Original Article

E-PodoFavalin-15999 (Atremorine®)-Induced Neurotransmitter and Hormonal Response in Parkinson's Disease

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Abstract

E-PodoFavalin-15999 (Atremorine®) is a novel bioproduct obtained by means of non-denaturing biotechnological procedures from structural components of the *Vicia faba* L. plant, for the prevention and treatment of Parkinsonian disorders. Preclinical studies have revealed that Atremorine is a powerful neuroprotectant with specific activity on dopaminergic neurons, reversing neurodegeneration and improving motor function in animal models of Parkinson's disease (PD). Clinical studies indicate that Atremorine is a powerful catecholaminergic enhancer with time- and genotype-dependent effects in PD. In the present study, we investigated the effects of Atremorine on the levels of neurotransmitters (dopamine, adrenaline, noradrenaline, serotonin) and hormones (adrenocorticotropic hormone (ACTH), growth hormone (GH), prolactin (PRL), follicle-stimulating hormone (FSH), luteinizing hormone (LH), cortisol, estrogens, testosterone) in Parkinsonian patients (n=119) 1 hour after Atremorine administration (5 g/day). These effects were also studied after stratification of PD patients according to their cardiovascular function. Atremorine induced a significant increase in the levels of dopamine (p<0.001), noradrenaline (p=0.004) and adrenaline (p=0.01), and a significant decrease in the levels of PRL (p<0.001), cortisol (p<0.001), and GH (p=0.002), with no apparent changes in the levels of serotonin, ACTH, FSH, LH, testosterone or estrogen. The levels of dopamine were significantly higher in patients with normal EKG than in patients with abnormal electrocardiogram (EKG); however, the levels of adrenaline, noradrenaline and serotonin tended to be lower in patients with normal EKG as compared to patients with abnormal EKG. There were also some differences in hormonal levels in patients with normal EKG, compared to abnormal EKG. These results clearly show that Atremorine is an effective enhancer of catecholaminergic neurotransmission, which contributes to optimization of hormonal regulation in PD.

Keywords: Atremorine; Dopamine; Adrenaline; Noradrenaline; Serotonin; Growth hormone; Prolactin; ACTH; Cortisol; Parkinson's disease.

Abbreviations: 5HT, serotonin; ACTH, adrenocorticotropic hormone; AD, adrenaline; COR, cortisol; DA, dopamine; EKG, electrocardiogram; FSH, follicle stimulating hormone; GH, growth hormone; L-DOPA, L-3,4-dihydroxyphenylalanine [(2-amino-3-(3,4-dihydroxyphenyl) propanoic acid); LH, luteinizing hormone; NA, noradrenaline; PRL, prolactin; T4, thyroxine; TSH, thyroid stimulating hormone.

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Introduction

Alzheimer's disease and Parkinson's disease (PD) are the most important neurodegenerative disorders afflicting today's elderly population. PD, in particular, shows a prevalence that ranges from 35.8 per 100,000 to 12,500 per 100,000, with an annual incidence ranging from 1.5 per 100,000 to 346 per 100,000 in different countries.[1–7] The prevalence of PD gradually increases in parallel with age, following an age-dependent trend (41 per 100,000 at 40–49 years; 107 at 50–59 years; 173 at 55–64 years; 428 at 60–69 years; 425 at 65–74 years; 1,087 at 70–79 years; and 1,903 per 100,000 at older than age 80).[6] PD is more prevalent in males (1,729 per 100,000, >65 yrs) than in females (1,644 per 100,000), with a peak prevalence in the age group of ≥90 years (4,633 cases per 100,000) and a mean prevalence of 1,680 per 100,000 in people >65 years of age.[7,8] The geographic distribution of PD is also characteristic, with a prevalence of 1,601 per 100,000 in North America, Europe and Australia, and a prevalence of 646 per 100,000 in Asia.[6]

The clinical onset of PD, characterized by resting tremor, rigidity and bradykinesia, is likely to occur several decades after the beginning of the progressive neurodegenerative process, which predominantly affects dopaminergic neurons in the substantia nigra pars compacta, and is accompanied by widespread involvement of other CNS structures and peripheral tissues.[9] PD is a form of multi-systemic α-synucleinopathy, with Lewy bodies deposits in the midbrain. Descriptive phenomena to explain, at least in part, this neuropathological phenotype include the following: (i) genomic factors, (ii) epigenetic changes, (iii) toxic factors, (iv) oxidative stress anomalies, (v) neuroimmune/neuroinflammatory reactions, (vi) hypoxic-ischemic conditions, and (vii) ubiquitin-proteasome system dysfunction; all these conditions lead to protein misfolding and aggregation and premature neuronal death.[10–17] Recent evidence also suggests that PD might be a prion-like disease.[12] Additionally, the catecholaldehyde hypothesis postulates an "autotoxicity"-inherent cytotoxicity of catecholamines and their metabolites in neurons, in which a long-term increase in 3,4-dihydroxyphenylacetaldehyde (DOPAL, the catecholaldehyde metabolite of dopamine) oligomerizes and aggregates alpha-synuclein, accelerating the eventual death of dopaminergic neurons.[18]

Classical therapeutic interventions for the symptomatic treatment of motor symptoms in PD include pharmacotherapy, deep brain stimulation, and physiotherapy.[19] L-3,4-dihydroxyphenylalanine [(2-amino-3-(3,4-dihydroxyphenyl) propanoic acid] (L-DOPA) has been the most representative treatment for PD since the 1960s.[20,21] In addition to L-DOPA, as a dopamine precursor,

other symptomatic treatments for PD include dopamine agonists, monoamine oxidase (MAO) inhibitors, and catechol-O-methyltransferase (COMT) inhibitors.[22] A common complication of L-DOPA and dopamine agonists, after long periods of treatment, is the "wearing-off" phenomenon[23,24], with motor fluctuations and dyskinesia.[20,25] Polypharmacy in PD and the co-administration of psychotropic drugs and/or any other drug category with potential interaction with dopaminergic neurotransmission may also contribute to severe complications.[26] It is also common to find gastrointestinal[27], cardiovascular[28], hormonal and neuropsychiatric problems associated with the chronic administration of conventional antiparkinsonian drugs.[22,27]

The present understanding of PD suggests the necessity for a change in the paradigm of PD management, including the following: (i) characterization of pathogenic factors (either genomic or environmental) and reliable biomarkers for early intervention in the population at risk, (ii) accelerating the search for novel modalities of therapeutic intervention devoid of side effects and/or delayed deleterious consequences for the quality of life and well-being of PD patients, and (iii) optimization of the present therapeutic resources in order to optimize pharmacological treatments by means of a personalized, pharmacogenetic approach.[10]

We have developed a research program in search of new preventive and/or therapeutic options for PD. The first bioproduct of this series is E-PodoFavalin-15999 (Atremorine®).[29] Atremorine is a novel biopharmaceutical compound, obtained by means of non-denaturing biotechnological procedures from structural components of the *Vicia faba* L. plant, for the prevention and treatment of PD.[29] Preclinical studies revealed that Atremorine is a powerful neuroprotectant in (i) cell cultures of human neuroblastoma SH-SY5Y cells, (ii) hippocampal slices under conditions of oxygen and glucose deprivation, and (iii) striatal slices under conditions of 6-hydroxydopamine (OHDA)-induced neurotoxicity.

