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Effects of a memory enhancing peptide on cognitive abilities of brain-lesioned mice: additivity with huperzine A and relative potency to tacrine

ZHIWEN XU, HUI ZHENG, SEK LUN LAW, DONNA DONG SO, YIFAN HAN and HONG XUE*

Department of Biochemistry, Hong Kong University of Science and Technology, Hong Kong, China

Abstract: Alzheimer's disease (AD) and related dementing disorders having cognitive manifestations represent an increasing threat to public health. In the present study, the effects of a memory enhancing NLPR tetra-peptide (MEP), huperzine A (Hup A), or a combination of the two on the cognitive abilities of brain-lesioned mice were evaluated and compared with tacrine in the passive avoidance and Y-water maze tests for the acquisition and retention aspects of cognitive functions. MEP at $\mu g\ kg^{-1}$ doses, and Hup A or tacrine at $mg\ kg^{-1}$ doses significantly reversed the cognition deficits induced by scopolamine. For acquisition ability, it was observed that mice administered with MEP ($4.0\ \mu g\ kg^{-1}$) spent less time escaping onto the platform in the water maze than those treated with tacrine ($1.5\ mg\ kg^{-1}$); whereas for memory retention, tacrine-administration resulted in a higher step-through latency in mice at the tested dose regime. In addition, co-administration of MEP ($2.0\ \mu g\ kg^{-1}$) and Hup A ($0.1\ mg\ kg^{-1}$) exhibited an additive effect resulting in considerable improvements in both acquisition and retention abilities of brain-lesioned mice. The results demonstrated that MEP was highly efficient in the rescue of cognitive abilities of brain-lesioned mice and in particular, the effective doses of MEP were about two orders of magnitude lower than that of tacrine, a therapeutic currently used in the treatment of AD. Moreover, MEP and Hup A were effective at reduced doses when the two were co-administered, providing a rationale for their combined usage in the treatment of cognitive deficits. Copyright © 2005 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: brain-lesioned mice; huperzine A; memory enhancing peptide; passive avoidance response; scopolamine; tacrine; Y-water maze

INTRODUCTION

Patients with AD gradually lose their cognitive and psychomotor abilities [1]. The decrease in the concentration of acetylcholine in the brain, resulting in a marked reduction of cholinergic neuronal functions [2], has been associated with the impairment of memory in AD patients [3]. Acetylcholinesterase inhibitors are effective in treating cognitive and memory deficits by delaying the hydrolytic action of acetylcholinesterase and thus increasing the concentration of active acetylcholine [4,5]. On the other hand, anti-cholinergic drugs, such as scopolamine, can disrupt cognitive function and memory in humans and animals [6,7].

Tacrine, a well-known and centrally acting acetylcholinesterase inhibitor [8], is currently used in the treatment of AD [9]. However, its clinical efficacy is limited by dose-dependent liver toxicity [10,11]. As a neurotransmitter/neuromodulator in the brain, arginine-vasopressin contributes to the stimulation of inositol phospholipid metabolism in rat hippocampus [12] and is involved in the modulation of memory [13]. It has been found to facilitate the acquisition and maintenance of cognitive ability and memory in rats, but

with side effects on peripheral receptors, affecting blood pressure, heart rate, urine flow and smooth muscle activity [14]. Therefore, it is desirable to search for new drugs with a high therapeutic index and minimal side effects.

Hup A is a naturally occurring alkaloid isolated from the club moss *Huperzia serrata* [15]. It is a reversible acetylcholinesterase inhibitor [16], and a concentration-dependent inhibitor of cholinesterase [17]. Hup A increases the concentration of acetylcholine at the neuronal synaptic cleft by the inhibition of acetylcholine hydrolysis and consequently improves neuronal transmission [15]. It is currently in phase III trials in China for the treatment of AD [18], and is marketed in USA as a dietary supplement [19]. Hup A has a long half-life, good penetratability through the blood–brain barrier and minimal side effects [19].

