in vitro genotoxicity assay. *Mutagenesis* 1992; **7**: 175-77.

**22.** Richardson C., Williams DA, Allen JA. Analysis of Data from In Vitro Cytogenetic Assays. In: Statistical Evaluation of Mutagenicity Test Data. Kirkland, DJ (ed) Cambridge University Press, Cambridge 1989; pp. 141-54.

**23.** Roncada T;. Vicentini VE. Mantovani MS. Possible modulating action of plant extract

on the chromosome breaking activity of MMC and ara-C in human lymphocytes in vitro. *Toxicol in Vitro* 2004; 18: 617-22

**24.** Gámez R, Gutiérrez A, Arencibia D, Curveco D, García H. In vitro genotoxic potencial of D-002 blood lymphocytes chromosomal aberration test. LAJP 2010; 29: 459-62.

**25.**Gámez R, Noa M, Mas R, Mendoza N, Pardo B, Pérez Y. Et al. Long-term carcinogenicity of D-003, a mixture of high molecular weight acids from sugarcane wax, in Sprague Dawley rats: a 24 months study. Food Chem Tox 2007;45:2352-2358

**26.**Noa M, Gámez R, Mas R, Gutiérrez A, Mendoza N, Pardo B, et al. Study of the long-term carcinogenicity potential of D-003, a mixture of high molecular weight sugarcane wax acids, in mice. Food Chem Tox 2009; 47(4): 687-92.

**Recibido: 03/10/12**

**Aceptado: 15/10/12**