In vivo studies have shown that Atremorine (i) protects against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced dopaminergic neurodegeneration, (ii) inhibits MPTP-induced microglia activation and neurotoxicity in substantia nigra, and (iii) improves motor function in mice with MPTP-induced neurodegeneration.[29,30] Clinical studies of untreated patients who received Atremorine for the first time (treatment-naïve for antiparkinsonian drugs) revealed that Atremorine can enhance dopaminergic neurotransmission and increase plasma dopamine levels by 200- to 500-fold. In patients who have been chronically treated with L-DOPA or other antiparkinsonian drugs, Atremorine was found to induce a dopamine response of similar magnitude to that observed in previously untreated patients. Moreover, this dopaminergic response was shown to be associated with the pharmacogenetic profile of the patients.[29,31]

In this paper we report, for the first time, the influence of Atremorine on different neurotransmitters (dopamine, noradrenaline, adrenaline, serotonin (5HT)), pituitary hormones (adrenocorticotropic hormone (ACTH), growth hormone (GH), prolactin (PRL), luteinizing hormone (LH), follicle-stimulating hormone (FSH)) and peripheral hormones (cortisol (COR), testosterone, estrogen) in PD patients.

Material and methods

Patients and treatment

Patients (n=119; age: 61.11±1.54 yrs) of both sexes (58 females,

Table 1. Sample features of patients with Parkinson's disease

Parameter	Total
N	119
Females	58
Males	61
Age (years)	61.11±1.54
Females	59.74±2.21
Males	62.42±3.16
Systolic blood pressure (mm Hg)	138.02±2.11
Diastolic blood pressure (mm Hg)	76.78±0.89
Pulse (bpm)	72.11±1.14
Weight (Kg)	68.89±1.24
Height (m)	1.62±0.008
BMI (Kg/m^2)	26.12±0.44
Glucose (mg/dL)	101.34±2.13
Cholesterol (mg/dL)	193.83±3.54
HDL-Cholesterol (mg/dL)	58.97±1.28
LDL-Cholesterol (mg/dL)	114.87±3.18
Triglycerides (mg/dL)	99.94±4.63
Urea (mg/dL)	41.84±1.29
Creatinine (mg/dL)	0.88±0.02
Uric acid (mg/dL)	4.51±0.12
Total Protein (g/dL)	6.99±0.04
Albumin (g/dL)	3.99±0.08
Calcium (mg/dL)	9.58±0.48
Phosphorus (mg/dL)	3.48±0.10
GOT/ASAT (IU/L)	21.19±1.06
GPT/ALAT (IU/L)	23.36±1.77
GGT (IU/L)	23.32±1.76
Alkaline phosphatase (IU/L)	80.69±7.54
Bilirubin (mg/dL)	0.67±0.10
CPK (IU/L)	277.31±186.67
LDH (IU/L)	289.21±25.84
Na^+ (mEq/L)	140.35±0.34
K^+ (mEq/L)	4.24±0.02
Cl^- (mEq/L)	102.80±0.54
Fe^{2+} (µg/dL)	78.86±2.60
Ferritin (ng/mL)	150.78±12.82
Folate (ng/mL)	17.76±0.63
Vitamin B_{12} (pg/mL)	715.27±35.97
TSH (µIU/mL)	1.80±0.12
T4 (ng/mL)	0.91±0.01
RBC (×10^6/µL)	4.58±0.04
HCT (%)	41.94±0.37
Hb (g/dL)	14.02±0.16

Table 1. Sample features of patients with Parkinson's disease - *(continued)*

Parameter	Total
VCM (fL)	91.96±0.38
HCM (pg)	30.91±0.14
CHCM (g/dL)	33.69±0.06
ADE (RDW)(%)	12.91±0.09
WBC ($\times 10^3/\mu L$)	6.57±0.17
%Neu	45.62±2.15
%Lin	32.04±0.78
%Mon	7.40±0.13
%Eos	2.80±0.12
%Bas	0.85±0.08
Platelets ($\times 10^3/\mu L$)	211.27±5.31
VPM (fL)	8.81±0.07
MMSE Score	24.35±0.76
ADAS-Cog-T	15.05±0.97
ADAS-NonCog	5.19±0.38
ADAS-T	20.26±1.20
Hamilton-A	11.31±0.45
Hamilton-D	10.94±0.43
GDS	2.77±0.10
UPDRS	47.71±5.06
Hoehn and Yahr Staging	1.90±0.22
Schwab and England ADL Scale	73.20±4.75

Data: mean ± standard error.

age: 59.74±2.21; 61 males, age: 62.42±3.16 yrs) with PD were recruited for this study. All patients underwent, under informed consent, the following protocol: (i) clinical (neurologic, psychiatric) examination, (ii) blood and urine analyses (Table 1), (iii) neuropsychological assessment [Mini-Mental State Examination (MMSE), Alzheimer's Disease Assessment Scale (ADAS), Hamilton-A/D, Geriatric Depression Scale (GDS), Unified Parkinson Disease Rating Scale (UPDRS), Hoehn and Yahr staging, Schwab and England 'activities of daily living' (ADL) scale] (Table 1), (iv) cardiovascular evaluation (EKG), (v) structural neuroimaging (brain magnetic resonance imaging), (vi) functional neuroimaging (brain mapping, brain optical topography), (vii) genetic assessment [apolipoprotein E (APOE)], and (viii) pharmacogenetic profiling (CYP2D6, CYP2C19, CYP2C9, CYP3A4/5).

All patients received a single oral dose of 5 g of E-PodoFavalin-15999 (Atremorine®) in the morning, to avoid circadian variations in biochemical and hormonal parameters, and blood samples were obtained prior to Atremorine intake and 60 minutes later.