In addition, memory enhancing peptides, analogues of arginine-vasopressin have been found to improve cognitive abilities in rats without the peripheral side effects, and are proposed as potential replacements of arginine-vasopressin [20–22]. For example, arginine-vasopressin fragment 4–9 was shown to stimulate acetylcholine release in the hippocampus of rats [23], and more recently, another peptide, arginine-vasopressin fragment 4–8 was shown to enhance nerve growth factor gene expression in the hippocampus and cerebral cortex [24]. Furthermore, Fujiwara *et al.*

* Correspondence to: H. Xue, Department of Biochemistry, Hong Kong University of Science and Technology, Clear Water Bay, Hong Kong, China; e-mail: hxue@ust.hk

reported that the efficacy of an arginine-vasopressin fragment was about 1000 times higher than arginine-vasopressin itself in recovering the scopolamine-induced disruption of spatial cognition [25].

A memory enhancing peptide, MEP, was previously reported to reverse memory-deficits in rats [16,26]. However, no comparative studies have been performed between MEP and the currently in-use therapeutics, tacrine, or between the therapeutic candidates MEP and Hup A. In the present study, brain-lesioned mice were administered with MEP, Hup A or tacrine, and their acquisition and retention abilities were examined in the step-through passive avoidance test and in the Y-water maze test. In addition, the effects of the co-administration of MEP and Hup A on the cognitive abilities of brain-lesioned mice were also investigated in these two behavioral models.

MATERIALS AND METHODS

Animals

Male Kunming mice weighing 16–25 g (4 weeks old) were used. Mice were housed five in a cage, given food and water *ad libitum*, and exposed to 12 h light/12 h dark cycles. The temperature was kept at $22° \pm 1°C$, and the relative moisture at 50%–55%.

Drugs

MEP, an analogue of arginine-vasopressin from which the active fragment contributing to the peripheral effects was removed, was synthesized in the Department of Biochemistry, Hong Kong University of Science and Technology. It is a tetra-peptide NLPR (Asn-Leu-Pro-Arg). Tacrine (9-amino-1,2,3,4-tetrahydroacridine) was purchased from Sigma Chemical Company. Hup A was isolated from the Chinese herb *Huperzia serrata* by the Shanghai Institute of Material Medica, Chinese Academy of Science, with a purity over 98%. Its structure was verified to be (5R,9R,1E)-5-amino-11-ethylidene-5,6,9,10-tetrahydro-7-methyl-5,9-methanocycloocta[b]-pyridin-2(1H)-one. The drugs were dissolved in saline (0.9% NaCl) at appropriate concentrations to give the required doses.

Scopolamine-induced Brain-lesion in Mice

Scopolamine hydrobromide, a muscarinic antagonist, was purchased from the Shanghai Hefeng Medicine Manufacture Company. To induce brain lesions, scopolamine (4.0 mg kg^{-1}) was administered i.p. to each mouse 40 min before testing [7]. Ten minutes after the injection of scopolamine, thus 30 min before testing, each group of ten mice were administered i.p. with respective drugs or saline. In addition, to evaluate the scopolamine effect and the effectiveness of the testing drugs in the rescue of cognitive functions, a non-lesioned group of ten mice was included in each trial, which were pretreated with saline only.

Step-through Passive Avoidance Test

The step-through passive avoidance apparatus consisted of two connected chambers, a dark chamber and an illuminated chamber. The dark chamber (17 cm in length × 17 cm in width ×25 cm in height) was made of dark grey plastic plates and with a floor of copper grid of 1.0 cm intervals. A round door of diameter 3.5 cm in one of the plastic plates connected to the illuminated chamber, which had a plastic platform floor (8.5 cm in length ×3.5 cm in width) and two sides of plastic plates (8.5 cm in length × 3.5 cm in height). The roof of the illuminated chamber was open, as was the side opposite the round door. The round door was the only entry/exit to the dark chamber. A 60 W lamp was positioned 30 cm above the floor of the illuminated chamber during the experiments.

