Research Article

Toxicological Evaluation of the Combined Therapy of D-004 and Finasteride in Male Rats

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ABSTRACT

The first-line drug therapy for the treatment of benign prostatic hyperplasia includes antagonists of α1-adrenoceptors and inhibitors of 5α-reductase. Combined therapy provides the benefits of both therapeutic classes. D-004 is a lipid extract from the cuban royal palm tree (*Roystonea regia*) fruits, containing a mixture of fatty acids. This mixture has shown to prevent prostatic hyperplasia induced with testosterone and phenylephrine in rodents. Combined therapy with repeated doses of D-004 and finasteride induced additional benefits in preventing testosterone-induced prostate enlargement compared with respective monotherapies. This study investigated the toxicological potential of combined therapy with high doses of D-004 and finasteride orally administered for 28 days to male Sprague Dawley rats. Animals were randomized into four groups of six animals each one: a control group treated only with the vehicle and three groups treated with D-004 (2000 mg/kg), finasteride (50 mg/kg), and the combined therapy D-004 (2000 mg/kg) + finasteride (50 mg/kg), respectively. The variables analyzed were: mortality, clinical signs, body weight, food consumption, hematology and blood chemistry parameters, relative organ weight and histopathological findings. No clinical signs of toxicity were observed throughout the study. Bodyweight, food consumption, blood biochemical and hematological parameters were similar in control and treated groups. Treatment with finasteride and combined therapy significantly reduced the relative organ weight of accessory sex organs in animals treated. In conclusion, the combined therapy D-004 and finasteride orally administered to male rats did not induce toxicological effects except the atrophy of the accessory sex organs related to finasteride administration. So, these results do not limit that D-004 can be administered in conjunction with finasteride.

Keywords: D-004, *Roystonea regia,* finasteride, combined therapy, toxicity.

INTRODUCTION

Benign prostatic hyperplasia (BPH) is a common disease in old men (> 50 years old)[1] resulting from a non- malignant uncontrolled growth of the prostate gland. Consequently, affected men may experience moderate to severe lower urinary tract symptoms (LUTS) that greatly affect their quality of life.[2]

The etiology of BPH, involves hormonal and non-hormonal factors that occur in the aging man.[3] The increased conversion of testosterone (T) to dihydrotestosterone (DHT) in prostate cells mediated through 5-α reductase enzyme is the main hormonal factor linked with BPH[4] and the increased tone of prostate and bladder smooth muscle cells mediated via activation of α1-adrenoreceptors (ADR) is the main non-hormonal factor that contributes to BPH etiology.[5,6]

Drug therapy is very common in the treatment of BPH, with prostate 5α- reductase inhibitors (finasteride, dutasteride)[7,8] and α1- adrenoceptor antagonists (alfuzosin doxazosin, prazosin, terazosin, tamsulosin)[5,9] being the standard pharmacological treatments for this pathological condition.

Taking into account that these therapeutic classes act by different mechanisms, management also include the combination of 5α- reductase inhibitors and α1-adrenoceptor antagonists[10-12] In such regard, a randomized, double-blind, placebo-controlled trial of 3087 patients with BPH showed that the combined therapy is more effective to improve the symptoms than respective monotherapies.[10] As well, subsequent studies have demonstrated the efficacy of combined therapy for the treatment of LUTS in men.[11,12]

Concomitant treatment with these drugs increases efficacy, but also the potential to produce adverse effects.[12,13] The α1-adrenoreceptors blockers frequently induce orthostatic hypotension, dizziness, fatigue, ejaculatory disorders and iris muscle disorders.[14-16] Meanwhile, 5α-reductase inhibitors produced mainly disorders of male sexual function (decreased libido, erectile dysfunction and ejaculation disorders).[17,18]

Pre or postnatal exposure to 5α-reductase inhibitors has been shown to alter male reproductive development and sexual function. Decreased prostate and seminal vesicle weights as well as effects on testicular descent have been reported following *in utero* exposure to finasteride.[19,20] Besides, in male rats *in utero* exposure to finasteride, decreased anogenital distance, increased nipple retention and incidence of cleft prepuce and ectopic testes in early postnatal life.[21]

