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# A novel prolyl endopeptidase inhibitor, JTP-4819, with potential for treatment of senile dementia

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## Introduction

Alzheimer's disease is a progressive neurodegenerative disorder, being characterized clinically by progressive cognitive deterioration, neuropathologically by neurofibrillary tangles and senile plaques, and neurochemically by degeneration of basal cholinergic neurons in the cerebral cortex and hippocampus. Investigation of damage to the forebrain cholinergic system has been a focus of attempts to understand the cognitive deficits in Alzheimer's disease and treatment has so far been primarily based on the use of cholinergic agents. However, several recent studies have shown that some neuropeptide-containing neurons populations are abnormal in Alzheimer's disease. Therefore, enhancement of peptidergic neurotransmission by the inhibition of certain peptidases may have therapeutic value in this disease. Many biological neuropeptides contain proline within their amino acid sequence, and prolyl endopeptidase (PEP) that hydrolyzes peptide bonds on the carboxyl side of L-proline residues has been proposed to play a role in the metabolism of proline-containing neuropeptides like substance P (SP), arginine-vasopressin (AVP), and thyrotropin-releasing hormone (TRH). There is considerable evidence that these proline-containing neuroactive peptides are capable of enhancing learning and memory, and that brain levels of these neuropeptides are significantly reduced in Alzheimer's disease. In investigations aimed at developing a treatment for senile dementia including Alzheimer's disease, and cerebrovascular dementia, we developed a novel PEP inhibitor, (S)-2-[[(S)-2-(hydroxyacetyl)-1-pyrrolidinyl]carbonyl]-N-(phenylmethyl)-1-pyrrolidinecarboxamide (JTP-4819).

## Materials and Methods

Male Wistar rats (250-280g) and male Fisher rats (3-monthe-old: 250-310; 24-month-old: 360-550g) were used.

*Assay of PEP activity* Rat brain samples were homogenized with 0.1 M phosphate buffer (pH 7.0). After centrifugation (10,000g, 20 min), the supernatant was preincubated with JTP-4819 at 30℃ for 30 min. The reaction was initiated by adding 200 $\mu$ M 7-(Suc-Gly-Pro)-4-methylcoumarinamide at 30℃ for 60 min and stopped by adding 1 M sodium acetate. The formation of 7-amino-4-methylcoumarinamide was determined by fluorescent spectrometer (ex. 370 nm; em. 460nm).

*Degradations of SP, AVP and TRH* Rat brain samples were homogenized in 10 volumes of 20 mM Tris-HCl buffer (pH 7.0) containing 0.32 M sucrose and centrifuged at 1,000 g for 10 min.The supernatant was centrifuged at 11,500 g for 20 min and the resulting supernatant were used to measure the degrading activity for SP, AVP, and TRH.The supernatant fractions were preincubated for 15 min at 37℃ with 80 $\mu$ l of 20 mM Tris-buffer (pH 7.0) plus 10 $\mu$ l of the buffer or JTP-4819. After preincubation, the reaction mixture was incubated with 10 $\mu$ l of 0.5 mM SP, 1.0 mM AVP, or 5.0 mM TRH for 15 min, 5 min, or 10 min, respectively. Then, the reaction was stopped by adding 20 $\mu$ l of 0.5% trifluoroacetic acid and mixture was let stand on ice for 15 min. After centrifugation at 11,500 g for 10 min, the supernatant was used for the assay of neuropeptides by high-performance liquid chromatography with ultraviolet detection.

*Determinations of SP, AVP and TRH* The enzyme immunoassays (EIA) for SP and AVP, respectively, were based on the double antibody method. A 10 mM phosphate-buffered saline solution (pH 7.2) containing 0.1% BSA, 200 KIU/ml aprotinin was used as the EIA buffer. The second antibody was diluted with 0.1M borate buffer (pH 8.6) containing 0.1% BSA and 4% PEG 6000. Anti-SP or anti-AVP antiserum, SP- or AVP- horseradish peroxide conjugate, and SP or AVP sample, respectively, were incubated for 24 hr at 4 ℃. After addition to the second antibody solution, the mixture was allowed to stand for 10 min at room temperature. Free and bound enzyme conjugates were separated by centrifugation (1,500 g, 50 min) at 4 ℃, and the precipitate was mixed with a substrate solution (2.8 mg/ml o-phenylenediamine in 0.1 M phosphate-citrate buffer, pH 5.0 containing 0.01% hydroperoxide). After 30 min incubation, the reaction was stopped by adding 1 N sulfuric acid. The absorbance was read in a dual-wavelength analyzer (492 nm). The radioimmunoassay for determination of TRH was performed. Namely, each sample was suspended in 20 mM phosphate-buffered saline (pH 7.4) containing 0.5% BSA and 0.03% Tween 20. Then, 0.1 ml each of sample, anti-TRH and $^{125}$I-TRH were mixed and incubated for 22 hr at 4 ℃. After incubation, 0.5 ml of Amerlex-M was added, and mixture was let stand for 30 min and centrifuged at 1,000 g for 10 min. Then the radioactivity of the precipitate was counted using a $\gamma$ −counter.