Each mouse was subjected to a training trial, an acquisition trial and a retention trial, successively. In the training trial, the mouse was placed on the platform in the illuminated chamber with its tail toward the round door. As soon as the mouse stepped into the dark chamber from the platform, the door was closed and a 0.3–0.4 mA electric shock was delivered through the grid floor for 5 s. The time each mouse took from standing on the platform to entering the dark chamber and receiving the initial electric shock was recorded and defined as latency. Mice that did not enter the dark chamber within 180 s were removed.

The acquisition trial and retention trial were performed 30 min and 48 h after the training trial, respectively, in a way similar to the training trial except that the electric shock (0.3–0.4 mA) was continuous. The latency and the number of electric shocks received within 5 min resulting from re-entering the dark chamber were recorded.

Y-Water Maze Test

The Y-water maze test was carried out 30 min after the administration of drugs or saline. The Y-water maze apparatus consisted of three connected open-roof arms (40 cm in length × 15 cm in width × 15 cm in depth) arrayed as 'Y'. The apparatus was filled with milk to a depth of 10 cm and at a temperature of $21°–23°C$. On one end of the three arms, a circular platform with a diameter of 3 cm was placed 0.6 cm under the milk, this being the only platform allowing the mice to rest above the milk.

Each mouse was subjected to a training trial, an acquisition trial and a retention trial, successively. In the training trial, each mouse was put into the milk in the Y-water maze at the end of the arm that did not have the platform. The time required for the mouse to swim and find the platform at one of the other two arms and escape from the milk was recorded and defined as the escaping time. The mouse was allowed to remain on the platform for 30 s. Mice that could not find the platform within 300 s were removed.

The acquisition trial and retention trial was performed 30 min and 72 h after the training trial, respectively, in a manner similar to the training trial. The escaping time required and the number of errors or times that a mouse went to the arm that did not have the platform within 5 min were recorded.

Statistical Analysis

Data are expressed as mean ± SEM. Comparisons between the non-lesioned mice and the brain-lesioned control group

were performed by Student's *t*-test. One-way ANOVA followed by Dunnett's multiple comparison test was used for statistical comparison between the drug-treated groups and the brain-lesioned control. To compare the therapeutic effects among different drug treatments, the values in the optimal dose groups were analysed by Tukey's multiple comparison tests. Values of $p < 0.05$ were considered significant.

RESULTS

Step-through Passive Avoidance Test

The brain-lesioned control group showed a significant ($p < 0.01$) decrease in step-through latency and an increase in the number of electric shocks received, compared with the non-lesioned mice, in both acquisition and retention trials of the passive avoidance test (Figures 1 and 2). Such scopolamine-induced cognition impairments in mice were reversed by treatment with MEP, Hup A or tacrine, as shown in Figures 1 and 2. The step-through latency and the number of electric shocks received for the brain-lesioned mice in the acquisition trial are presented in Figure 1A and 1B, respectively. As shown in Figure 1A, mice treated with MEP at 4.0 µg kg^{-1} or Hup A at 0.2 and 0.3 mg kg^{-1} exhibited significantly enhanced ($p < 0.05$) step-through latency in comparison with the brain-lesioned control, indicative of an improved cognitive ability. Although tacrine at a dose of 0.5–1.5 mg kg^{-1} also increased the average step-through latency, the increment was not statistically different from the control ($p > 0.05$). As shown in Figure 1B, mice administered with 1.5 mg kg^{-1} tacrine, 0.1–0.3 mg kg^{-1} Hup A and 2.0–6.0 µg kg^{-1} MEP all displayed a considerable reduction ($p < 0.05$) in the number of electric shocks received compared with the saline-treated brain-lesioned mice.

