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Short Communication

Effects of D-003 on Hepatic Drug-Metabolizing Enzyme Activities in Rats

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ABSTRACT D-003 is a mixture of very-long-chain aliphatic acids with cholesterol-lowering and concomitant anti-platelet effects. The microsomal cytochrome P-450 system comprises a superfamily of proteins present in hepatic and extrahepatic tissues that is responsible for the metabolism of many drugs. The present study was undertaken to investigate the effects of D-003 on *in vivo* drug-metabolizing hepatic enzymes. Two experimental series ($n = 6$ animals/group) were performed. In the first series rats were randomly distributed in one control and two groups treated with D-003 at 1,000 and 2,000 mg/kg for 14 days. In the second one they were distributed in one control and three groups treated with D-003 (250, 500, and 1,000 mg/kg) for 6 months. All treatments were orally administered by gastric gavage. Control rats were orally treated only with acacia gum/water vehicle. The content of microsomal P-450, b_5 cytochromes, total sulfhydryl groups, nonprotein sulfhydryl groups, and protein-bound sulfhydryl groups as well as the activities of NADPH cytochrome c reductase, aminopyrine demethylase, dimethylnitrosamine *N*-demethylase, 7-ethoxyresorufin *O*-deethylation, 7-pentoxyresorufin *O*-dependentylation, and cytosolic glutathione *S*-transferase were assessed. D-003 administered up to 2,000 mg/kg or 1,000 mg/kg during 14 days or 6 months did not affect the activities of the hepatic drug-metabolizing enzymes investigated. It is concluded that D-003 is not metabolized by the liver cytochrome system and that potential risk derived from drug-to-drug interactions between D-003 and concomitant drugs appears to be low.

KEY WORDS: • ***anti-platelet effects*** • ***cholesterol-lowering effects*** • ***D-003*** • ***microsomal cytochrome P-450 system***

INTRODUCTION

THE MICROSOMAL CYTOCHROME (CYT) P-450 system comprises a superfamily of proteins present in hepatic and extrahepatic tissues that is responsible for the metabolism of most drugs, playing a role in both detoxification and metabolic activation process. Thus, the influence on this system is relevant to predict the influence of any drug in the metabolism of others.[1,2]

There are multiple forms of CYT P-450 enzymes, which react with different substrates. Administration of drugs to animals can inhibit or increase monooxygenase activity, which is important because the monooxygenase system is relevant in drug-mediated toxicity.[3,4]

D-003 is a mixture of very-long-chain aliphatic acids purified from sugar cane (*Saccharum officinarum* L.) wax,[5] wherein 1-octacosanoic acid is the major component, followed by 1-triacontanoic, 1-dotriacontanoic, and 1-tetratriacontanoic acids. In addition, hexacosanoic, nonacosanoic, hentriacontanoic, tritriacontanoic, pentatriacontanoic, and hexatriacontanoic acids are present as minor components.

Pharmacological experimental studies conducted in rabbits showed that D-003 orally administered (5–200 mg/kg) to rabbits significantly reduced serum total cholesterol (TC) and low-density lipoprotein-cholesterol (LDL-C) levels in a dose-dependent manner, whereas it significantly increased high-density lipoprotein-cholesterol (HDL-C) values.[6–9] Also, D-003 administered from 5 to 50 mg/day has shown cholesterol-lowering effects in healthy volunteers and patients with Type II hypercholesterolemia causing reduction of serum LDL-C and TC, while markedly raising HDL-C.[10–12]

On the other hand, D-003 orally administered also induces anti-platelet effects in animal models and human beings,[12–17] which appears to be mediated through reduced thromboxane A_2 levels and increased prostacyclin values.[15,16]

Thus, D-003 shows a pharmacological profile suggesting that it could be a promising agent for treating atherothrombotic diseases, as expected from its cholesterol-lowering and anti-platelet effects. Nevertheless, both cholesterol-lowering and anti-platelet drugs are indicated as long-term treatments, which reinforces the point that their safety and tolerability profiles are crucial aspects for their definitive acceptance.