*Morris water maze* The water maze consisted of a circular pool (diameter: 144 cm, height: 45 cm) with a featureless blue inner surface. The pool was filled to a depth of 25 cm with 23 ℃ water. The hidden platform was a clear Plexiglas stand (diameter: 10 cm) submerged 2 cm below the water surface so that it was invisible at water level. At the begining of the experiment, the rats were allowed to swim freely for 1 min to become habituated to the apparatus. From the next day, each animal underwent one acquisition trial, and were allowed to rest on the platform for 30 sec after they found it. Rats which failed to find the platform in the time allowed were placed onto it by the experimenter. The platform's position and start point were kept constant during the acquisition trials. Each session was performed at 1 hr after JTP-4819 administration to aged rats and before the administration to middle cerebral artery occuluded rats, respectively. A video Image Analyzer was used to measure the escape latency and swimming path length to the platform.

*Passive avoidance studies* A one-trial passive avoidance test was performed. Scopolamine (0.5 mg/kg) was administered s.c. 30 min prior to the acquisition trial. JTP-4819 was administered p.o. 1 hr before the acqusition trial or the retention trial. SP was administered s.c. immediately after the acquisition trial, and AVP and TRH, respectively, were administered s.c. 1 hr prior to retention trials.

*Microdialysis study* The rats were anesthetized with pentobarbital sodium (young rats : 40 mg/kg, i.p.; aged rats: 20 mg/kg, i.p.). The skull was exposed and a stainless steel guide cannula was implanted into the left frontal cortex (A +3.2, L -3.0, V -1.5 mm) or left hippocampus (A -6.5, L -4.9, V -3.5 mm), according to the coordinates in the atlas of Paxinos and Watson (1986). Perfusion experiments with a unilateral probe inserted into the each brain region were carried out between 26-48 hr after surgery. Perfusion was performed at a constant rate of 2 $\mu$ l/min with Ringer's solution (mM: NaCl 147; $CaCl_2$ 3.4; KCl 4.0; pH 6.1) containing 10 $\mu$ M physostigmine.

Results and discussion

JTP-4819 strongly and specifically inhibited PEP activity in the rat brain, with an $IC_{50}$ value of 0.8 nM, and exhibited a high degree of *in vitro* stability in plasma. Enzyme activity studies performed *ex vivo* showed that JTP-4819 (3 mg/kg, p.o.) significantly inhibited PEP activity in the cerebral cortex , hippocampus, corpus striatum, etc. of rats. JTP-4819 also inhibited the degradation of SP, AVP, and TRH by PEP in rat cortical supernatants (with $IC_{50}$ values of 4.0, 2.1, and 1.4 nM, respectively) and rat hippocampal supernatants (with $IC_{50}$ values of 3.3, 2.8, and 1.9 nM, respectively). Hippocampal SP-like immunoreactivity (SP-LI) was found to be significantly decreased in aged rats, and oral administration of JTP-4819 (1 mg/kg) for 21 days produced a significant increase of SP-LI. Cerebral and hippocampal TRH-LI were also significantly decreased in aged rats. Oral administration of JTP-4819 at a dose of 1 mg/kg caused a significant increase of cortical TRH-LI, while hippocampal TRH-LI was increased significantly at doses of 0.3 and 1 mg/kg. A study using the Morris water maze task demonstrated that oral administration of JTP-4819 (1.0 mg/kg, p.o.) improved memory deficits in aged rats after 14 days and in rats with middle cerebral artery occulusion after 10 days. In addition, in the one-trial passive avoidance test in rats with scopolamine-induced amnesia, oral JTP-4819 significantly prolonged the retention time when doses of 1 and 3 mg/kg were given 1 hr before acquisition or doses of 3 and 10 mg/kg were given 1 hr before retention. Furthermore, coadministration of JTP-4819 and SP, AVP, or TRH (at dose levels where each agent alone did not prolong the retention time) improved the retention time in scopolamine-treated rats, suggesting that its action may be based on the activation of peptidergic neurons. In view of the reported hypofunction of cholinergic neurons in Alzheimer's disease, a microdialysis study was performed to clarify the effects of JTP-4819 on central cholinergic neurons. Oral doses of 1 and 3 mg/kg caused a significant increase of ACh release from the frontal cortex and hippocampus, suggesting that JTP-4819 also activates cholinergic neurons in the brain.

Taken together, these unique and potent pharmacological actions of JTP-4819 suggest that it may be of therapeutic value for senile dementia related to Alzheimer's disease.