In the retention trial, the results demonstrated that tacrine at 1.0 and 1.5 mg kg^{-1} caused significant increases ($p < 0.05$) in step-through latency of the brain-lesioned mice (Figure 2A). A similar effect was observed with Hup A at 0.3 mg kg^{-1}, but not with MEP at a dose of 2.0–6.0 µg kg^{-1}. All the tacrine and MEP-treated groups showed a marked reduction ($p < 0.05$) in the number of electric shocks received (Figure 2B). Hup A at 0.2 and 0.3 mg kg^{-1}, but not at the lower concentration of 0.1 mg kg^{-1}, also induced significant decreases ($p < 0.05$) in the number of shocks compared with the brain-lesioned control.

Within the three subgroups of the MEP-treated brain-lesioned mice, the optimal therapeutic effects, indicated by the highest latency and the fewest shocks in mice, were observed with MEP administration at 4.0 µg kg^{-1}, in both the acquisition (Figure 1) and retention (Figure 2) trials. Similarly, the optimal doses for Hup A and tacrine treatment at the tested dose regime were 0.3 mg kg^{-1} and 1.5 mg kg^{-1}, respectively. Upon comparison of the results for the optimal dose
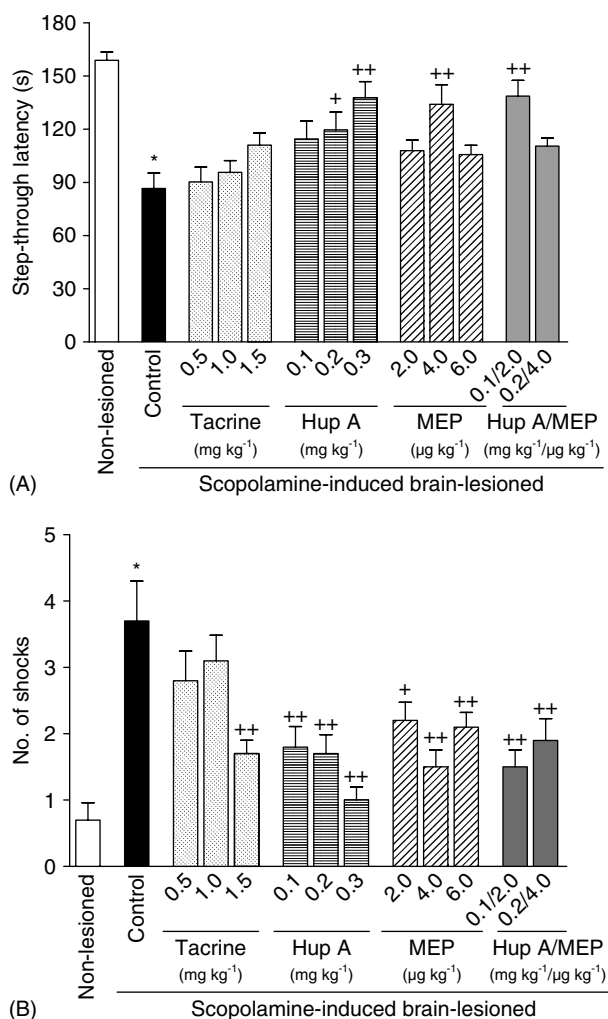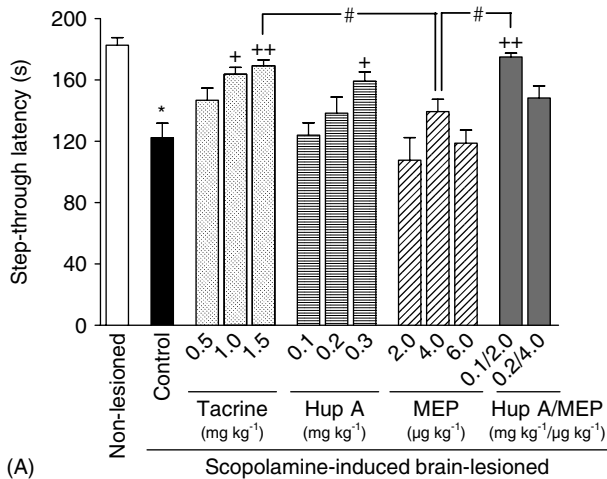