D-004, a lipid extract of the royal palm (*Roystonea regia*) fruits, also contains a mixture of fatty acids, wherein oleic, lauric, palmitic and myristic acids are the most abundant. *In vitro* studies have demonstrated that D-004 inhibits prostate 5α-reductase activity[22] and α1-ADR-mediated responses.[23] Oral administration of D-004 has

been shown to ameliorate testosterone (T)- and phenylephrine-induced prostate hyperplasia,[24-27] and to produce antioxidant effects in rodents.[28] D-004 has been effective to reduce plasma oxidative variables in healthy and BPH men,[29,30] and to decrease LUTS in BPH men in accordance to the significant reduction of the International Prostate Symptom Score (IPSS).[30,31]

Experimental toxicology studies of single or repeated oral doses (up to 2000 mg/kg) of D-004 in rodents did not demonstrate treatment related toxicity.[32-34] In particular, long-term (12 months) oral treatment with D-004 (800-2000 mg/kg) did not show evidences of D-004-related toxicity in rats, and the highest dose (2000 mg/kg) was a non-observable adverse effect level (NOAEL).[34] Genotoxicity studies did not reveal D-004-related cytotoxic or genotoxic potential,[35] and administered to 1000 mg/kg did not induce foetal or reproductive toxicity.[36] Likewise, clinical studies have demonstrated that D-004 is safe and well tolerated.[29-31]

A preclinical pharmacology study demonstrated that administration of D-004 (200 mg/kg) and finasteride (0.5 mg/kg) induced additional benefits in preventing T-induced prostate enlargement compared with each treatment administered alone.[37]

This study investigated the oral toxicological potential of combined therapy with D-004 (2000 mg/kg) + finasteride (50 mg/kg) administered for 28 days to male SD rats.

MATERIALS AND METHODS

Animals and housing conditions

Young adult (5 weeks) male Sprague Dawley (SD) rats (70-100 g) were purchased from the National Centre for Laboratory Animals Production (CENPALAB, Cuba). Rats were adapted for 7 days to the experimental conditions: controlled temperature (20-25 ^{o}C) and relative humidity (60 ± 10%) and 12-h light/dark cycles.

Rats were housed in plastic shoeboxes and bedding (processed hardwood chips) was changed and sterilized in autoclave. Free access to tap water and food (CENPALAB rodent chow) was allowed during the study.

At treatment completion, rats were fasted for 12 h prior to the sacrifice. Animals were handled in accordance to the Cuban Ethical Regulations for Animal Care and the Cuban Code of Good Laboratory Practices. Study protocol was approved by the Institutional Board of Animal Use. The study was periodically audited by the Quality Assurance Unit.

Test substance

The batch used in the study (Purity 90.9%), supplied by the Chemistry Department of the Centre of Natural Products (Havana, Cuba) and assessed with a validated gas chromatography method, had the following free fatty acid composition: caprylic (0.2%), capric (0.6%), lauric (22.5%), palmitic (12.1%), myristic (10.5%), palmitoleic (0.3%), stearic (2.6%) and oleic (42.1%) acids. Finasteride tablets (Merck Sharp (Dohme, Mexico) were used.

D-004 was emulsified in Tween-65/water vehicle (2%) and finasteride tablets were crushed and suspended in arabic gum/water vehicle (1%). Substances were prepared daily before the administration and concentrations were adjusted weekly according to bodyweight gain.

Rats were randomised into four groups of six animals each one: a control group treated only with the vehicle and three groups treated with D-004 (2000 mg/kg), finasteride (50 mg/kg), and the combined therapy D-004 (2000 mg/kg) + finasteride (50 mg/kg), respectively. Treatments were given by oral gavage (2 mL/kg), once daily (5 days per week) for 28 days.

