·Original Article·

A novel derivative of xanomeline improved memory function in aged mice

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Abstract: Objective To characterize the function of a new xanomeline-derived M1 agonist, 3-[3-(3-florophenyl-2-propyn-1-ylthio)-1,2,5-thiadiazol-4-yl]-1,2,5,6- tetrahydro-1-methylpyridine Oxalate (EUK1001), the acute toxicity and the effects on synaptic plasticity and cognition of EUK1001 were evaluated. **Methods** To examine the median lethal dose (LD50) of EUK1001, a wide dose range of EUK1001 was administered by p.o. and i.p. in aged mice. Furthermore, novel object recognition task and *in vitro* electrophysiological technique were utilized to investigate the effects of EUK1001 on recognition memory and hippocampal synaptic plasticity in aged mice. **Results** EUK1001 exhibited lower toxicity than xanomeline, and improved the performance of aged mice in the novel object recognition test. In addition, bath application of 1 μmol/L EUK1001 directly induced long-term potentiation in the hippocampus slices. **Conclusion** We conclude that EUK1001 can improve the age-related cognitive deficits.

Keywords: xanomeline; EUK1001; LD50; hippocampus; long-term potentiation; memory

1 Introduction

Age-related cognitive decline diseases, such as Alzheimer's disease (AD, the most common form of degenerative dementia), are characterized by progressive impairment in memory and cognitive function during mid- and late-adult life[1]. The loss of basal forebrain cholinergic neurons is likely the cause of profound memory deficit in AD patients[2].

The cholinergic approach to treatment of AD involves compensating cholinergic deficiency by pharmacological intervention to increase cholinergic transmission. Inhibitors of acetylcholinesterase (AChE), such as tacrine, were used to be an effective attempt to correct cholinergic deficiency in some AD cases[3]. But this approach is restricted due to the progressive loss of cholinergic neurons[4]. It has been reported that there are no changes in the number of muscarinic receptors in AD[5]. In addition, considerable evidence has demonstrated the important role of M1 muscarinic receptors in modulating AD pathology[6]. M1 muscarinic receptor agonists could directly activate muscarinic receptors to ameliorate cognition dysfunction and modulate beta-amyloid together with tau phosphorylation. This unique property of M1 agonists to alter different aspects of AD pathogenesis could represent the most remarkable clinical value of such compounds. The first generation of muscarinic agonists, such as arecoline, pilocarpine, bethanechol and oxotremorine, failed to demonstrate consistent clinical efficacy, mainly due to the narrow safety margins and low intrinsic activity[4,7]. Based on these, the second generation of M1 agonists is being designed. Among them, xanomeline showed distinguished potential in treatment of AD. Binding studies demonstrated that this compound was a subtype of selective M1 agonists with high affinity and remarkable potency in the interaction with M1 receptors[8,9]. *In vitro* investigation also showed its notable efficacy in AD-like pathology[10]. In phase II of clinical trials in AD patients, xanomeline significantly improved several measures of cognitive function, however, unwanted side effects were observed[11]. Therefore, the clinical utility of this compound was limited.

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In order to obtain compounds that can exhibit greater potency and lower toxicity, a series of modifications on the basic structure of xanomeline was performed. Based on our preliminary *in vitro* test, a novel derivative of xanomeline, 3-[3-(3- florophenyl-2-propyn-1-ylthio)-1,2,5-thiadiazol-4-yl]-1, 2,5,6-tetrahydro-1- methylpyridine Oxalate (EUK1001), was chosen for further study to evaluate its toxicity property as well as its effects on memory and hippocampal synaptic plasticity in aged mice.

2 Material and methods

2.1 Compounds EUK 1001 and xanomeline were synthesized in the laboratory of Dr Xiao-Ping LEI at College of Medicine, Peking University. All other chemicals were purchased from Sigma Chemical Co. (St. Louis, USA).

2.2 Animal care Eight weeks old Imprinting Control Region (ICR) mice (n = 190), weighing (20 ± 4) g, were used in the toxicity studies, while 15-18 months old male C57 BL/6J mice (n = 36), weighing about 30 g, were used in the behavioral and electrophysiological studies. All mice were healthy.

