Brain-region responsiveness to DT56a (Femarelle) administration on allopregnanolone and opioid content in ovariectomized rats

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Abstract

Objective: The natural selective estrogen receptor modulator DT56a (Femarelle), derived from soybean, has been shown to relieve menopausal vasomotor symptoms with no effect on sex steroid hormone levels or endometrial thickness. The purpose of the present study was to evaluate the neuroendocrine effect of DT56a administration through the evaluation of brain content of allopregnanolone (AP), an endogenous neurosteroid γ-aminobutyric acid agonist with anxiolytic properties, and through the assessment of β-endorphin (β-END), the endogenous opioid implicated in pain mechanism, emotional state, and autonomic control.

Methods: Five groups of Wistar ovariectomized (OVX) rats received one of the following treatments: oral DT56a administration at doses of 6, 12, 60, and 120 mg kg^{-1} day^{-1} or estradiol valerate (E_2V) at a dose of 0.05 mg kg^{-1} day^{-1} for 14 days. One group of fertile and one group of OVX rats receiving placebo were used as controls. The concentration of AP was assessed in the frontal and parietal cortex, hippocampus, hypothalamus, anterior pituitary, and serum, whereas the content of β-END was evaluated in the frontal and parietal cortex, hippocampus, hypothalamus, neurointermediate lobe, anterior pituitary, and plasma.

Results: DT56a increased AP levels in all brain areas analyzed and in serum, with a classical dose-related curve in comparison with OVX rats. In some brain areas, such as the frontal cortex, the parietal cortex, and the anterior pituitary, positive results were found even with the administration of a lower DT56a dose of 60 mg kg^{-1} day^{-1}, attaining AP levels in the range of those in animals treated with E_2V. Similarly, β-END levels were enhanced in selected brain areas such as the hippocampus, the hypothalamus, the neurointermediate lobe, and the anterior pituitary in comparison with those in OVX rats, in which the increase of the opioid was dose related and in the range of those in rats treated with E_2V.

Conclusions: This study demonstrated that DT56a positively affects brain neurosteroidogenesis and the opiatergic system: DT56a exerts an estrogen-like effect on selective areas related to mood, cognition, and homeostasis control, presenting a specific pattern of interaction with the brain function. These findings may, in part, explain the clinical effect of DT56a on menopausal symptoms.

Key Words: DT56a – Brain – β-Endorphin – Allopregnanolone.

Estrogen therapy (ET) is well known for its beneficial effect on climacteric brain-derived symptoms, such as hot flashes, sleep disturbance, mood changes, and sexual dysfunction, and a protective role for early estrogen use on cognitive impairment and dementia in late menopause has also been postulated.[1-3] Estrogen receptors (ERs), in their various forms, are present throughout the brain, and it is well documented that estrogens affect directly the biology of neurons and glia cell, modulating the synthesis/release of neurotransmitters and neurotrophins.[4,5] Moreover, ET enhances the brain content of allopregnanolone (AP), a 3α-5α reduced neuroactive metabolite of progesterone and produced by the neuron-glia functional unit.[6-8] AP acts mainly as an agonist on the γ-aminobutyric acid receptor, modulating stress, mood, and behavior with anxiolytic, sedative, and antiepileptic effects.[9] In addition, ET improves the activity of the endogenous opioid system. β-Endorphin (β-END) is the endogenous opiate that has generated the most research interest: it is involved in response to stress, emotional

regulation, pain mechanism, and reward system.[7,10-12] β-END might play a major role in the mechanism of sexual arousal and pleasure in both sexes.[13,14]

DT56a (Femarelle; Se-cure Pharmaceuticals, Yavne, Israel) is a compound derived from processed soybeans, producing a unique active complex as found in tofu.[15] It has been demonstrated to increase bone mineral density in postmenopausal women[16] and to relieve vasomotor symptoms with no effect on sex hormone levels or endometrial thickness.[17] DT56a selectively stimulates ERs in skeletal tissues but not in the uterus.[18] Thus, DT56a acts as a selective estrogen receptor modulator (SERM), exerting a tissue-selective mechanism of action and affecting menopausal symptoms and the bone tissue, without affecting the breast and uterus.[15,18]