Figure 1 Effects of MEP, Hup A, tacrine and MEP plus Hup A on cognitive ability of brain-lesioned mice in the acquisition trial of the step-through passive avoidance test. Scopolamine-induced brain-lesioned mice ($n = 10$) were treated with saline (control), tacrine, Hup A, MEP or Hup A plus MEP. Values represent mean ± SEM of the step-through latency (A) and the number of electric shocks received (B) for each group of mice. *$p < 0.01$ significantly different from non-lesioned mice, Student's *t*-test; $^+p < 0.05$ and $^{++}p < 0.01$ significantly different from brain-lesioned control, one-way ANOVA followed by Dunnett's multiple comparison test. When the values in the optimal dose groups were analysed by Tukey's multiple comparison test, no statistical significance ($p > 0.05$) was found between the different drug treatments.

of the different drugs, significant variance ($p < 0.05$) in the step-through latency was observed between the MEP-treated and tacrine-treated mice (Figure 2A). This suggested that regarding memory retention, tacrine improved the passive avoidance response of mice better than the test doses of MEP.

Y-Water Maze Test

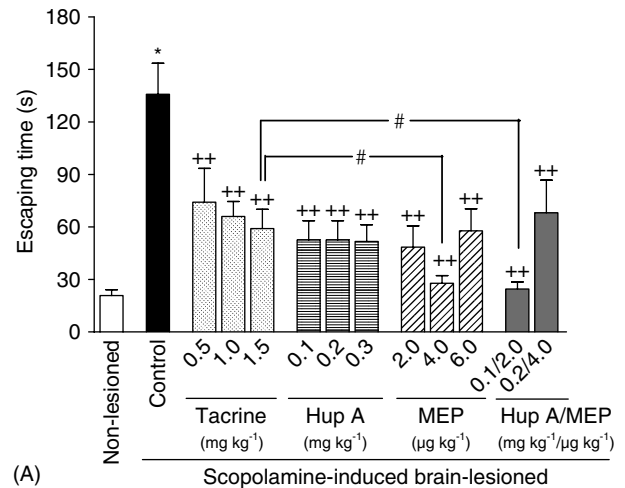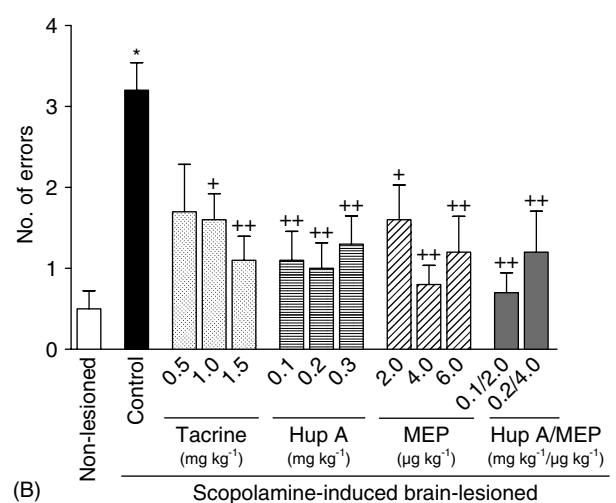The results of the Y-water maze test are shown in Figures 3 and 4 for the acquisition and retention trials,
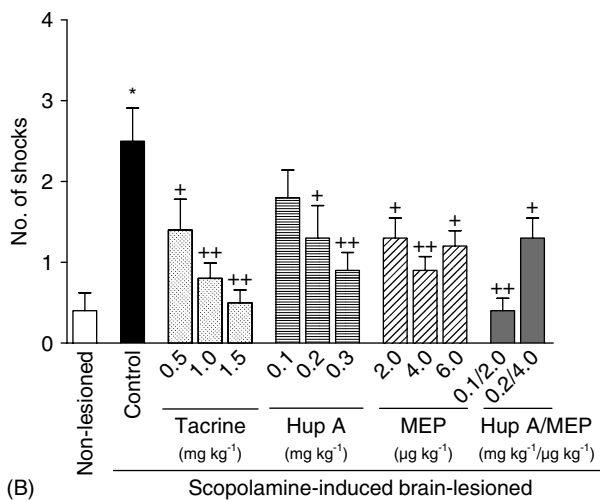