Analytical methods

Venous blood samples were taken after overnight fasting, with patients in supine position. Blood for the analysis of serum total testosterone, FSH, LH, PRL, COR, GH, estradiol (E2), 5HT and thyroid hormones [thyroid-stimulating hormone (TSH) and free-thyroxine (FT4)] was collected in BD Vacutainer serum separation tubes, while blood for analysis of plasma catecholamine (noradrenaline, adrenaline and dopamine) and ACTH was collected in EDTA-containing tubes. Specimens for catecholamine and ACTH analyses were immediately placed on ice and centrifuged at 3000 rpm, at 4°C, for 10 minutes, soon after venipuncture.[32] Serum tubes were allowed to clot at room temperature for 30 minutes before processing, and centrifuged within 60 minutes of sampling under the same conditions as the EDTA tubes. After refrigerated centrifugation, serum and plasma were removed from blood cells[33] and placed in a suitable sample container.

Serum levels of testosterone, FSH, LH, PRL, COR, GH and E2 and plasma levels of ACTH were measured by electrochemiluminescence on the same day of venipuncture, using the Immulite System (Siemens, Malvern, PA, USA). The Immulite 1000 is an automated, random-access immunoassay analyzer with a solid-phase washing process and a chemiluminescent detection system.[34–37] Access2 Immunoassay System (Beckman Coulter, Brea, CA, USA), an automated system with chemiluminescent signal, was used to analyze serum concentrations of ultrasensitive TSH (us-TSH; 3rd generation) and FT4.[38] Plasma aliquots for fractionated catecholamine determinations were stored at −20°C (1) and purified with albumin until their analysis by High Performance Liquid Chromatography (HPLC) with electrochemical detection.[39,40] The HPLC system consisted of a pump (515; Waters Corp., Milford, MA, USA), an autosampler (717; Waters Corp.), a chromatographic column (Resolve C18; Waters Corp.), an electrochemical detector (2465; Waters Corp.), and the Empower2 chromatography data software (Waters Corp.). Serum aliquots for serotonin analysis were stored at −20°C until measurement by a commercial ultrasensitive ELISA kit (Labor Diagnostika Nord, Nordhorn, Germany) after quantitative acylation[41]; the ELISA was performed according to the manufacturer's instructions and each reaction was monitored at 450 nm using the Sunrise Microplate Absorbance Reader (Tecan, Grödig, Austria).

Statistical analysis

Data were analyzed by using IBM SPSS Statistics 20 and SigmaPlot 10.0 software. Comparisons between groups were studied by *t*-test, Mann-Whitney rank sum test, chi-square test without Yates correction and Fisher's exact test, and Pearson's correlation analysis (nonlinear regression, Durbin-Watson statistic, normality test, constant variance test, 95% confidence). All values are expressed as mean ± SE, and the degree of significance was considered to have been met when $p<0.05$.

Results

Neurotransmitters

One hour after Atremorine administration, basal dopamine levels increased from 762.28±296.94 pg/mL (range: 8–30,318 pg/mL) to 4,556.51±678.95 (range: 54–40,603 pg/mL) ($p<0.001$) (Table 2; Fig. 1), with a similar response in females and males ($p<0.001$) (Table 2). A significant increase was observed in noradrenaline (286.52±21.88 pg/mL *vs.* 360.72±22.54 pg/mL) ($p=0.004$) (Table 2; Fig. 2) and adrenaline (21.83±1.31 pg/mL *vs.* 30.69±3.70) ($p=0.01$) (Table 2; Fig. 3), with no significant changes in serotonin (Table 2; Fig. 4). The noradrenaline response to Atremorine was similar in females ($p=0.05$) and males ($p=0.01$); however, although adrenaline responded to Atremorine in both females and

Table 2. Atremorine-induced neurotransmitter and hormonal changes

Parameter	Cases	Basal levels	Atremorine	p
Noradrenaline (pg/mL)	T	286.52±21.88	360.72±22.54	0.004 A *vs* B
	F	281.19±26.75	371.70±31.15	0.01 A *vs* B; 0.91 BF *vs* BM
	M	295.50±34.48	350.27±32.70	0.05 A *vs* B; 0.48 AF *vs* AM
Adrenaline (pg/mL)	T	21.83±1.31	30.69±3.70	0.01 A *vs* B
	F	18.62±0.89	28.93±6.97	0.22 A *vs* B; 0.04 BF *vs* BM
	M	24.88±2.35	32.37±2.95	0.03 A *vs* B; 0.008 AF *vs* AM
Dopamine (pg/mL)	T	762.28±296.94	4556.51±678.95	<0.001 A *vs* B
	F	232.05±107.33	3431.03±676.71	<0.001 A *vs* B; 0.03 BF *vs* BM
	M	1266.44±564.98	5626.83±1147.08	<0.001 A *vs* B; 0.26 AF *vs* AM
Serotonin (ng/mL)	T	171.71±14.43	171.31±14.22	0.92 A *vs* B
	F	158.10±18.07	157.84±19.64	0.98 A *vs* B; 0.58 BF *vs* BM
	M	182.10±22.29	184.13±20.55	0.77 A *vs* B; 0.32 AF *vs* AM
Prolactin (ng/mL)	T	11.94±1.76	7.32±1.38	<0.001 A *vs* B
	F	12.89±2.27	7.60±1.74	<0.001 A *vs* B; 0.01 BF *vs* BM
	M	11.08±2.67	7.20±2.12	0.003 A *vs* B; 0.19 AF *vs* AM
Cortisol (µg/dL)	T	14.47±0.54	10.64±0.46	<0.001 A *vs* B
	F	15.29±0.96	10.40±0.71	<0.001 A *vs* B; 0.29 BF *vs* BM
	M	13.73±0.54	10.35±0.62	<0.001 A *vs* B;<0.001 AF *vs* AM
ACTH (pg/mL)	T	31.29±4.12	29.69±3.95	0.77 A *vs* B
	F	35.10±8.21	31.92±7.49	0.54 A *vs* B; 0.67 BF *vs* BM
	M	28.35±2.93	27.71±3.48	0.99 A *vs* B; 0.35 AF *vs* AM
GH (ng/mL)	T	1.24±0.20	0.43±0.08	0.002 A *vs* B
	F	1.72±0.35	0.52±0.12	0.003 A *vs* B; 0.003 BF *vs* BM
	M	0.80±0.20	0.38±0.13	0.16 A *vs* B; 0.05 AF *vs* AM
FSH (mIU/mL)	T	30.69±3.50	29.62±3.57	0.89 A *vs* B
	F	54.00±5.52	52.36±5.84	0.80 A *vs* B; <0.001 BF *vs* BM
	M	9.58±1.38	9.56±1.40	0.88 A *vs* B; <0.001 AF *vs* AM
LH (mIU/mL)	T	14.78±1.61	13.63±1.46	0.68 A *vs* B
	F	24.52±2.71	22.05±2.48	0.54 A *vs* B; <0.001 BF *vs* BM
	M	5.96±0.65	6.21±0.74	0.90 A *vs* B; <0.001 AF *vs* AM
Testosterone (ng/dL)	M	379.79±24.54	331.18±20.68	0.13 A *vs* B
Estrogen (pg/mL)	F	59.23±12.44	58.27±11.83	0.69 A *vs* B