We previously demonstrated that two SERMs, raloxifene and EM-652, affect the brain and serum content of AP and β-END differentially in relation to the dose used and the brain area analyzed, showing a dissimilar estrogenic activity in the central nervous system (CNS) of ovariectomized (OVX) rats.[19-22]

Because DT56a is considered a phytoSERM and clinical data suggest a neuroactive role for this molecule,[17] we have been speculating that DT56a could modify brain neurosteroidogenesis and the endogenous opioid milieu. The effects of oral administration of DT56a on AP and β-END in selected brain areas related to mood, cognition, and homeostasis control of gonadectomized female animals were assessed to test this hypothesis. In addition, the effects of DT56a were compared with those produced by placebo and by the oral administration of estradiol valerate (E₂V).

METHODS

Animals

Forty-six female Wistar rats (weight, 150-200 g), purchased from Harlan Nossan, Italy, were used in the present study. They were divided into groups of eight rats and were housed together for 14 days in a climate-controlled room: the access to food and water was completely free, and rats had 14 hours of illumination (light on at 6 AM and light off at 8 PM).

Bilateral ovariectomy was performed under tiletamine plus zolazepam anesthesia (Zoletil, 1 mg/rat IM), with the only exception of the fertile control group. Rats were ovariectomized on the day of proestrus, as indicated by vaginal smear (evaluated daily). The treatments started 2 weeks after the surgery. OVX animals were treated with oral E₂V (0.05 mg kg^{-1} d^{-1}) or different doses of oral DT56a: 6, 12, 60, and 120 mg kg^{-1} day^{-1}; one group of eight fertile and one group of eight OVX rats were used as controls receiving only the vehicle (sesame oil). Rats were then treated for 14 days and at the end of the treatment, all groups were killed by decapitation. The fertile animals were killed in the morning of proestrus, as verified by a vaginal smear. The following brain regions were taken and weighed: frontal and parietal cortex, hippocampus and hypothalamus, anterior and neurointermediate pituitary, and the adrenal

glands. After all tissues were weighed, they were collected in 2.5 mL solution of 4% acetic acid and were homogenized at ice-cold temperature. The homogenate was centrifuged at 1,200g for 15 minutes at 4°C; it was likewise divided in two aliquots (1.25 mL each) and assayed in duplicate for AP and β-END. A blood specimen was immediately drawn after decapitation from each rat and collected into heparinized plastic tubes. At the end of the process, β-END levels were determined in frontal and parietal cortex, hippocampus, hypothalamus, anterior pituitary, neurointermediate pituitary, and plasma, whereas AP levels were measured in frontal and parietal cortex, hippocampus, hypothalamus, anterior pituitary, serum, and adrenal gland. Animal treatments were carried out in compliance with state laws and guidelines of the Institutional Animal Care and Use Committee at the University of Pisa. The local research ethics committee approved this protocol.

AP assay

The supernatant of tissue homogenates and serum was passed through a C-18 Sep-Pak cartridge, previously equilibrated with homogenizing buffer. The cartridge was sequentially washed with homogenizing buffer, 50% aqueous methanol, and the unconjugated steroid fraction was eluted with absolute methanol and brought to dryness under nitrogen. Analytical grade solvents were purchased from Merck
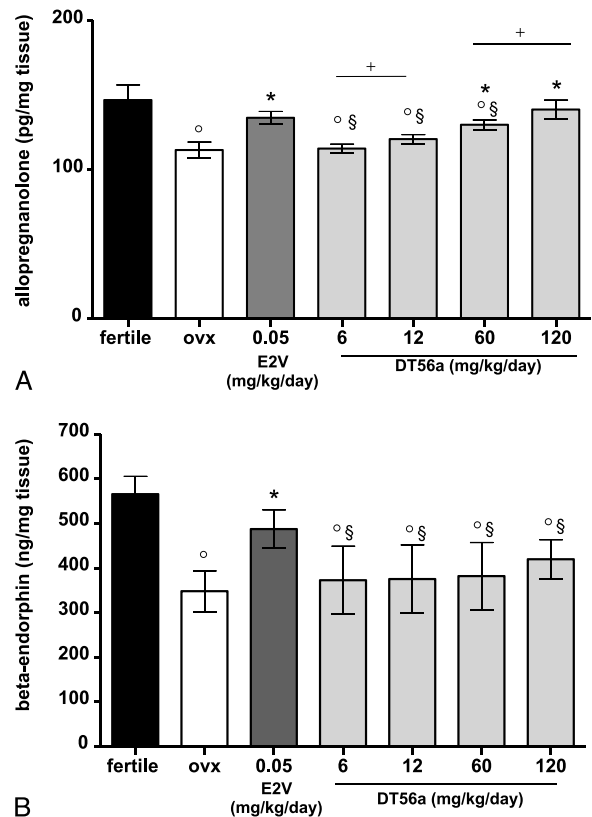