Dose levels of D-004 (2000 mg/kg) and Finasteride (50 mg/kg) are 10 and 100 times higher, respectively, than the effective doses of D-004 (200 mg/kg) and Finasteride (0.5 mg/kg) used in the study of the effects of combined therapy on prostate hyperplasia (PH) induced with testosterone in rats.[37]

Clinical symptoms, haematology and blood biochemistry

Animals were observed daily (8:00–11:30 a.m.) during the whole study, including their aspect and overt behaviour, so that changes in the skin and fur, eyes and mucous membranes, faeces and motor activity and occurrence of salivation, lacrimation, tremors, convulsions, piloerection and stereotypes were registered. Observations were conducted up to the day prior to the sacrifice.

Body weight was determined at baseline (a day prior to starting the treatment) and then weekly during the whole study. Food consumption was assessed similarly.

Euthanasia under ether anaesthesia was performed to moribund animals and to those with appreciable body reduction (≥10%) or with clinical symptoms indicating risk of death. These animals were subjected to complete necropsy, in which all cavities and organs were carefully observed.

At study completion, survivors were isolated in individual cages, fasted for 12 h with free access to water, anesthetised under ether atmosphere and sacrificed by complete bleeding. Rats were randomized in similar groups for the daily sacrifice.

Blood was drawn from the abdominal aorta and samples were collected for serum biochemical and hematological determinations. Samples were placed at room temperature for 30 min and centrifuged at 3 000 rpm for 10 min. Samples were processed the same day of blood sampling.

Serum aliquots were taken to assess the values of glucose, urea, triglycerides, cholesterol, creatinine, total protein, alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatine phosphokinase (CPK), and alkaline phosphatase. Serum biochemical parameters were determined by using reagent kits (SPINREACT;

Crumlin Co., Antrim, UK), except blood acetylcholinesterase, determined according to Voss and Sacsse (1970).[38] In turn, hematological parameters (hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular haemoglobin concentration, and counts of red blood cells, platelets and white blood cells were determined in an hematological equipment (model KX-21N; SySMEX, Kobe, Japan).

Pathology

During the necropsy, the abdominal, thoracic and cranial cavities of all animals were examined. The liver, heart, spleen, lungs, thymus, kidneys, adrenal glands, testis, prostate, epididymis, seminal vesicles (with coagulating glands and seminal fluid), levator-ani-bulbocavernosous (LABC) muscle, and bulbourethral glands were weighed (Sartorius Universal Scale, Goettingen, Germany). The organ to body weight ratios were determined and expressed as percent.[39] Samples of the organs mentioned above and other such pituitary, thyroid and mammary glands were preserved in 10% buffered formaldehyde.[40]

Aliquot of testis (0.5 g) were taken and gently homogenized with 150 mM of buffer tris (pH 7.4) in an ice-cold bath, with an Ultra-Turrax homogenizer for determining biochemical parameters (ALT, AST, alkaline and acid phosphatases and total protein). The homogenates were stored at -20 ^{o}C until use.

Samples from all animals with macroscopic lesions, and from the control and highest dose groups, were taken and embedded in paraffin, sectioned with a rotary microtome (Leitz microtome, Wetzlar, Germany), stained with haematoxylin and eosin, and examined by light microscopy. A Zeiss Primo Start microscope (Zeiss Optical Co., Ltd. Tokyo, Japan) was used for these observations.

Statistical analysis

Data were analysed following the recommendations for toxicological studies.[41, 42] Continuous data (bodyweight, blood parameters, food consumption and organ weight percentage) were analysed with analysis of variance (ANOVA) and categorical data (mortality, histological lesions) with the Fisher's Exact Probability test. An alpha value of 0.05 was a priori established. Tests were two tailed and the statistical analyses were performed independently by sex using the STATISTIC data analysis software (StatSoft, Inc. 2003; Tulsa, OK, USA, version 6. (www.statsoft.com).

RESULTS AND DISCUSSION

No clinical signs of toxicity were observed throughout the study. No differences on body weight were found between animals from treated and control groups (Table 1). Likewise, analysis of food consumption did not show significant differences (data not shown for simplicity).