Data: mean ± standard error.
Abbreviations: A: atremorine (60 minutes after treatment with 5g Atremorine, p.o.); AF: Atremorine-females; AM: Atremorine-males; B: basal levels (prior to treatment with Atremorine); BF: basal-females; BM: basal-males; F: females; M: males; T: total sample.

males, this response was only significant in males (p=0.05) (Table 2).

Hormones

Atremorine induced a significant decrease in the levels of prolactin (11.94±1.76 ng/mL *vs.* 7.32±1.38 ng/mL) (p<0.001) (Table 2; Fig. 5), GH (1.24±0.20 *vs.* 0.43±0.08 ng/mL) (p=0.002) (Table 2; Fig. 6) and COR (14.47±0.54 *vs.* 10.64±0.46 µg/mL) (p<0.0001) (Table 2; Fig. 7). No significant changes were observed in ACTH, FSH, LH, testosterone and estrogen levels in response to Atremorine (Table 2). Basal levels of PRL were higher in females than in males (p=0.05), and both sexes responded significantly to Atremorine in a similar manner (Table 2). GH levels were found to be higher in females than in males (p=0.003). In addition, females exhibited a significant response to Atremorine (p=0.003), while the response in males was poorer (Table 2). Basal COR levels were similar in females and males, and patients of both sexes responded to Atremorine in an identical manner (p<0.001) (Table 2). As expected, both FSH and LH levels were significantly higher in females than in males (p<0.001), with no apparent response to

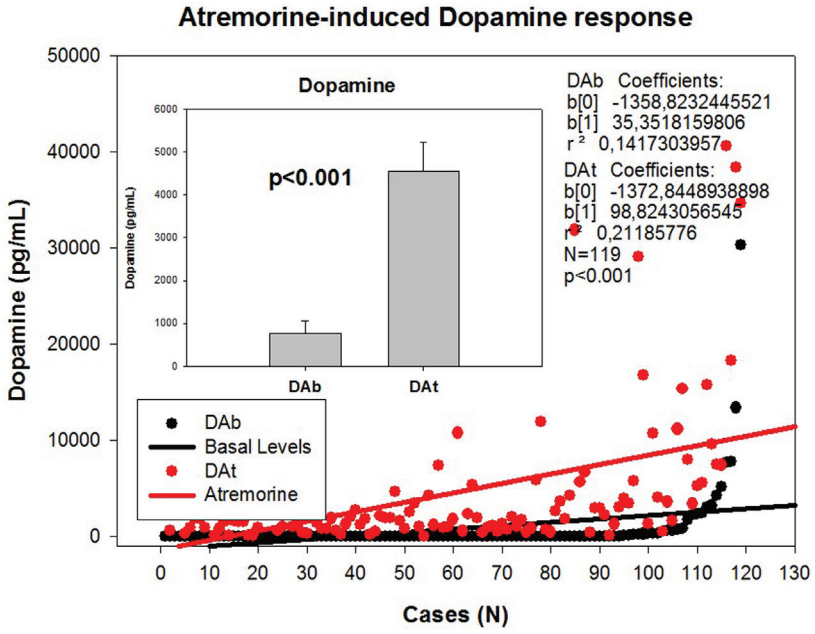

Fig. 1. Atremorine-induced dopamine (DA) response in patients with Parkinsonian disorders. DAb: Basal dopamine levels; DAt: Plasma dopamine levels at 1 hour after Atremorine administration (5 g, p.o.).

Atremorine in either of the sexes (Table 2).

EKG-related Atremorine-induced changes in neurotransmitters and hormones

Important differences were identified in the peripheral concentration of some neurotransmitters and hormones in PD patients according to their cardiovascular condition, as assessed by EKG recording (Table 3). Basal dopamine levels were found to be significantly higher in patients with a normal EKG as compared with those patients with an abnormal EKG (p=0.01). However, the dopamine response to Atremorine was significantly higher in the three conditions (normal EKG, borderline EKG, abnormal EKG) as compared with their respective basal levels (p<0.001) (Table 3). In contrast, basal noradrenaline levels were lower in cases with

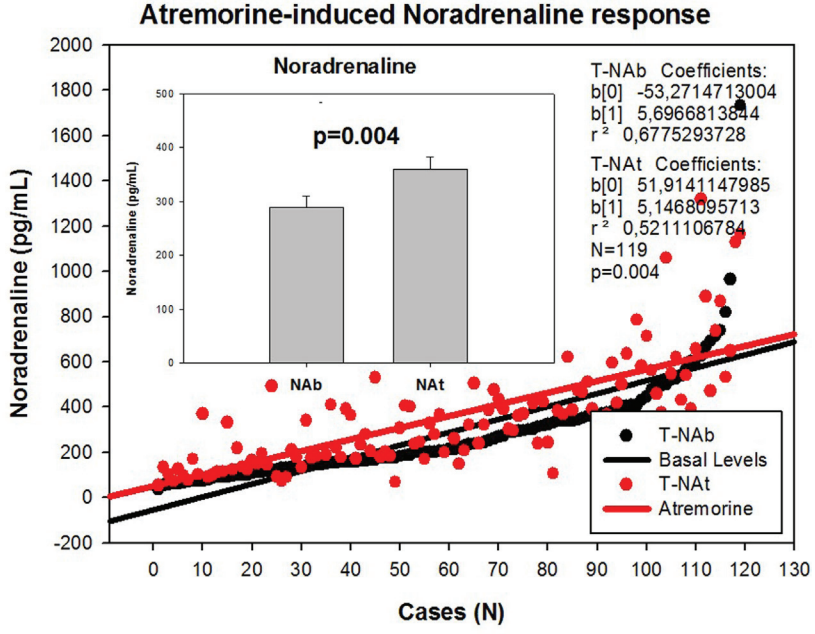

Fig. 2. Atremorine-induced noradrenaline (NA) response in patients with Parkinsonian disorders. NAb: Basal noradrenaline levels; NAt: Plasma noradrenaline levels at 1 hour after Atremorine administration (5 g, p.o.).