FIG. 1. Allopregnanolone (*A*) and β-endorphin (*B*) levels in frontal cortex of seven groups of eight animals each. Fertile, fertile rats; OVX, ovariectomized rats; E2V, estradiol valerate. °, vs fertile (°*P* < 0.05); *, vs OVX (**P* < 0.05); +, vs other treatment dose (+*P* < 0.05); §, vs E2V (§*P* < 0.05).
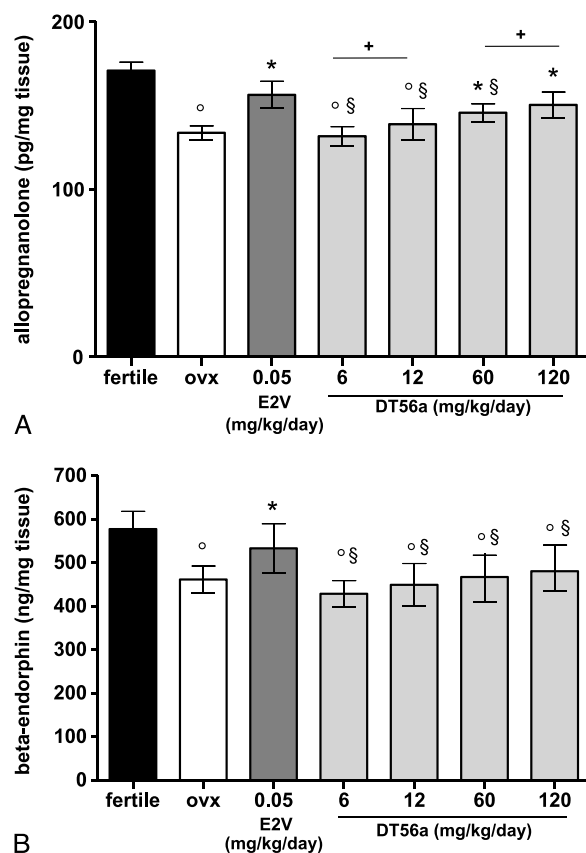
FIG. 2. Allopregnanolone (*A*) and β-endorphin (*B*) levels in parietal cortex of seven groups of eight animals each. Fertile, fertile rats; OVX, ovariectomized rats; E2V, estradiol valerate; °, vs fertile (°*P* < 0.05); *, vs OVX (**P* < 0.05); +, vs other treatment dose (⁺*P* < 0.05); §, vs E2V (§*P* < 0.05).

(Darmstadt, Germany); Waters Corporation (Milford, MA) provided C-18 Sep-Pak cartridges. AP contents were measured through a radioimmunoassay method previously described, using an antiserum that Dr. R.H. Purdy (San Diego, CA)[23,24] kindly provided. The sensitivity of this assay was 18 pg/mL, the recovery after extraction and chromatography was 88.5 ± 12.7% (mean ± SEM), and the intra-assay and interassay coefficients of variation were 6% and 8%, respectively.

β-END assay

The supernatant of tissue homogenates and plasma were passed through a C-18 Sep-Pak cartridge, previously equilibrated with 50% aqueous methanol, whereas the unconjugated fraction was eluted with absolute methanol and brought to dryness under vacuum. β-END levels were measured by a previously described specific radioimmunoassay,[24] using camel β-END as standard (Sigma Chemicals, St. Louis, MO).

The antiserum (supplied by Dr. P. Sacerdote, Milano, Italy) was used at the final dilution of 1:130,000, and analytical grade solvents were purchased from Merck; C-18 Sep Pak cartridges were supplied by Waters Corporation. The sensitivity of this assay was 10 pg/mL, the recovery after acetic acid extraction and chromatography corresponded to

85 ± 11% of the total amount, and the intra-assay and interassay coefficients of variation were 6% and 8%, respectively.