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Previous studies of oral toxicity of D-003 in rodents (acute, subchronic, and chronic) have shown that it did not induce drug-related toxicity.[18–20] Similarly, the *in vitro* and *in vivo* assessment of cytotoxic and/or genotoxic potential effects of D-003 showed that D-003 was devoid of cytotoxic or genotoxic potential,[21,22] and the lack of reproductive toxicity has been also documented for D-003.[23]

Nevertheless, information about putative drug-to-drug interactions between D-003 and other drugs commonly consumed is scarce, being limited to isolated experiences of pharmacodynamic interactions.[16,17] Thus, the investigation of the effects of D-003 on hepatic drug-metabolizing activities is justified as a first approach to such a target.

Considering this background, this study was conducted to investigate whether D-003 affects the activity of phase I and phase II biotransformation enzymes in male Sprague–Dawley rats, a species used extensively in toxicological studies.

MATERIALS AND METHODS

Chemicals

All chemicals were of analytical reagent grade. Phenobarbital, glucose 6-phosphate, bovine serum albumin, and NADP were obtained from BDH Chemicals Ltd. (Poole, Dorset, UK). Acacia gum, (−)-naphthoflavone, glucose-6-phosphate dehydrogenase, CYT *c*, NADPH, aminopyrine, dimethylnitrosamine, 7-ethoxyresorufin, 7-pentoxyresorufin, resorufin, benzo[*a*]pyrene, 1-chloro-2,4-dinitrobenzene, 3,3-dithiobis(2-nitrobenzoic acid), reduced glutathione (GSH), H_2O_2, and sodium dithionite were purchased from Sigma Chemical Co. (St. Louis, MO).

Animals

Young adult male Sprague–Dawley rats weighing 150–200 g were obtained from the Centre for Laboratory Animal Production (CENPALAB, Havana, Cuba). Animals were adapted to experimental conditions (temperature 25 ± 5°C, relative humidity 65 ± 5%, and 12-hour dark/light cycles). Rat standard chow supplied by CENPALAB and tap water was provided *ad libitum*.

The present study was conducted under the Cuban Good Laboratory Practices Code, which is consistent with updated standard international guidelines, and following the ethical standard for animal care and the standard operational procedures for each manipulation.

Administration and dosage

D-003 was supplied by the Chemistry Department of the Centre of Natural Products after its purity was checked through gas chromatography. D-003 was suspended in acacia gum/water (10 mg/mL) and orally administered by gastric gavage (5–10 mL/kg). Acacia gum/water was selected as the vehicle, as recommended for insoluble substances administered orally by gastric gavage.[4] Suspensions were weekly prepared, after corroboration of the stability of the substance in such conditions.

Two experimental series were conducted. In the first experimental series, animals (six animals per group) were randomly distributed in three groups: a control and two groups treated with D-003 at 1,000 and 2,000 mg/kg, with all treatments administered for 14 days. In the second one, animals (six animals per group) were distributed in four groups: a control and three groups receiving D-003 at 250, 500, and 1,000 mg/kg, with treatments being administered for 6 months. Control rats received equivalent volumes of acacia gum/water vehicle by gastric gavage.

Liver removal

Twenty-four hours after the last dose, rats were weighed, anesthetized, and sacrificed. Then, livers were removed and weighed. The relative weight of the liver relative to body weight was expressed in a percentage and calculated as follows: % = (liver weight/body weight) × 100.

Preparation of microsomes

Liver samples of 5 g were taken from three animals and homogenized in 3 volumes of ice-cold 1.15% KCl using a Polytron homogenizer. In the 6-month study, one part of the homogenate was used for determining the acid-soluble sulfhydryl content, and the rest for obtaining the microsomes. The cell debris, nuclei, and mitochondria were removed by centrifugation at 9,000 *g* for 20 minutes at 0–4°C. The supernatant was ultracentrifuged (100,000 *g* for 60 minutes) at 0–4°C. The pellet was suspended again in ice-cold 0.1 mol/L Tris-HCl buffer (pH 7.4) containing 0.175 mmol/L KCl and 0.2 mmol/L EDTA. Aliquots (1 mL) of microsomal fractions were quickly stored at −80°C until usage.