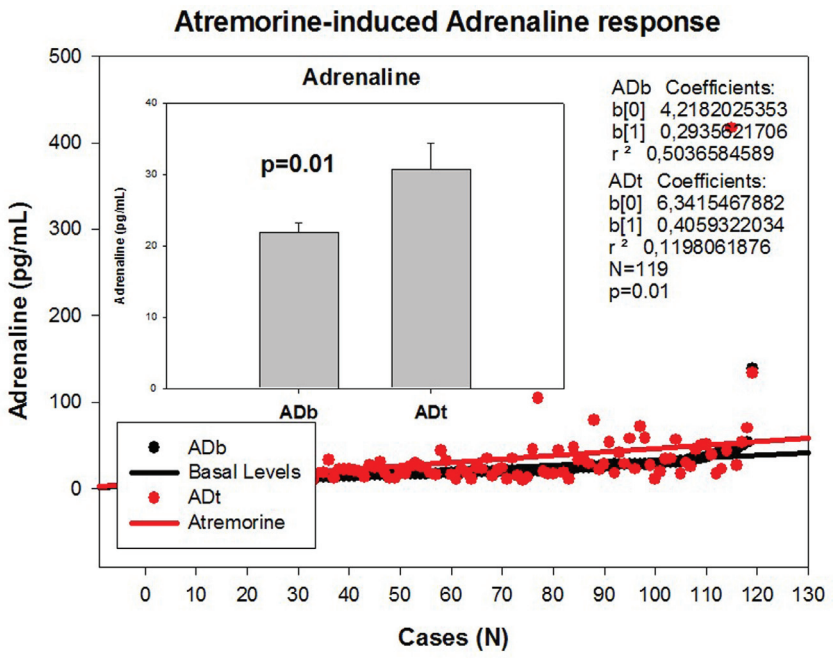

Fig. 3. Atremorine-induced adrenaline (AD) response in patients with Parkinsonian disorders. ADb: Basal adrenaline levels; ADt: Plasma adrenaline levels at 1 hour after Atremorine administration (5 g, p.o.).

normal EKG than in patients with abnormal EKG (p=0.01), and only cases with a defective EKG significantly responded to Atremorine (Table 3). Basal adrenaline levels tended to be higher in patients with an abnormal EKG, and the Atremorine-induced adrenaline response was more modest, but still significant, especially in cases with dysfunctional EKG (Table 3). No relevant differences were detected in either basal levels or Atremorine-induced serotonin response in PD patients (Table 3).

The PRL response to Atremorine was completely unrelated to cardiovascular function, with a significant response of PRL to Atremorine (p<0.001), and no differences in basal levels associated with cardiovascular function (Table 3). GH levels responded in a similar fashion to Atremorine with a more significant response in cases with either abnormal (p=0.04) or borderline EKG

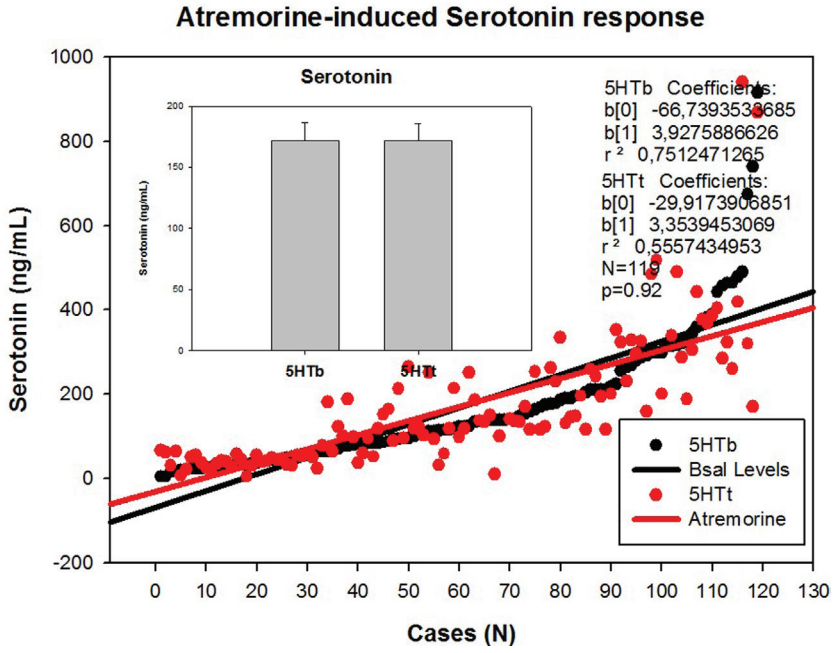

Fig. 4. Atremorine-induced serotonin (5-hydroxy-tryptamine, 5HT) response in patients with Parkinsonian disorders. 5HTb: Basal serotonin levels; 5HTt: Serum serotonin levels at 1 hour after Atremorine administration (5 g, p.o.).

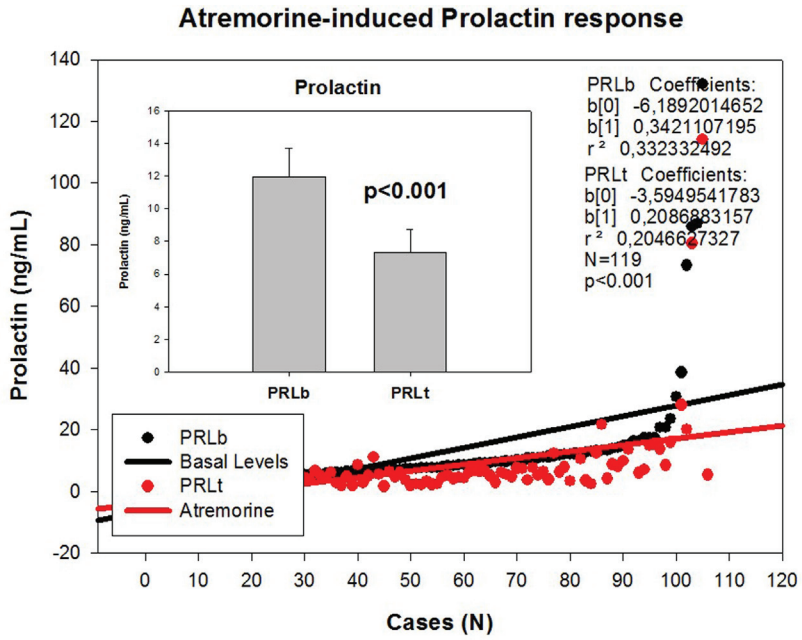

Fig. 5. Atremorine-induced prolactin (PRL) response in patients with Parkinsonian disorders. PRLb: Basal prolactin levels; PRLt: Serum prolactin levels at 1 hour after Atremorine administration (5 g, p.o.).