Statistical analysis

In accordance with previously reported data, AP levels in all brain areas and tissues analyzed were expressed in nanograms per milligram of tissue, and AP levels in serum were expressed in nanograms per milligram. β-END levels were expressed in nanograms per organ or nanograms per milligram of tissue and in nanograms per milliliter in serum. All data were reported as mean ± SD. Data obtained were studied by one-way analysis of variance, and the Bonferroni multiple comparison test was used to compare treatment groups. *P* < 0.05 was considered significant. Comparisons were made between control groups (fertile, OVX, E₂V) and animals receiving different doses of DT56a. All these comparisons are indispensable for proper analysis of the activity of this phytoSERM on brain function. However, using the Bonferroni correction for multiple comparisons, there is an additional chance that some differences among groups could actually be significant but pronounced not significant. The SPSS for MAC software package was used in the statistical analysis.
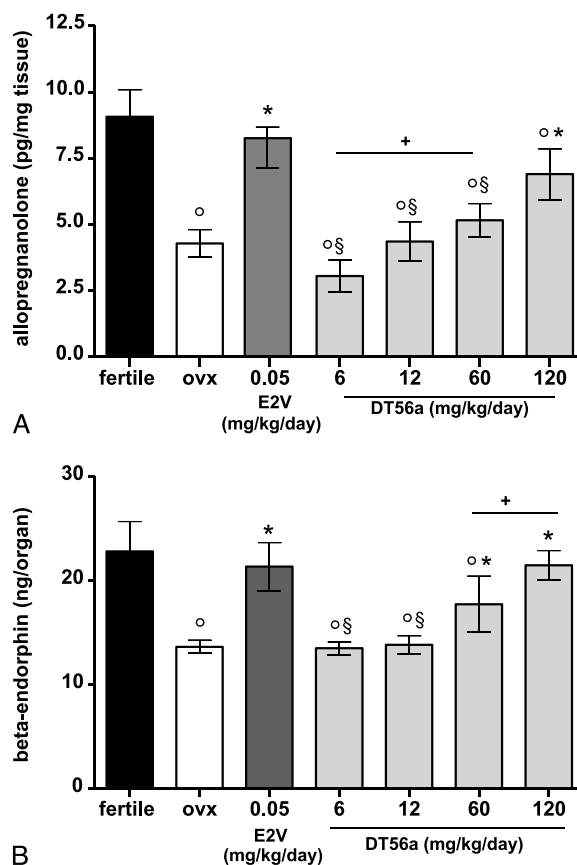

FIG. 3. Allopregnanolone (*A*) and β-endorphin (*B*) levels in hippocampus of seven groups of eight animals each. Fertile, fertile rats; OVX, ovariectomized rats; E2V, estradiol valerate; °, vs fertile (°*P* < 0.05); *, vs OVX (**P* < 0.05); +, vs other treatment dose (⁺*P* < 0.05); §, vs E2V (§*P* < 0.05).

RESULTS

Ovariectomy

Ovariectomy induced an important and significant reduction of AP in all regions examined (except for the adrenal glands) and serum. Similarly, β-END was reduced in all brain areas analyzed along with plasma after gonadectomy (Figs. 1-6).

### E$_2$V (0.05 mg kg^{-1} d^{-1}) administration to OVX rats

E$_2$V administration increased AP in all areas examined except for the adrenal glands and increased β-END levels in all areas examined. There was no statistically significant difference between the level of AP of the fertile group compared with that of the group treated with E$_2$V in the frontal cortex, parietal cortex, and hippocampus. Furthermore, E$_2$V increased β-END in all brain areas analyzed and in plasma to values in the range of those in fertile controls (Figs. 1-6).