All mice were kept in a vivarium at (22 ± 3) ºC, 40-70% humidity on a 12-h light/dark cycle, and had free access to standard chow and water. All animal procedures were approved by the East China Normal University Committee for the Care and Use of Laboratory Animals.

2.3 Toxicity studies It was reported the median lethal dose (LD50) of xanomeline was 100 mg/kg p.o. and 75 mg/kg i.p.[12] To assess the LD50 of EUK1001, single dose acute toxicity study was applied. All procedures were designed following the principle of guidance for industry provided by Center for Drug Evaluation and Research (CDER). Eight weeks old ICR mice were observed for 1 week prior to testing, and were fasted by withholding food for 12 h prior to dosing. Mice were administered EUK1001 either through p.o. by gavage with vehicle control and doses (in mg/kg) of 1000, 800, 640, 512, 496, 396, 360, 345, 320 and 256, or through i.p. injection with vehicle control and doses (in mg/kg) of 156, 125, 100, 80, 51.2, 49.6 and 39.7. Ten mice (5 mice per gender) were included in each dose. The initial dose and the subsequent doses were selected according to the preliminary experiment. Drugs were dissolved in physiological saline with 2% tweenum-80. The mice administered only saline with 2% tweenum-80 were used as control. The volume via both i.p injection and p.o. administration of drug for each mouse was 0.4 mL.

The animals were observed individually every day after dosing for one week and were recorded with mortality and clinical signs. Particular attention was paid to symptoms associated with hyper-activation of peripheral cholinergic systems, including tremors, lacrimation, salivation and diarrhea.

2.4 Novel object recognition test Novel object recognition test was performed as described by Cao X *et al*.[13] Briefly, mice were handled individually and habituated to an open field box (500* 500* 250 mm) for 5 min daily for 3 d. During the training session, two objects were placed in the center of the box and each mouse was given 5 min to explore. The time spent in exploring each object was recorded. Then the mouse was returned to its home cage. During the retention test, the trained mouse was again placed in the center of the box, wherein one of the familiar objects was replaced by a novel object, and was given 5 min to explore. The ratio of the amount of time spent in exploring the novel object to the total time spent in exploring both objects (preference index) was calculated to evaluate the recognition memory of each mouse.

2.5 Hippocampal slice recording Hippocampal slices were prepared from the aged C57BL/6J mice according to procedures previously described[13]. Briefly, the mice were rapidly decapitated and brain slices containing the hippocampus were extracted using a vibratome. The slice preparations were then incubated in a recovery chamber for at least 1 h at 30 ºC and an additional hour at 25 ºC. The chamber was filled with the oxygenated (95% O_2 and 5% CO_2) artificial cerebrospinal fluid (ACSF) in pH 7.4 consisting of (in mmol/L) NaCl 124, KCl 4.4 , $CaCl_2$ 2.0, $MgSO_4$ 1.0, $NaHCO_3$ 25, Na_2HPO_4 1.0, and glucose 10. Prior to recording, the chamber was heated to 30 ºC and superfused at a rate of 2.5 mL/min with ACSF. A stimulating electrode was placed in stratum radiatum of CA3 and stimulation was delivered at 0.033 Hz with a current intensity set to evoke half maximum field excitatory postsynaptic potential (fEPSP) amplitude. An extracellular recording electrode filled with sky blue (1-5 MΩ tip resistance) was placed in the stratum radiatum of CA1 region for recording of fEPSPs. Data were pooled and normalized with respect to the steady baseline values (Baseline = 100%).

2.6 Statistical analysis Data were expressed as mean±SEM. In the toxicity studies, LD50 value was calculated by Bliss method. The significance of differences in the behavioral

test was determined by one-way ANOVA, followed by Dunn's (Bonferroni) *post hoc* test for multiple comparisons. A student's *t*-test was applied to determine the effects of drugs on fEPSPs in the electrophysiological experiment. $P < 0.05$ was considered statistically significant.