No significant differences or trends on haematological variables (Table 2) or biochemical parameters on serum (Table 3) and testes homogenate (Table 4) were found between control and treated groups.

Organ to bodyweight ratios showed atrophy of different structures of the reproductive system and sexual accessory organs in animals treated with finasteride, consistent with the significant reduction in the relative weight of the prostate and ventral prostate, seminal vesicle-coagulating gland and the bulbourethral glands of these groups with respect to the control group.

In contrast, no significant differences of organ to bodyweight ratios between treated with D-004 and control groups were found (Table 5).

At necropsy, no macroscopic changes were observed in the cavities and organs in animals treated with D-004, which was consistent with histopathological analysis.

On the other hand, the microscopic analysis of animals treated only with finasteride showed that five of six animals (83.3%) had a decrease (shrink) of tissue, which it is more obvious in prostate, seminal vesicles and bulbourethral glands. A similar situation occurred in the group treated with the combined therapy. This analysis also revealed that four animals [three treated with finasteride (50%) and one with combined therapy] showed a reduction of sperm in the epididymis, which also exhibited a decreased sperm counting and thickness of the germinal epithelium of the testes.

The results of this study related to monotherapies were as expected. However, there is no history of the combined therapy (D-004 + finasteride) at high doses.

A preclinical pharmacology study demonstrated that a combined therapy with minimal effective doses of both D-004 (200 mg/kg) and finasteride (0.5 mg/kg) induced benefits with respect the same doses of both drugs on T induced PH in rats, since the inhibition induced with such combined therapy (63.7%) was nearly the double than that induced with D-004 (32.9%) or finasteride (30.6%) alone, in an additive, rather than a synergic manner.[37]

During 28 days of administration of D-004 + finasteride to male SD rats, no evidences of treatment related toxicity were found, in accordance to daily observations, food consumption, weight gain, biochemical and haematological indicators. Besides, individual values of tested parameters were within normal limits.[34,40,43,44]

Different from finasteride,[21,45] D-004 did not affect the relative weight of accessory sex organs,[33] which are very vulnerables to dramatic changes in the levels of androgens in newborn and young rats. On the contrary, combined therapy showed a similar effect on accessory sex organs as described for finasteride, so D-004 did not change the effect of finasteride. Thus, the lack of effect of D-004 on the relative organ weight, is consistent with the data of the previous results of toxicological studies in this species.[32,34,36]

Tissue shrinkage of organs of animals treated with finasteride is consistent with observations of other

authors, who attributed this effect to the antiandrogenic action of finasteride.[45,46] These findings also were observed in animals treated with the combined therapy.

Several studies have proposed that one of the earliest changes leading to ventral prostate shrinking following finasteride treatment is a decrease of prostate vascularization.[45,47] Reduction in the weight of prostatic and seminal vesicles in the finasteride-treated groups could also be attributed to a decrease in DHT since homeostasis of these organs are partially dependent on DHT.[46]

Table 1: Effects of treatments on the rat bodyweight values (g) (mean ± SD)

Treatment (mg/kg)	Baseline	Day 7	Day 14	Day 21	Day 28
Control	89,7 ± 4,63	120,0 ± 14,21	129,8 ± 18,17	136,8 ± 16,70	143,0 ± 17,84
D-004 (2000)	89,7 ± 5,13	121,7 ± 12,91	132,3 ± 17,93	140,5 ± 23,16	149,7 ± 27,27
FIN (50)	89,7 ± 4,68	124,2 ± 9,77	133,7 ± 10,65	142,5 ± 10,60	143,2 ± 13,38
D-004 +FIN	89,7 ± 5,57	125,7 ± 12,82	144,5 ± 19,19	146,3 ± 20,80	153,5 ± 19,95

SD, standard deviation; FIN, finasteride

Table 2: Effects of treatments on the rat hematological parameters (mean ± SD)