Effects of DT56a administration at doses of 6, 12, 60, and 120 mg kg^{-1} day^{-1} on AP brain and serum levels in OVX rats

In the frontal cortex and parietal cortex, the administration of DT56a at a dose of 60 mg kg^{-1} day^{-1} increased AP
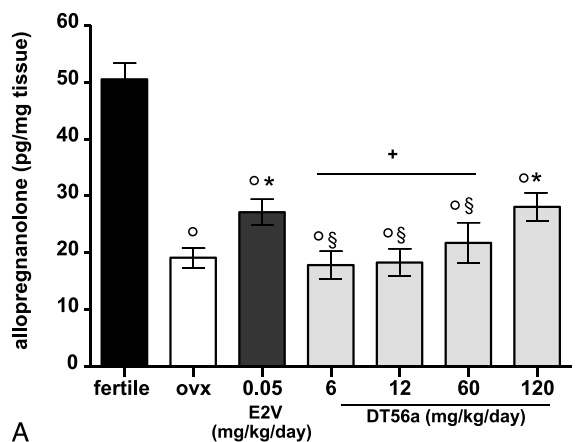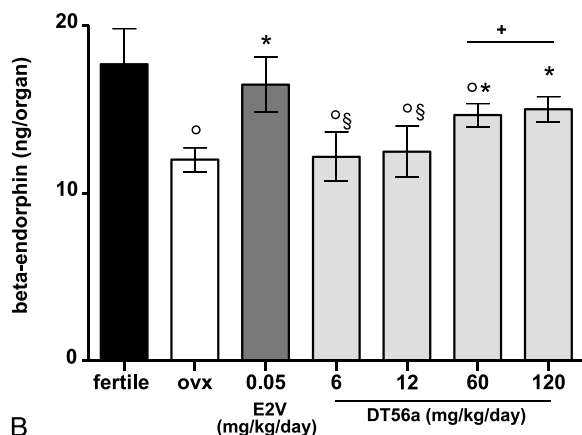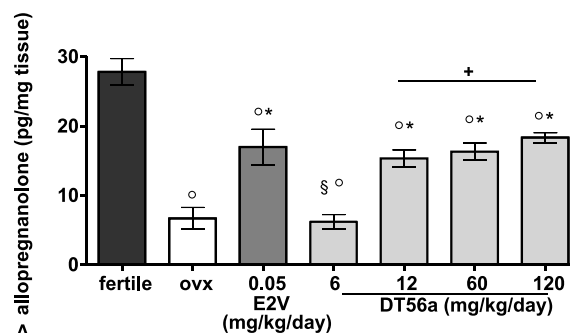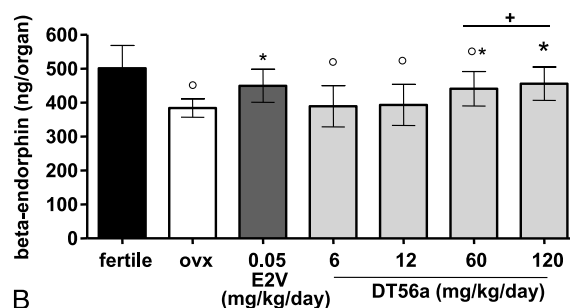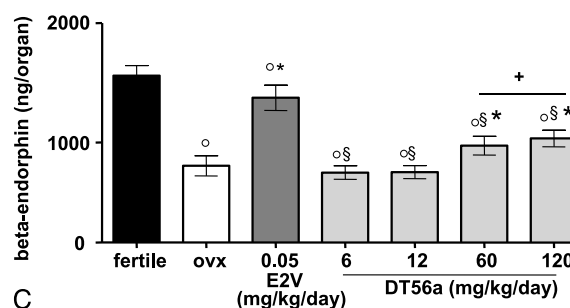

FIG. 4. Allopregnanolone (*A*) and β-endorphin (*B*) levels in hypothalamus of seven groups of eight animals each. Fertile, fertile rats; OVX, ovariectomized rats; E2V, estradiol valerate; °, vs fertile (°*P* < 0.05); *, vs OVX (**P* < 0.05); +, vs other treatment dose (+*P* < 0.05); §, vs E2V (§*P* < 0.05).