(p=0.005) than in patients with a normal EKG (Table 3). Significant differences were found in the basal FSH levels of patients with abnormal EKG versus borderline EKG (p=0.006) and in those with normal EKG versus borderline EKG (p=0.003). These differences persisted after Atremorine administration (Table 3). A similar pattern was found regarding basal LH levels, which were found to be significantly higher in borderline EKG cases than in patients with abnormal (p=0.04) or normal EKG (p=0.01) (Table 3). Atremorine did not alter these differences (Table 3). Both ACTH and testosterone levels did not show any relevant variability (Table 3); however, basal levels of estrogen were the highest in cases with normal EKG, intermediate in patients with borderline EKG, and the lowest in those cases exhibiting an abnormal EKG (Table 3).

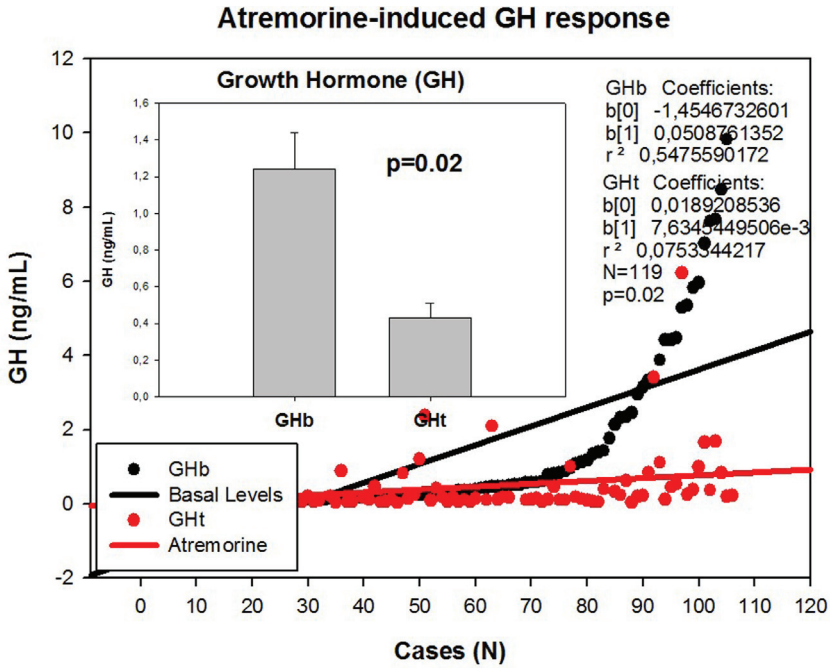

Fig. 6. Atremorine-induced growth hormone (GH) response in patients with Parkinsonian disorders. GHb: Basal growth hormone levels; GHt: Serum growth hormone levels at 1 hour after Atremorine administration (5 g, p.o.).

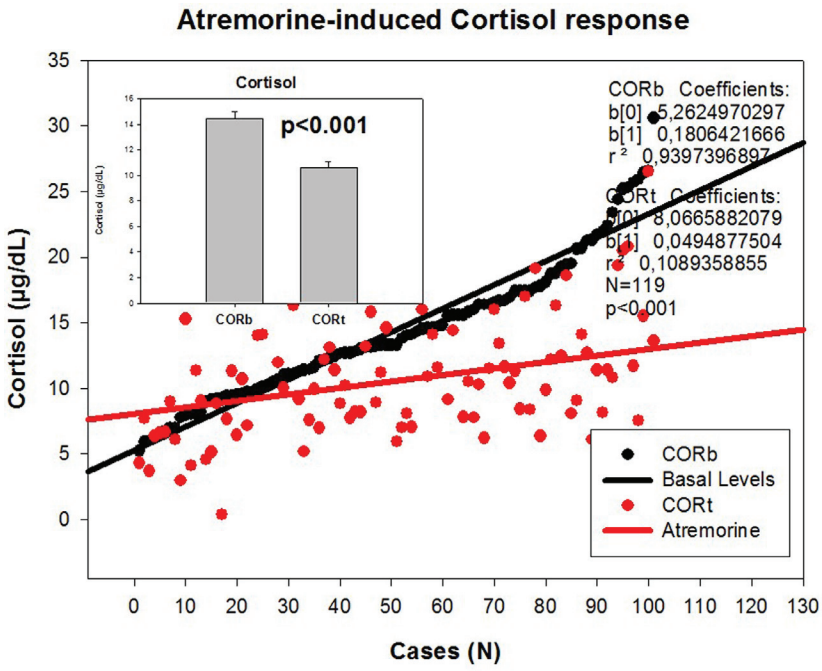

Fig. 7. Atremorine-induced cortisol (COR) response in patients with Parkinsonian disorders. CORb: Basal cortisol levels; CORt: Serum cortisol levels at 1 hour after Atremorine administration (5 g, p.o.).