Protein content was determined by the method of Lowry *et al.,*[24] using bovine serum albumin as the standard. The content of microsomal P-450 and b_5 CYTs was measured according to the established method.[25]

The activities of drug-metabolizing hepatic microsomes were assayed by established methods. Thus, aminopyrine demethylase activity was assessed as formaldehyde formation according to Nash,[26] dimethylnitrosamine *N*-demethylase following Weibel *et al.*,[27] and 7-ethoxyresorufin *O*-deethylation (EROD) and 7-pentoxyresorufin *O*-depentylation (PROD) as reported by Donato *et al.,*[28] whereas NADPH CYT c reductase activity was determined as described by Williams and Kamin.[29]

The Ellman method was used for determining total sulfhydryl groups (T-SH), nonprotein sulfhydryl groups (NP-SH), and protein-bound sulfhydryl groups (PB-SH),[30] while the activities of cytosolic glutathione *S*-transferase (GST) and catalase were determined as described.[31,32]

Statistical analysis

Data are presented as mean ± standard error (SEM) values. Comparisons between groups were determined using

TABLE 1. EFFECTS OF D-003 ORALLY ADMINISTERED FOR 14 DAYS TO MALE SPRAGUE-DAWLEY RATS ON HEPATIC MICROSOMAL DRUG-METABOLIZING ENZYME ACTIVITIES

Treatment	*Liver body weight (%)*	*Protein (mg/kg)*	*NADPH CYT c reductase (nmol/mg/minute)*	*CYT P-450 (nmol/mg)*	*CYT b_5 (nmol/mg)*	*EROD (pmol/minute/mg)*	*PROD (pmol/minute/mg)*	*Aminpyrine demethylase (nmol/g of liver)*	*Dimethylnitrosamine dealkylase (nmol/mg/minute)*	*GST (nmol/minute/mg)*	*Catalase (U/mg)*
Control	3.69 ± 0.10	25.58 ± 3.7	11.87 ± 2.9	0.504 ± 0.14	0.313 ± 0.12	7.68 ± 2.03	2.73 ± 0.62	0.261 ± 0.09	0.075 ± 0.01	544.3 ± 108.9	6.95 ± 2.0
D-003 (mg/kg)											
1,000	3.63 ± 0.15	24.32 ± 2.3	13.16 ± 2.5	0.520 ± 0.12	0.323 ± 0.17	8.42 ± 2.44	3.07 ± 0.97	0.254 ± 0.08	0.072 ± 0.01	529.8 ± 55.0	7.23 ± 1.1
2,000	3.66 ± 0.29	23.48 ± 3.4	14.15 ± 2.0	0.485 ± 0.17	0.345 ± 0.10	7.49 ± 1.80	2.70 ± 0.95	0.280 ± 0.10	0.074 ± 0.01	569.2 ± 163.6	7.16 ± 1.7

Data are mean ± SEM values. All comparisons were not significant (Mann–Whitney test).

TABLE 2. EFFECTS OF D-003 ORALLY ADMINISTERED FOR 6 MONTHS TO MALE SPRAGUE-DAWLEY RATS ON HEPATIC MICROSOMAL DRUG-METABOLIZING ENZYME ACTIVITIES

Treatment	*Liver body weight (%)*	*Protein (mg/kg)*	*NADPH CYT c reductase (nmol/mg/minute)*	*CYT P-450 (nmol/mg)*	*CYT b_5 (nmol/mg)*	*EROD (pmol/minute/mg)*	*PROD (pmol/minute/mg)*	*Aminpyrine demethylase (nmol/g of liver)*	*Dimethyl-nitrosamine dealkylase (pmol/mg/minute)*	*GST (pmol/minute/mg)*	*Catalase (U/mg)*	*T-SH*	*μmol/g of tissue*	
													NP-SH	*PB-SH*
Control	2.53 ± 0.22	21.39 ± 0.79	14.14 ± 2.89	0.53 ± 0.19	0.327 ± 0.10	9.09 ± 1.87	2.83 ± 0.81	0.301 ± 0.12	0.050 ± 0.002	670 ± 10	27.89 ± 1.42	38.44 ± 3.04	9.44 ± 0.97	29.33 ± 1.89
D-003 (mg/kg)														
250	2.31 ± 0.17	21.93 ± 1.21	15.07 ± 2.21	0.53 ± 0.002	0.332 ± 0.02	8.68 ± 1.85	2.66 ± 0.69	0.311 ± 0.11	0.059 ± 0.004	693 ± 50	7.40 ± 1.39	39.10 ± 2.23	9.29 ± 0.68	27.66 ± 4.11
500	2.37 ± 0.22	21.22 ± 1.23	14.92 ± 2.46	0.54 ± 0.04	0.322 ± 0.05	9.08 ± 1.45	2.65 ± 0.51	0.280 ± 0.11	0.054 ± 0.005	786 ± 115	8.22 ± 0.56	36.14 ± 3.76	9.14 ± 0.87	27.07 ± 3.15
1,000	2.38 ± 0.09	21.36 ± 0.98	13.10 ± 2.89	0.53 ± 0.24	0.360 ± 0.14	9.75 ± 1.91	2.27 ± 0.57	0.303 ± 0.11	0.054 ± 0.007	622 ± 240	7.35 ± 1.90	39.73 ± 1.68	9.63 ± 0.71	29.80 ± 2.27