3 Results

3.1 Toxicity of EUK 1001 in mice Single dose acute toxicity testing for EUK1001 was examined in mice during the first 24 h after dosing. All mice treated by both p.o. and i.p. with a wide range of EUK1001 doses manifested typical cholinergic side effects, i.e. tremors, lacrimation, salivation, diarrhea and rearing. The results of p.o. (Table 1) and i.p. (Table 2) acute toxicity studies were summarized, and LD50 value was calculated using Bliss method (Fig. 1). The LD50 of EUK1001 by p.o. administration was determined to be 307.27 mg/kg (Feiller correct: 95% confidence limit = 266.63–332.86 mg/kg) (Fig. 1A), while the LD50 of EUK1001 by i.p. injection was determined to be 91.094 mg/kg (Feiller correct: 95% confidence limit = 69.177–136.65 mg/kg) (Fig. 1B).

All surviving animals were observed daily for one week after drug dosing. During the consecutive 7 d, the body weight of survived mice showed no significant changes. Death was observed in each dose both i.p and p.o. in the following 7 d period.

3.2 Effects of drugs on object recognition memory in aged mice To evaluate the effects of EUK1001 on age-related cognition decline, a novel object recognition task was conducted. Based on the toxicological and pharmacological data, an atoxic dose (0.5 mg/kg) was chosen. Aged C57 BL/6J mice were given daily i.p. injections with saline, 0.5 mg/kg

Table 1 Acute toxicity of EUK 1001 (p.o.)

Dosage (mg/kg)	Log Dosage	Animal (*n*)	Died (*n*)	Mortality (%)	Probability unit	Conf. limit
496	2.6955	10	10	100	-	7.2842
396	2.5977	10	9	90	6.2817	6.2101
360	2.5563	10	9	90	6.2817	5.7555
345	2.5378	10	7	70	5.524	5.5525
320	2.5051	10	3	30	4.476	5.1936
256	2.4082	10	3	30	4.476	4.1291

Table 2 Acute toxicity of EUK 1001 (i.p.)

Dosage (mg/kg)	Log Dosage	Animal (*n*)	Died (*n*)	Mortality (%)	Probability unit	Conf. limit
156	2.1931	10	9	90	6.2817	5.6388
125	2.0969	10	5	50	5	5.3758
100	2	10	5	50	5	5.1108
80	1.9031	10	4	40	4.7471	4.8458
51.2	1.7039	10	3	30	4.476	4.3158
49.6	1.6955	10	2	20	4.1585	4.2781
39.7	1.5988	10	2	20	4.1585	4.0137