Discussion

In the first clinical study of Atremorine in patients with Parkinsonian disorders, the powerful effect of this novel bioproduct on plasma dopamine was clearly demonstrated in practically 100% of the cases receiving a single oral dose (5 g/day) of Atremorine, with a genetically-dependent response observed in both treatment-naïve patients (never treated before with anti-PD drugs) and patients chronically treated with conventional antiparkinsonian drugs.[31] This pro-dopaminergic effect can be attributed to the rich content of natural L-DOPA (average concentration 20 mg/g) in the composition of Atremorine. However, the neuroprotective effect of this nutraceutical product on dopaminergic neurons, as demonstrated in *in vitro* studies[29] and in animal models of PD[30], cannot be attributed to L-DOPA alone, but to other intrinsic constituents of the compound.[29,31]

We postulated that Atremorine might be an option to minimize the "wearing-off" phenomenon, extending the therapeutic effect of conventional antiparkinsonian drugs, and reducing potential side effects, since the co-administration of Atremorine with other antiparkinsonian drugs allows a dose reduction of the conventional drugs by 25–50%, with enhancement of clinical benefits and reduction of short- and long-term adverse drug reactions.[10,31] Although the dopaminergic surge induced by Atremorine is proportional to basal DA levels in treatment-naïve patients and in patients chronically treated with conventional antiparkinsonian drugs, with a potential 200- to 500-fold increase over basal levels, its real potency and pharmacodynamic and pharmacokinetic properties are highly influenced by genetic and pharmacogenetic factors.[29,31]

In the present study, we demonstrate that Atremorine is a powerful enhancer of plasma catecholamines (noradrenaline, adrenaline, dopamine) (Table 2; Fig. 1–3), with no apparent effect on serotonin (Table 2; Fig. 4). Catecholamines are processed by three main nuclei (A8-retrobulbal, A9-substantia nigra pars compacta, A10-ventral tegmental area) arranged in the mesencephalic region where the mesostriatal, mesolimbic and mesocortical pathways are organized.[42,43] Midbrain dopaminergic neurons in the ventral tegmental area and noradrenergic neurons in the locus coeruleus are major sources of dopamine and noradrenaline to the prefrontal cortex, where these amines regulate cognition, behavior, and psychomotor function.[44,45] Noradrenaline, adrenaline, dopamine and serotonin play a central role in CNS and gut pathophysiology. Dopamine and noradrenaline are involved in the chemical structure of neuromelanins in the substantia nigra and the locus coeruleus, respectively. Dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), 3,4-dihydroxyphenylethanol (DOPE), and 3,4-dihydroxyphenylalanine (DOPA) are mainly responsible for the structure of neuromelanin from substantia nigra, while noradrenaline, 3,4-dihydroxymandelic acid (DOMA), and 3,4-dihydroxyphenylethylene glycol (DOPEG) are responsible for the structure of neuromelanin from locus coeruleus.[46] Deficiencies in these monoamines are currently found in PD.[47,48]

Monoamine transporters that facilitate the reuptake of noradrenaline, dopamine and serotonin are sodium-coupled transport proteins belonging to the neurotransmitter symporter family, which have also been implicated in PD.[49,50] Hypoactivity of the dopaminergic and noradrenergic systems in the brain stem are related to non-motor and motor symptoms in PD.[51,52] Dysregulation of these neurotransmitters is also involved in a variety of gastrointestinal symptoms in PD[53], and all of them appear to contribute to neurotransmitter and autonomic dysfunctions in PD[51], including mechanisms of L-DOPA-induced dyskinesia[53,54] and cardiovascular dysautonomia.[55] Therefore, appropriate doses of Atremorine alone or in combination with low doses of conventional anti-PD drugs[56] may benefit PD patients in whom the biosynthetic apparatus of the catecholaminergic system is damaged, including tyrosine hydroxylase (TH), the tetrahydrobiopterin (BH4) cofactor of TH, and the activity of the BH4-synthesizing enzyme, GTP cyclohydrolase

Table 3. Atremorine-induced neurotransmitter and hormonal changes in parkinsonian patients with normal, borderline and abnormal EKG

Parameter		Abnormal	Borderline	Normal	P
Noradrenaline (pg/mL)	B	340.76±39.49	265.30±47.87	249.32±29.00	0.08 A *vs* B; 0.01 A *vs* N
	T	405.89±32.61	386.87±56.18	305.12±35.58	0.04 Ab *vs* At; 0.04 Bb *vs* Bt
Adrenaline (pg/mL)	B	24.42±2.86	18.60±1.42	20.85±1.40	0.02 Ab *vs* At
	T	31.21±3.21	22.02±2.83	33.89±8.37	0.04 A *vs* B
Dopamine (pg/mL)	B	676.57±253.94	824.91±614.33	815.10±621.06	0.01 A *vs* B; 0.008 A *vs* N
	T	5984.76±1276.53	4533.43±1686.01	3197.63±750.64	<0.001 Ab *vs* At, Bb *vs* Bt, Nb *vs* Nt
Serotonin (ng/mL)	B	167.80±24.95	189.91±30.72	160.12±21.08	NS
	T	176.51±22.24	199.82±41.25	152.95±19.28	NS
Prolactin (ng/mL)	B	10.04±1.91	11.61±3.93	13.81±3.36	0.002 Ab *vs* At; 0.05 Bb *vs* Bt
	T	5.56±0.90	5.42±0.94	9.65±3.02	<0.001 Nb *vs* Nt
Cortisol (µg/dL)	B	10.93±0.93	14.00±1.24	14.24±0.79	0.009 Ab *vs* At; 0.05 Bb *vs* Bt
	T	11.72±4.53	10.69±0.98	9.67±0.73	<0.001 Nb *vs* Nt
ACTH (pg/mL)	B	30.09±6.49	38.88±16.48	29.74±3.47	NS
	T	31.11±7.41	36.55±13.53	25.65±3.22	NS
GH (ng/mL)	B	1.00±0.26	1.67±0.42	1.24±0.37	0.04 Ab *vs* At
	T	0.53±0.18	0.44±0.18	0.34±0.09	0.05 Bb *vs* Bt
FSH (mIU/mL)	B	25.86±5.09	51.08±9.00	26.08±5.16	0.006 A *vs* B; 0.003 B *vs* N
	T	26.01±5.45	46.98±9.65	25.78±5.12	0.01 A *vs* B; 0.03 B *vs* N
LH (mIU/mL)	B	11.60±1.61	23.59±4.17	13.83±2.90	0.01 B *vs* N
	T	11.24±1.55	23.01±4.59	11.95±2.26	0.04 A *vs* B; 0.03 B *vs* N
Testosterone (ng/dL)	B	380.36±37.93	407.16±61.97	390.60±41.12	NS
	T	316.95±29.18	353.00±49.60	353.40±36.28	NS
Estrogen (pg/mL)	B	28.12±3.62	44.10±17.96	95.63±26.44	NS
	T	28.13±3.88	48.15±20.52	86.33±23.40	NS

Data: mean ± standard error.
Abbreviations: A: abnormal EKG; B: borderline EKG; b: basal levels; N: normal EKG; t: 60 minutes after treatment with Atremorine (5g); NS: non-significant differences.