Treatment (mg/kg)	Hemoglobin (g/dL)	Hematocrit (%)	Platelet count (x 10^9/L)	RBC x 10^{12}/L
Control	16,04 ± 2,28	48,56 ± 5,96	840,20 ± 120,09	8,17 ± 0,79
D-004 (2000)	13,75 ± 0,96	41,88 ± 2,23	742,50 ± 191,99	7,08 ± 0,43
FIN (50)	14,30 ± 1,81	44,33 ± 5,40	711,00 ± 223,33	7,62 ± 0,92
D-004 + FIN	14,20 ± 1,59	43,17 ± 3,40	876,00 ± 117,99	7,31 ± 0,61
	MCV (fl)	**MCH (pg)**	**MCHC (g/L)**	**WBC (x 10^9/L)**
Control	59,34 ± 2,08	19,58 ± 1,12	32,98 ± 0,85	5,62 ± 1,40
D-004 (2000)	59,25 ± 1,94	19,45 ± 1,14	32,80 ± 1,22	6,02 ± 1,61
FIN (50)	58,17 ± 0,24	18,78 ± 0,69	32,25 ± 1,14	4,67 ± 1,98
D-004 + FIN	59,10 ± 1,09	19,40 ± 0,75	32,83 ± 1,15	5,88 ± 1,54
	Lymphocytes (%)	**Neutrophils (%)**		
Control	78,12 ± 3,69	21,88 ± 3,69		
D-004 (2000)	81,72 ± 1,10	18,28 ± 1,10		
FIN (50)	76,48 ± 4,82	23,52 ± 4,82		
D-004 + FIN	77,50 ± 8,70	22,50 ± 8,70		

SD, standard deviation; FIN, finasteride; WBC, white blood cell count; RBC, red blood cell count; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration

Table 3: Effects of treatments on the rat blood biochemical parameters (mean ± SD).

Treatment (mg/kg)	Glucose (mmol/L)	Creatinine (mol/L)	Triglycerides (mmol/L)	Cholesterol (mmol/L)
Control	5,03 ± 1,11	52,74 ± 6,06	0,84 ± 0,39	1,01 ± 0,12
D-004 (2000)	5,17 ± 0,88	47,29 ± 5,24	0,68 ± 0,20	0,88 ± 0,21
FIN (50)	4,58 ± 0,68	48,92 ± 7,02	0,80 ± 0,23	0,81 ± 0,09
D-004 +FIN	4,84 ± 1,78	48,03 ± 4,53	0,53 ± 0,15	0,87 ± 0,12
	AP (U/L)	**CPK (U/L)**	**ALT (U/L)**	**AST (U/L)**
Control	537,00 ± 159,81	201,50 ± 79,38	50,50 ± 18,06	223,67 ± 68,16
D-004 (2000)	472,50 ± 79,19	300,00 ± 56,00	45,17 ± 7,03	193,67 ± 54,47
FIN (50)	424,83 ± 117,57	204,33 ± 56,34	51,67 ± 5,61	225,83 ± 0,36
D-004 +FIN	386,83 ± 96,05	219,67 ± 76,90	42,67 ± 5,01	233,67 ± 55,06
	AChE (µmol/L)	**Total protein (g/dL)**	**Urea (mmol/L)**	
Control	0,45 ± 0,02	5,77 ± 0,56	5,87 ± 0,91	
D-004 (2000)	0,45 ± 0,02	5,60 ± 0,47	4,89 ± 1,22	
FIN (50)	0,44 ± 0,05	5,53 ± 0,37	5,24 ± 1,31	
D-004 +FIN	0,46 ± 0,04	5,63 ± 0,53	4,71 ± 0,80	

SD, standard deviation; FIN, finasteride; AChE, acetylcholinesterase; CPK, creatine phosphokinase; ALT, alanine aminotransferase; AST, aspartate aminotransferase, AP, alkaline phosphatase.

Table 4: Effects of treatments on the rat biochemical parameters in testis homogenate (mean ± SD).