FIG. 5. Allopregnanolone (*A*) and β-endorphin (*B*) levels in anterior pituitary and β-endorphin (*C*) levels in neurointermediate lobe of seven groups of eight animals each. Fertile, fertile rats; OVX, ovariectomized rats; E2V, estradiol valerate; °, vs fertile (°*P* < 0.05).* vs OVX (**P* < 0.05). +vs other treatment dose (†*P* < 0.05). § vs E2V (§*P* < 0.05).

content compared with OVX rats (*P* < 0.05), reaching values that were lower than fertile controls (*P* < 0.05 vs fertile) and lower than rats receiving E$_2$V only in the frontal cortex (*P* < 0.05 vs E$_2$V). DT56a at a dose of 120 mg kg^{-1} day^{-1} induced a significant increase of AP compared with OVX rats (*P* < 0.05 vs OVX) and with rats administered DT56a at a dose of 60 mg kg^{-1} day^{-1} (*P* < 0.05), attaining values in the range of fertile controls and rats receiving E$_2$V. No differences were observed between OVX rats and animals receiving DT56a at doses of 6 and 12 mg kg^{-1} day^{-1}; however, when the 6 and 12 mg kg^{-1} day^{-1} groups were compared to each other a significant change was shown (*P* < 0.05; Figs. 1 and 2A). In the hippocampus and in the hypothalamus, the administration of DT56a at a dose of 120 mg kg^{-1} day^{-1} increased AP content with respect to OVX rats (*P* < 0.05 vs OVX) to a level that was in the range of animals receiving E$_2$V, but still lower than fertile controls (*P* < 0.05 in the hippocampus and *P* < 0.05 in the hypothalamus).
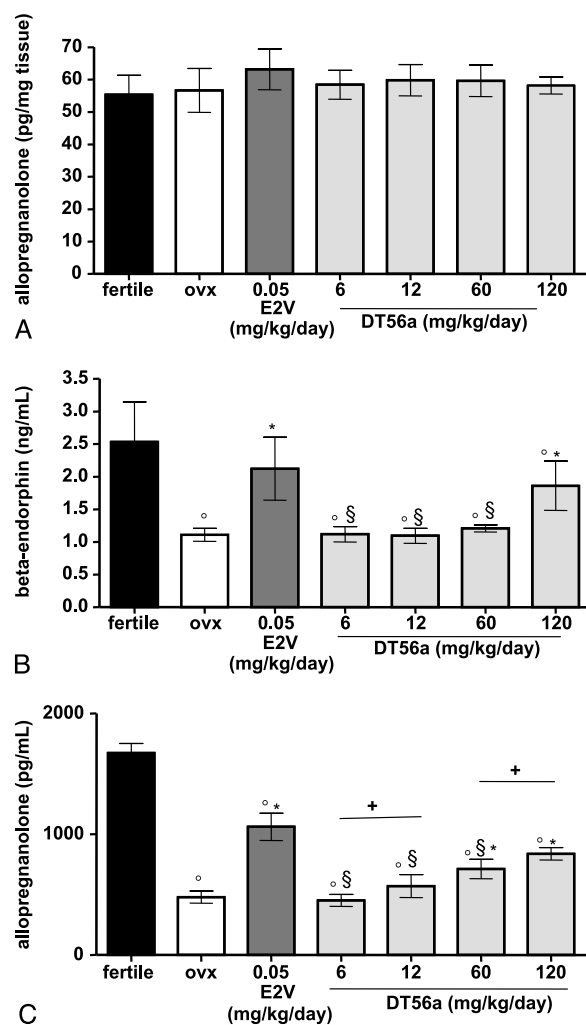
FIG. 6. Allopregnanolone (*A*) levels in adrenal glands. β-Endorphin and allopregnanolone circulating levels (*B, C*) of seven groups of eight animals each. Fertile: fertile rats; OVX, ovariectomized rats; E2V, estradiol valerate; °, vs fertile (°$P < 0.05$); *, vs OVX (*$P < 0.05$); +, vs other treatment dose (+$P < 0.05$); §, vs E2V (§$P < 0.05$).