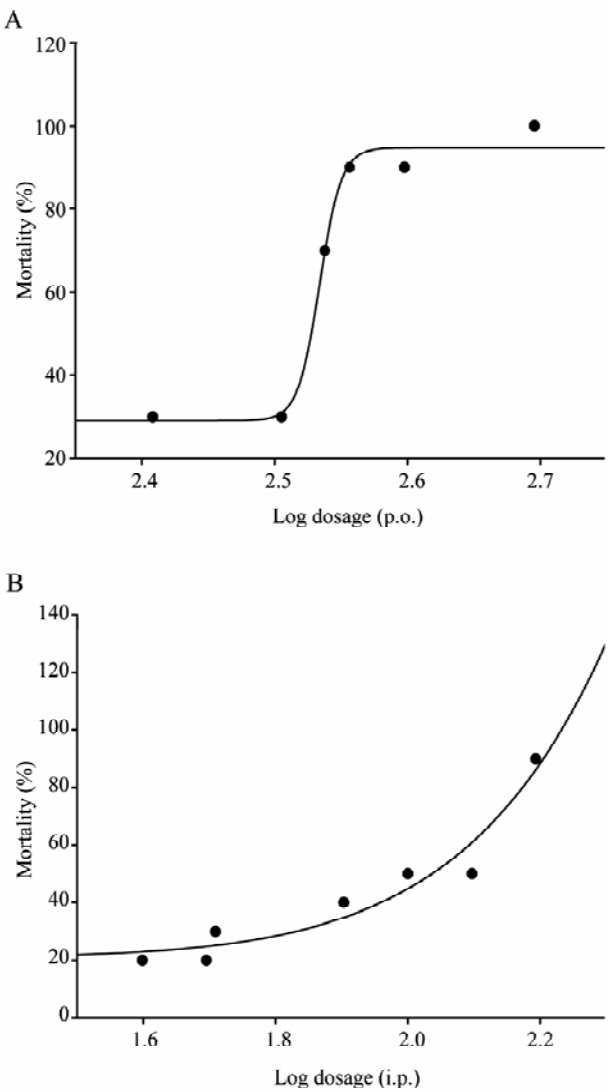

Fig. 1 Mortality was calculated by a regression equation to a standard curve to assess the LD50 value in acute toxicity study. A: LD50 of EUK1001 (p.o.) was calculated by Bliss method ($n = 60$). Regression equation is y (Probit) = –22.323 + 10.984 Log x (Dosage). B: LD50 of EUK1001 (i.p.) was calculated by Bliss method ($n = 70$). Regression equation is y (Probit) = –0.35801 + 2.7344 Log x (Dosage).

xanomeline or 0.5 mg/kg EUK1001 for 15 d prior to behavioral testing. Initially, the locomotor activities of all mice were assessed by measuring the distance traveled and the total time of movement during a 15-min period. There was no significant difference in the distance traveled and the total time of movement among the groups (data not shown).

During training session of novel object recognition, treatment with xanomeline (0.5 mg/kg) or EUK1001 (0.5 mg/kg) did not significantly (0.5 mg/kg) alter time spent in exploring objects compared to saline-injected controls [Fig. 2A, $F(2, 24) = 0.98$, $P > 0.05$]. However, *post hoc* analysis indicated that aged mice treated with EUK1001 (0.5 mg/kg) or xanomeline (0.5 mg/kg) had the significantly stronger exploratory preference for the novel object than control group in the one-hour retention test [Fig. 2B, $F(2, 24) = 7.2$, $P < 0.01$]. In one-day retention test, only those aged mice treated with EUK1001 enhanced the exploratory preference significantly (Fig. 2C, Bonferroni test, $P < 0.05$). There was no significant difference in exploratory preference among three groups during two-day retention test (Fig. 2D). These data demonstrate that EUK1001, like xanomeline, can significantly improve cognitive function in aged mice.

3.3 Effect of EUK1001 on hippocampal synaptic plasticity in aged mice To understand the potential mechanism by which EUK1001 enhanced memory function of aged mice, we studied the effects of EUK1001 on plasticity at CA3-CA1 synapses of hippocampal slices in aged mice. The effects of the muscarinic agonist EUK1001 on fEPSPs of CA3-CA1 were characterized in submerged slices of mice hippocampus by extracellular recording method. Superfusion with 1 μmol/L EUK1001 for 20 min induced a gradual, long lasting increase in the slope of the fEPSP compared to the baseline slope [Fig. 3, (153.6 ± 15.4)%, $n = 9$; $P < 0.01$, *vs* baseline]. The result demonstrates that EUK1001 can directly induce LTP.