Treatment (mg/kg)	Total protein (g/dL)	ALT (U/ L)	AST (U/ L)	AP (U/L)	Acid phosphatase (U/L)
Control	64,34 ± 10,71	3,70 ± 0,94	5,96 ± 2,08	4,91 ± 1,40	4,89 ± 0,97
D-004 (2000)	54,91 ± 16,40	3,57 ± 1,07	5,49 ± 2,52	5,19 ± 2,26	5,73 ± 1,52
FIN (50)	71,18 ± 13,12	3,53 ± 0,68	5,07 ± 1,77	4,28 ± 0,81	4,63 ± 0,79
D-004 +FIN	71,99 ± 19,98	3,21 ± 1,34	4,84 ± 1,37	3,82 ± 0,99	4,49 ± 0,85

SD, standard deviation; FIN, finasteride; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AP, alkaline phosphatase.

Table 5: Effects of treatments on the rat relative organ weights[a] (mean ± SD).

Organ (g)	Control	D-004 (2000 mg/kg)	Finasteride (50 mg/kg)	D-004 + FIN
Thymus	0,14 ± 0,02	0,14 ± 0,05	0,15 ± 0,03	0,16 ± 0,04
Heart	0,33 ± 0,02	0,34 ± 0,02	0,36 ± 0,04	0,34 ± 0,03
Lungs	0,51 ± 0,06	0,53 ± 0,04	0,46 ± 0,04	0,51 ± 0,02
Spleen	0,23 ± 0,02	0,21 ± 0,03	0,21 ± 0,03	0,20 ± 0,02
Liver	3,84 ± 0,62	3,87 ± 0,83	3,92 ± 0,75	3,43 ± 0,63
Right kidney	0,40 ± 0,03	0,39 ± 0,03	0,38 ± 0,02	0,40 ± 0,02
Left kidney	0,40 ± 0,03	0,38 ± 0,03	0,39 ± 0,03	0,37 ± 0,02
Right adrenal	0,009 ± 0,003	0,009 ± 0,002	0,009 ± 0,001	0,009 ± 0,002
Left adrenal	0,010 ± 0,002	0,009 ± 0,001	0,009 ± 0,002	0,008 ± 0,003
Prostate	0,15 ± 0,02	0,12 ± 0,03	0,07 ± 0,02** [d]	0,06 ± 0,03** [d]
Ventral prostate	0,10 ± 0,02	0,07 ± 0,02	0,04 ± 0,02** [c]	0,03 ± 0,02** [d]
Seminal V. and CG	0,19 ± 0,03	0,15 ± 0,04	0,06 ± 0,01** [e]	0,06 ± 0,03** [e]
Right testis	0,83 ± 0,07	0,78 ± 0,08	0,79 ± 0,05	0,71 ± 0,14
Left testis	0,81 ± 0,09	0,78 ± 0,08	0,77 ± 0,09	0,72 ± 0,15
Right epididymis	0,14 ± 0,04	0,13 ± 0,02	0,11 ± 0,03	0,11 ± 0,05
Left epididymis	0,14 ± 0,04	0,13 ± 0,03	0,10 ± 0,03	0,11 ± 0,05
LABC muscle	0,17 ± 0,01	0,16 ± 0,05	0,13 ± 0,03	0,13 ± 0,06
Bulbourethral glands[b]	0,022 ± 0,007	0,018 ± 0,005	0,009 ± 0,002* [c]	0,009 ± 0,006* [c]

SD, standard deviation; FIN, finasteride; Seminal V., seminal vesicles; CG, coagulating gland; LABC, levator ani bulbocavernosous.; [a]Expressed as organ-to-body-weight percentage ratio.; [b]Paired weights.; *p < 0.01, **p < .001 compared with the control group by analysis of variance.; [c]p< 0,05,[d]p< 0,01, [e]p< 0,001 compared with the D-004 group by analys00 of variance.

CONCLUSION

Combined therapy D-004 (2000 mg/kg) and finasteride (50 mg/kg) orally administered to male rats did not induce toxicological effects except the atrophy of the accessory sex organs related to finasteride administration. So, these results do not limit that D-004 can be administered in conjunction with finasteride.

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