Data are mean ± SEM values. All comparisons were not significant (Mann–Whitney test).

the Mann–Whitney test; $\alpha = .05$ was *a priori* selected for statistical significance. Statistical analyses were performed using the software Statistics for Windows.

RESULTS AND DISCUSSION

The present study is the first reporting the effects of D-003, a mixture of very-high-molecular-weight aliphatic acids, on *in vivo* drug-metabolizing liver microsomal enzymes. The results presented here demonstrate that D-003 administered orally at very high doses (1,000 and 2,000 mg/kg) did not affect any of the variables tested, and the same was true when D-300 was administered orally from 250 to 1,000 mg/kg for 6 months.

Tables 1 and 2 summarize the results. D-003 did not affect the relative liver weight, which is consistent with results of previous toxicological studies, and no significant differences or trends with the doses related with any indicator were observed.

D-003 did not change CYT P-450 content and NADPH-dependent reduction of CYT *c*, thus indicating that it neither directly interacts with CYT P-450 nor interferes with the electron flow to the CYTs, respectively. Likewise, the forms of the CYT P-450 enzymes responsible for the biotransformation of the substrates used also remained unchanged by the treatment. In this case, the activity of CYP 1A1, CYP 2B 1,2, CYP 3A, and CYP 2E1 were determined since they are among the most relevant isoenzymes of this superfamily of proteins with toxicological or clinical implications.[33]

The treatment administered here was long term, since repeated administration for 6 months has been accepted for oral chronic toxicity in rodents. Thus, an acute induction could take place, but short-term (2 days) treatment revealed similar results (authors' unpublished data), thus eliminating such a possibility.

A reduction in the activity of phase II enzyme activities (catalase and GST) has been reported for other cholesterol-lowering drugs,[34] but in contrast D-003 did not change significantly the activity of GSTs, which catalyze the addition of glutathione to numerous electrophilic compounds.[35] In addition, D-003 administered for 6 months at up to 1,000 mg/kg did not change the content in animal livers of sulfhydryl groups, an indirect surrogate of GSH constituting most (>95%) of the liver NP-SH.[36]

Previous studies have shown that D-003 orally administered (5–200 mg/kg) to rats similarly inhibited "*in vitro*" and "*in vivo*" lipid peroxidation induced by both enzymatic and non-enzymatic systems.[36–38] The results suggest that the antioxidant effect of D-003 is not associated with inhibition of Fe^{2+} regeneration through the P450 system. D-003 also inhibited lipid peroxidation induced by CCl_4,[39] suggesting that its antioxidant effects are not related to changes in the activity of hepatic NADPH CYT c reductase.

Phase I/Phase II liver enzymatic systems are major pathways of biotransformation or detoxification of most drugs, which involve the activities of one or more families. Thus, the assessment of the effects of a new substance on this system is relevant for predicting putative drug-to-drug interactions,[40] which are a cause of relevant adverse events or/and treatment failures. One of the mechanisms leading to pharmacokinetic drug-to-drug interactions is the disturbance of one or multiple hepatic enzymes of the CYT P-450 system. Thus, the results reported here indicate that the potential risk for pharmacokinetic drug-to-drug interactions between D-003 and concomitant drugs appears to be low.

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