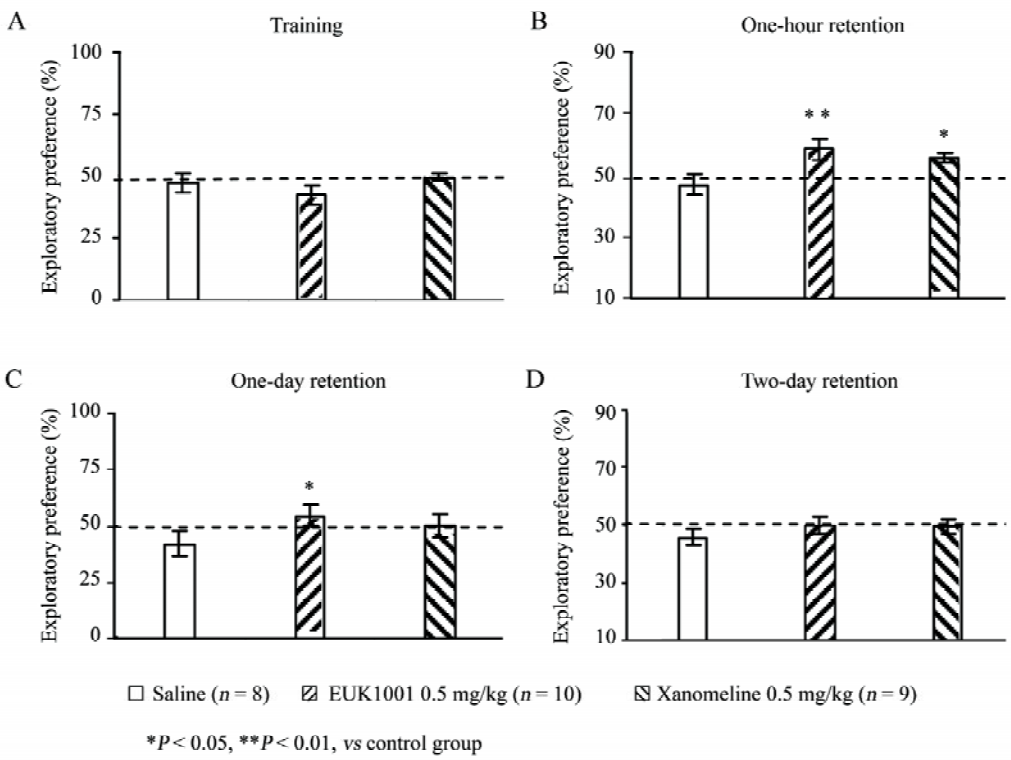

Fig. 2 Effects of xanomeline and EUK1001 on novel object recognition task in aged mice. A: Both xanomeline (0.5 mg/kg) and EUK1001 (0.5 mg/kg) had no effects on basal exploratory behavior of mice. B: Both xanomeline (0.5 mg/kg) and EUK1001 (0.5 mg/kg) improved object recognition memory in one-hour retention test in aged mice. C: EUK1001 (0.5 mg/kg) significantly improved object recognition memory while xanomeline (0.5 mg/kg) dose not show any improvement in one-day retention test in aged mice. D: Neither EUK1001 (0.5 mg/kg) and xanomeline (0.5 mg/kg) showed significant differences in object recognition memory in two-day retention test in aged mice compared to saline control group. *$P < 0.05$, **$P < 0.01$ *vs* control, one-way ANOVA and *post hoc* analysis.

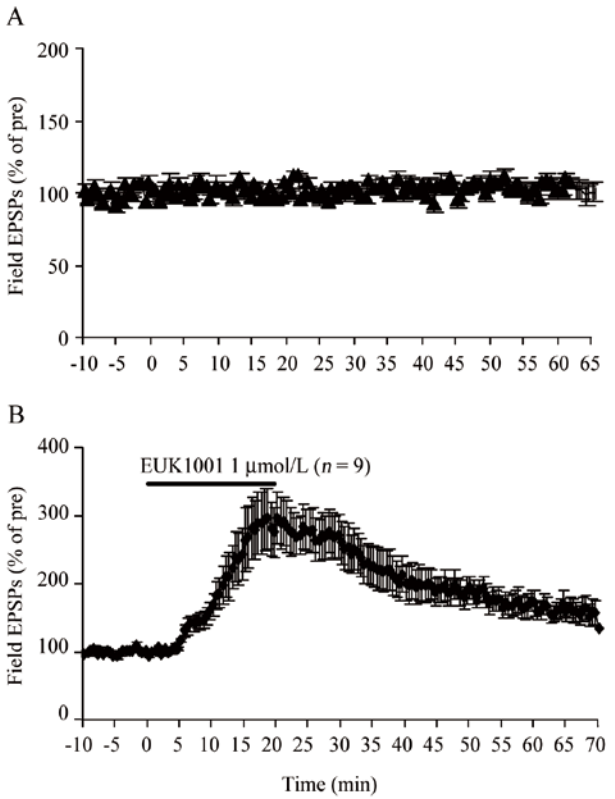

Fig. 3 Effects of EUK1001 on EPSPs of hippocampal CA3-CA1 in aged mice. A: EPSPs were evoked from CA3-CA1 of mice hippocampal slices submerged in normal ACSF without drugs. B: EUK1001 (1 μmol/L) added to hippocampal slices induced enhancement of the EPSPs slope [(153.6±15.4)%, n = 9, P < 0.01 *vs* baseline, Student's *t*-test].