Although the administration of DT56a at doses of 6, 12, and 60 mg kg^{-1} day^{-1} did not modify AP content with respect to OVX rats in these two brain regions, treatment with 60 mg kg^{-1} day^{-1} enhanced AP as compared with 6 mg kg^{-1} day^{-1} ($P < 0.05$ in the hippocampus and $P < 0.05$ in the hypothalamus; Figs. 3 and 4A). In the anterior pituitary, treatment with 12, 60, and 120 mg kg^{-1} day^{-1} increased AP in comparison with OVX rats ($P < 0.05$ vs OVX), arriving at values similar to E2V, which were lower than fertile controls ($P < 0.05$ vs fertile). A dose-related difference was evidenced between 12 and 120 mg kg^{-1} day^{-1} (Fig. 5A). In the adrenal gland, the administration of DT56a, at any dose, did not affect AP content (Fig. 6A). The serum level of AP was increased in comparison with OVX rats after the administration of DT56a at doses of 60 and 120 mg kg^{-1} day^{-1} ($P < 0.05$ vs OVX) in a dose-related manner ($P < 0.05$, 60 mg kg^{-1} d^{-1} vs 120 mg kg^{-1} d^{-1}). At 120 mg kg^{-1} day^{-1} of DT56a, AP levels reached the range of E2V treatment (Fig. 6C).

Effects of DT56a administration at doses of 6, 12, 60, and 120 mg kg^{-1} day^{-1} on β-END brain and serum levels in OVX rats

In frontal and parietal cortex, the administration of DT56a, at any different dose, did not affect β-END content (Figs. 1 and 2B). In the hippocampus, hypothalamus, and anterior pituitary, treatment with DT56a at doses of 6 and 12 mg kg^{-1} day^{-1} did not induce any changes in β-END compared with OVX rats. The administration of DT56a at 60 mg kg^{-1} day^{-1} enhanced β-END content with the same amplitude of E$_2$V ($P < 0.05$ vs OVX) but still lower than the fertile controls. DT56a at 120 mg kg^{-1} day^{-1} further increased β-END ($P < 0.05$ vs 60 mg kg^{-1} d^{-1} in the hippocampus, hypothalamus, and anterior pituitary) to values in the range of fertile animals (Figs. 3-5B). In the neurointermediate lobe, 60 and 120 mg kg^{-1} day^{-1} of DT56a determined a dose-related rise of β-END content in comparison with OVX ($P < 0.05$ vs OVX) ($P < 0.05$ 60 vs 120 mg kg^{-1} d^{-1}) reaching levels that were, however, lower than fertile rats and E$_2$V therapy (Fig. 5C). β-END circulating levels developed in OVX animals after administration of DT56a at a dose of 120 mg kg^{-1} day^{-1}, in comparison with ovariectomy.

DISCUSSION

The present data demonstrated that the administration of DT56a enhances the content of AP and β-END in selected brain areas in OVX rats. Because AP and β-END affect the brain function directly, the present data might support hypothesis of a neuroactive role of DT56a.

In particular, DT56a increased the level of AP compared with placebo in all brain regions analyzed and in the serum, using doses ranging from 12 to 120 mg kg^{-1} day^{-1}. The activity of DT56a was similar to the administration of E$_2$V, consequently evidencing an estrogen-like profile of action on brain neurosteroidogenesis. These data support the concept that DT56a acts as an estrogen-agonist molecule in CNS, at least in part, for AP synthesis and also sustain the hypothesis for its definition as a phytoSERM. Further studies using estrogen receptor knockout animals or an estrogen receptor antagonists might elucidate this "estrogen-like" action of DT56a in the brain.

Additional features characterized the result of DT56a administration on brain AP in OVX rats: the dose-dependent effect on AP synthesis and the specific brain region responsiveness. The dose-dependent increase of AP has been shown in all brain areas analyzed, comparing low doses of DT56a in the frontal and parietal cortex, hippocampus, and hypothalamus and high doses of DT56a in the frontal cortex, anterior pituitary, and serum.

Moreover, the administration of DT56a at the dose of 120 mg kg^{-1} day^{-1} increased the AP level to levels in the range of animals receiving E$_2$V in all brain areas analyzed, with the exception of the anterior pituitary. The anterior pituitary seemed to be a well-characterized region, highly receptive to DT56a: 12 mg kg^{-1} day^{-1}, a dose 10 times lower than the

active dose in all the other brain regions (120 mg kg^{-1} d^{-1}), was properly adequate to increase AP content in the range of E$_2$V treatment. These data support the concept of different brain region responsiveness to DT56a administration.