Figure 2 Effects of MEP, Hup A, tacrine and MEP plus Hup A on the cognitive ability of brain-lesioned mice in the retention trial of the step-through passive avoidance test. Values represent mean ± SEM of the step-through latency (A) and number of electric shocks received (B) for each group of mice. $^*p < 0.01$ significantly different from non-lesioned mice, Student's t-test; $^+p < 0.05$ and $^{++}p < 0.01$ significantly different from the brain-lesioned control, one-way ANOVA followed by Dunnett's multiple comparison test; $^{\#}p < 0.05$ significantly different, Tukey's multiple comparison test.
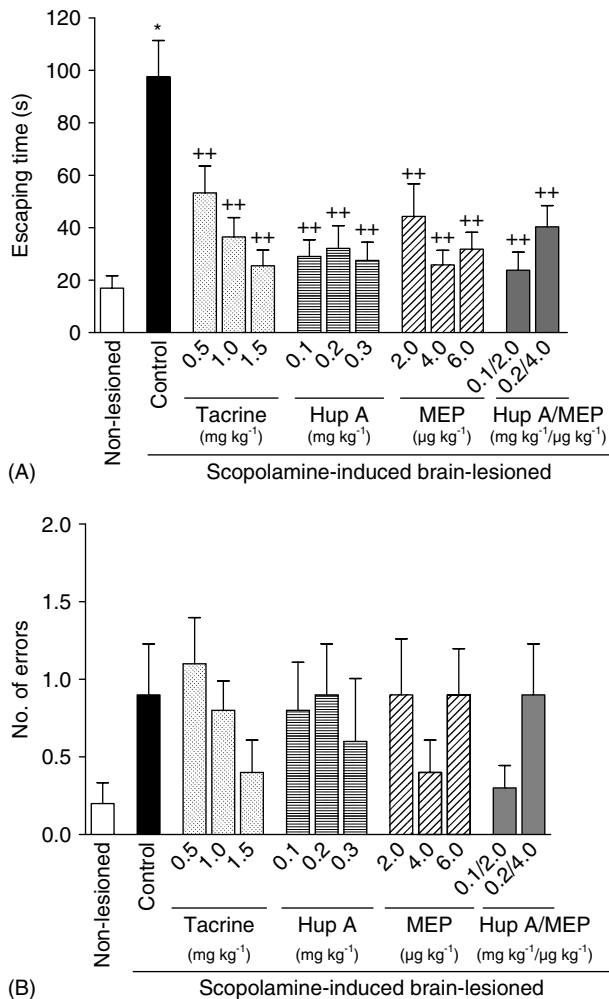
Figure 3 Effects of MEP, Hup A, tacrine and MEP plus Hup A on cognitive ability of brain-lesioned mice in the acquisition trial of the Y-water maze test. Scopolamine-induced brain-lesioned mice ($n = 10$) were treated with saline (control), tacrine, Hup A, MEP or Hup A plus MEP. Values represent mean ± SEM of the escaping time (A) and number of errors made in escaping (B) by each group of mice. $^*p < 0.01$ significantly different from non-lesioned mice, Student's t-test; $^+p < 0.05$ and $^{++}p < 0.01$ significantly different from the brain-lesioned control, one-way ANOVA followed by Dunnett's multiple comparison test; $^{\#}p < 0.05$ significantly different, Tukey's multiple comparison test.