I, as well as the activities of aromatic L-amino acid decarboxylase (DOPA decarboxylase), dopamine beta hydroxylase, and phenylethanolamine N-methyltransferase, which synthesize dopamine, noradrenaline, and adrenaline, respectively.[57] Atremorine may also neutralize the apoptosis of nigro-striatal dopamine neurons which, in postmortem studies, show increased levels of pro-inflammatory cytokines (TNF-α, IL-6), increased levels of apoptosis-related factors (p55, soluble Fas, bcl-2, caspases 1-2), and decreased levels of neurotrophins (BDNF).[57]

The increase in noradrenaline induced by Atremorine may contribute to clinical improvement and neuroprotection, since noradrenergic neuronal loss in the locus coeruleus is exacerbated in PD. Noradrenaline exerts critical effects in the modulation of different types of behavior (sleep-wakefulness cycle, depression, anxiety), psychomotor function, anti-inflammatory responses in glial cells, neurotrophic activity, and neuroprotection against oxidative stress-related free radical formation.[58,59] Preclinical studies showed that Atremorine displays powerful anti-oxidant, anti-inflammatory, and neuroprotective effects[29,30], some of which might be associated with its effect as a noradrenergic enhancer. Premature noradrenaline deficiency resulting from selective degeneration of neurons of the locus coeruleus and sympathetic ganglia has also been postulated as a praecox event in PD, even prior to selective dopaminergic neurodegeneration.[60,61] In this regard, the noradrenergic effects of Atremorine may also explain, in part, its clinical and biochemical benefits.

The midbrain dopaminergic system is regulated by the central adrenergic system.[62] The moderate increase in adrenaline levels observed after Atremorine administration (Table 2; Fig. 3) may also contribute to enhancing dopaminergic neurotransmission in PD.

Neuroendocrine dysfunction and alterations in circadian rhythms are frequently seen in patients with PD, but most results are contradictory, with no clear definition between basal conditions and drug-induced modifications in hypothalamus-pituitary neuropeptides and hormones.[63–65] Furthermore, PRL and GH secretion are directly regulated by hypothalamic and supra-hypothalamic dopaminergic mechanisms.[66–68] Some studies reported higher levels of PRL and GH in PD patients, as compared with controls.[65,69] In patients with multiple system atrophy, in whom there is a reported loss of hypothalamic dopamine, basal PRL levels are elevated, L-DOPA increases GH secretion, and the neuroendocrine response to L-DOPA is unclear[70], differing from endocrine responses in PD patients.[71,72] 6-Pyruvoyl-tetrahydropterin synthase deficiency is a BH4 deficiency with hyperphenylalaninemia, which is treated with L-DOPA/carbidopa, 5-hydroxytryptophan and BH4. In these patients, serum PRL levels are elevated due to their hypodopamin-

ergic condition, and the administration of L-DOPA reduces PRL secretion.[73] In healthy subjects, acute L-DOPA and exercise release GH, but in PD patients this response is delayed.[74] In animal studies, L-DOPA causes a decrease in PRL response, whereas COR levels tend to increase.[75] It has also been postulated that peripheral noradrenergic terminals may contribute to regulating PRL secretion.[76]

In our study, Atremorine induced a significant decrease in PRL and GH levels (Table 2; Fig. 5,6), and a significant decrease in COR levels (Table 2; Fig. 7). The PRL and GH response to Atremorine can be directly attributed to the effect of L-DOPA on dopamine and noradrenaline synthesis and release, with the consequent increase in central and peripheral dopamine and noradrenaline levels (Fig. 1). In contrast, the effect on COR might be primarily influenced by a direct effect of dopamine, noradrenaline and adrenaline on the adrenal gland, and secondarily by pituitary and/or hypothalamic regulation of ACTH, which in plasma did not show any significant changes (Table 2). In our opinion, neuroendocrine function in PD is still poorly understood and the investigation of differences in PD-related basal neuroendocrine conditions versus anti-PD-drug-induced neuroendocrine changes deserves further studies.

Cardiovascular dysfunction is a common finding in PD patients. Low plasma levels of noradrenaline and adrenaline, secondary to the loss of catecholaminergic neurons in the rostral ventrolateral medulla, together with loss of nigral dopaminergic neurons, may be responsible for reduced sympathetic activity[77] and cardiovascular dysautonomia in PD.[55] Our findings, in part, agree with this postulate. We found that PD patients with abnormal EKG have lower levels of dopamine, and the basal levels of noradrenaline are substantially different between cases with abnormal versus normal EKG (Table 3). In any case, the administration of Atremorine tends to increase the plasma levels of the three catecholamines, with the highest impact on dopamine levels, and a minimum effect on adrenaline levels (Table 3). Other non-motor symptoms present in PD, such as constipation and other alterations in gastrointestinal motility mediated via catecholaminergic mechanisms[78], might also be alleviated by Atremorine. However, further investigation is needed on the central and peripheral effects of Atremorine, especially taking into account that the effects of Atremorine are genotype-related, involving both pathogenic genes associated with neurodegeneration, and genes of the cytochrome P450 family associated with drug metabolism.[31]

All these data together clearly indicate that Atremorine is a very safe bioproduct for PD patients, capable of exerting a powerful effect on catecholamines, especially dopamine and, to a lesser extent, noradrenaline. The effect of Atremorine on adrenaline is very modest, and no effect on serotonin levels could be detected at 1 hour after oral administration. The effect of Atremorine on PRL and GH is likely to result from the primary consequence of hypothalamic regulation mediated via dopaminergic and noradrenergic neurotransmission, and secondarily as a direct effect on the pituitary gland. In contrast, the effect on COR might result primarily from a direct effect on the adrenal gland, and secondarily from the hypothalamus-hypophyseal regulation of ACTH. According to these results, Atremorine may help to optimize neuroendocrine function in PD, especially in those patients with somatotropinergic, lactotropinergic and corticotropinergic dysregulation. The effects of Atremorine on plasma catecholamines might also be beneficial for PD patients with cardiovascular dysautonomia.

Conflict of interest

All authors are staff members of EuroEspes at EuroEspes Biomedical Research Center (RC, MA, LN, CF, PC, JCC) and EuroEspes Biotechnology (LFN, RA, IC). RC is the inventor of the product, and EuroEspes Co. is the owner of the patent.

Author contributions

Writing the paper and acting as the IP in clinical studies (RC), monitoring and management of patients in the clinical trial (MA, LN, CF, PC), performing biochemical and analytical studies (LC, SR), in charge of genomic and pharmacogenomic studies (JCC), performing chemical studies and monitoring quality control of the product in preclinical and clinical studies (LFN, RA, IC).

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