We previously showed that the raloxifene analogue LY-117018 and EM-652 reversed the ovariectomy-induced changes in AP levels in cerebral areas, pituitary, and adrenals in OVX rats.[19,21] However, the particular brain regions, which responded to the administration of DT56a, are unique, thus showing specific features of this phytoSERM.

The mechanism by which estradiol and SERMs modulate circulating and tissue levels of AP is unclear. Estrogens can increase the activity of the enzymatic pathway (5α-*R*)-3-hydroxysteroid-oxidoreductase, which converts progesterone into 5-dihydroprogesterone and AP, respectively.[8,25] It has been postulated that raloxifene, DT56a, and estradiol have a common receptor(s) and mechanism of action in the bone and vascular tissue.[15,26-28] Thus, the same mechanism of action, mediated by the ER(s), might be recruited for the modulation of AP content in the brain and serum. However, additional factors, such as the desensitization of ER(s) or their rearrangement or changes in coactivators and/or corepressors, might explain, to a certain extent, differences between DT56a, estradiol, and other SERMs on AP modulation.[28]

Clinical data evidence a neuroactive role of DT56a: all CNS-related symptoms in the Kupperman Index were improved after 12 weeks of treatment, which were sustained throughout the 12 months of treatment. Moreover, 76% of the women using the standard dose of DT56a reported a decrease in hot flashes, which was sustained after 12 months of treatment.[17]

The drop in 5α-reduced metabolites of progesterone plays a pivotal role in pathophysiology of CNS-related symptoms during the menopausal transition.[29] A rat model of mood disorders and anxiety-induced behavior showed a strong region-specific dysregulation of AP homeostasis in brain: reduced levels of AP in the brain are associated with high anxiety scoring and depressive behavior.[30,31]

Thus, improvement of mood symptoms and anxiety evaluated after DT56a administration might be the clinical expression, to a certain extent, on the growth content of brain AP.

As for β-END, we observed that the administration of DT56a enhanced β-END levels in selected brain areas (hippocampus, hypothalamus, neurointermediate lobe, and anterior pituitary). The above results were found starting at 60 mg kg^{-1} day^{-1} of DT56a reaching levels in the range of E$_2$V treatment with the maximal dose of 120 mg kg^{-1} day^{-1} and showing a dose-related effect. Similarly, plasma levels of this opioid increased after the administration of DT56a at the dose of 120 mg kg^{-1} day^{-1} in the same amplitude of E$_2$V treatment. Thus, in the brain regions where DT56a was active, DT56a evidenced an estrogen-like activity. Nevertheless, this phytoSERM was not active on cortical areas, where the administration of E$_2$V restored β-END levels in the range of fertile animals. Because DT56a increased the hypothalamus/neurointermediate lobe content of β-END, which are the main sites of synthesis and release of this peptide in rat brain,[32,33] DT56a directly affects the cell systems deputed to endorphin synthesis/release. However, the lack of direct effects in cortical areas proves, to a certain extent, a distinct biological response of the endorphin system, between E$_2$V and DT56a treatment. Whether the basis of this difference is related to the recruitment of ERs or to the activity of their coregulators or whether it is a function of dose and length of treatment is still to be further analyzed. The fact that the raloxifene analogue LY-117018 failed to increase hippocampal opioid content in OVX rats, whereas EM-652 showed an estrogen-like activity in sovra-hypothalamic regions,[20,21] confirms the hypothesis of a specific interaction with the opioid milieu for each SERM analyzed.

All of the previously described results showed that using the Bonferroni correction for multiple comparisons, as indicated in the statistical section, should suggest prudent interpretation of data.

CONCLUSIONS

In conclusion, DT56a evidenced a selective effect on β-END synthesis and release. Although the effect was generally in the amplitude of E$_2$V administration, certain differences with estrogen activity have been shown. Moreover, DT56a administration results in a wide positive modulation of endogenous AP milieu throughout the central structures analyzed and evidencing an estrogen-like activity. Particularly, DT56a action on certain parts of the limbic system (hippocampus and hypothalamus) and of the neocortex for AP supports the hypothesis that this phytoSERM might affect the autonomic control, the emotional state, and higher brain functions (thought and action). Although raloxifene, EM-652, and DT56a are considered SERMs with an estrogen agonist activity on CNS, the highlighted differences between these compounds further support and characterize the existence of specific effects for each molecule on brain biology.

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