4 Discussion

In this report we have provided evidence that administration of a novel xanomeline derivative, EUK1001, can increase cognitive function and directly induce LTP in hippocampal slices in aged mice. Previous reports have shown that M1 agonists, such as xanomeline[12], WAY-132983[14], CI979[15] and WAL2014[16], could significantly ameliorate memory decline in aged mice and cognition-impaired mice. The usefulness of these drugs has been limited, however, by the significant side effects caused by hyper-activation of peripheral cholinergic pathways[12,14-16], including salivation, lacrimation, tremors and diarrhea[12].

Due to the shortcomings of the clinical value of these drugs, we set about generating the new derivatives of xanomeline. Moreover, in acute toxicity studies, we found that the LD50 of EUK1001 were 307.27 mg/kg p.o. and 91.09 mg/kg i.p., which were higher than the LD50 of xanomeline (100 mg/kg p.o. and 75 mg/kg i.p.)[12], which suggests that EUK1001 may produce lower toxicity. Our additional study has shown that EUK1001 had less side effects than xanomeline (This result will be published in Cell Research)[17].

Novel object recognition task is a particularly useful method for assessing memory function in aged mice because it does not require food or water deprivation and keep the animals under relatively normal physiological conditions. Our study has shown that 0.5 mg/kg EUK1001 by i.p. injection on mice, which did not cause any significant side effect typically associated with hyper-activation of peripheral cholinergic pathways, improved the recognition memory of aged mice in one-hour retention test of novel object recognition task. In particular, compared to xanomeline, EUK1001 exhibited significantly improved effect on recognition memory of aged mice in one-day retention test.

Novel object recognition task could test hippocampus-dependent memory function[18]. The modulatory effects of acetylcholine on hippocampal synaptic plasticity have been documented previously. For example, activation of M1 receptors in the dentate gyrus of rats could facilitate the induction of LTP[19]. Furthermore, the selective M1 agonist, SDZ ENS 163, enhanced LTP in area CA1 of hippocampus[20], while the muscarinic antagonist atropine suppressed associative LTP[21]. In order to understand the mechanism of EUK1001-induced enhancement of memory function in aged mice, effects of EUK1001 on hippocampal synaptic plasticity were examined. Perfusion of 1 μmol/L EUK1001 onto hippocampal slice preparations induced a LTP-like response, which suggests that there is the physiological correlation between enhancement of memory function and synaptic plasticity by EUK1001.

It has been demonstrated that M1 muscarinic receptors are relatively well preserved in brains of AD patients[22] and therefore M1 receptors are considered to be an attractive therapeutic target for treatment of the disease. Similar to human aging and AD, rodents also show the considerable age-related cognitive decline associated with depreciation of cholinergic function[23]. Thus aged mice provide a suitable system for evaluating strategies to supplement cholinergic malfunction in basal forebrain nuclei[24]. Taken together, our results suggest that EUK1001, the new high-affinity M1 agonist, may represent a promising therapeutic agent for the treatment of AD and age-related memory disorders.

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一种新的咕诺美林衍生物改善老年小鼠的记忆能力

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摘要：目的 为了分析 EUK1001- 新的咕诺美林衍生物的功能性质，本实验以老年小鼠为实验材料，研究了该化合物的急性毒理以及对突触可塑性和识别记忆的影响。**方法** 通过口服及腹腔注射途径，对小鼠进行梯度剂量的毒理学实验，测定EUK1001的半致死剂量(median lethal dose, LD50)；采用新奇物体识别任务和离体脑片电生理学技术研究EUK1001对老年小鼠识别记忆和海马突触可塑性的影响。**结果** EUK1001 比咕诺美林呈现出更小的毒副作用。在新奇事物识别实验中，EUK1001 能够显著改善老年小鼠在识别记忆任务中的表现。此外，海马脑片灌流 1 μmol/L 的 EUK1001，能直接诱导产生长时程突触增强(long-term potentiation)。**结论** EUK1001 能够改善正常老龄化过程中学习记忆能力的衰退。

关键词：咕诺美林；EUK1001；半致死剂量；海马；长时程增强；记忆