respectively. The brain-lesioned control group showed obvious increases in the escaping time and errors made compared with the non-lesioned mice, indicative of scopolamine-induced impairments in cognitive abilities (Figures 3 and 4). The changes in escaping time were significant ($p < 0.01$) in both acquisition and retention trials, whereas increases in the number of errors were statistically significant ($p < 0.01$) in the acquisition but not retention trials. In the acquisition trial, all the drug-treated mice escaped much quicker ($p < 0.01$) than the brain-lesioned control group (Figure 3A), and apart from the subgroup administered with 0.5 mg kg^{-1} tacrine, made fewer errors ($p < 0.05$) in escaping (Figure 3B). Similarly, in the retention trial, the time

required for mice escaping onto the platform was obviously shorter ($p < 0.01$) in all the drug-treated groups compared with the brain-lesioned control (Figure 4A). However, none of the drug-treated groups showed a significant variation in the number of errors made in escaping (Figure 4B).

Among the three MEP-treated subgroups, the greatest improvements in the cognitive ability of the brain-lesioned mice, indicated by the shortest time and the fewest errors in escaping, were observed for mice administered with 4.0 µg kg^{-1} MEP, in both the acquisition (Figure 3) and retention (Figure 4) trials. The

(A)

(B)

Figure 4 Effects of MEP, Hup A, tacrine and MEP plus Hup A on cognitive ability of brain-lesioned mice in the retention trial of the Y-water maze test. Values represent mean $\pm$ SEM of the escaping time (A) and the number of errors made in escaping (B) by each group of mice. *$p < 0.01$ significantly different from non-lesioned mice, Student's t-test; $^+p < 0.05$ and $^{++}p < 0.01$ significantly different from brain-lesioned control, one-way ANOVA followed by Dunnett's multiple comparison test. When the values in the optimal dose groups were analysed by Tukey's multiple comparison test, no statistical significance ($p > 0.05$) was found between the different drug treatments.

optimal dose of Hup A was 0.3 mg kg^{-1} and of tacrine was 1.5 mg kg^{-1}. As shown in Figure 3A, mice administered with 4.0 µg kg^{-1} MEP took less time to escape onto the platform ($p < 0.05$) than those treated with 1.5 mg kg^{-1} tacrine, indicating that MEP had better therapeutic effects on the memory acquisition ability of the brain-lesioned mice than the test doses of tacrine.

Co-administration of MEP and Hup A

As shown in the figures, mice administered with 2.0 µg kg^{-1} MEP plus 0.1 mg kg^{-1} Hup A exhibited better cognitive abilities than the control in both acquisition and retention trials, as indicated by a higher

step-through latency and fewer electric shocks received in the passive avoidance test ($p < 0.01$) (Figures 1 and 2), and shorter escaping time and fewer errors made in the Y-water maze test ($p < 0.01$) (Figures 3 and 4A). However, mice treated with higher doses of MEP (4.0 µg kg^{-1}) and Hup A (0.2 mg kg^{-1}) showed fewer changes in the parameters compared with the low dose group (Figures 1–4).

The current results demonstrated that mice administered with 2.0 µg kg^{-1} MEP plus 0.1 mg kg^{-1} Hup A had better cognitive abilities than those treated with either drug alone (Figures 1–4). Moreover, when the optimal effects of the different drug treatments were compared, this co-administration induced comparable or better therapeutic effects on the brain-lesioned mice than the optimal tacrine treatment. In particular, in the passive avoidance test, the average step-through latency of the mice co-administered with MEP and Hup A (2.0 µg kg^{-1} and 0.1 mg kg^{-1}) was higher than those treated with tacrine (1.5 mg kg^{-1}) (Figure 1A), and in the retention trial of the Y-water maze test, the co-administered mice took less time ($p < 0.05$) to escape onto the platform than the tacrine-treated mice (Figure 3A).

DISCUSSION

Patients with AD progressively lose their cognitive ability and memory [1]. The biochemical changes in the brains of AD patients have been found to include a decrease of acetylcholine activity and a decline in the number of muscarinic and nicotinic receptors, thus resulting in reduced activity in the cholinergic system [2,4]. To correct the cognitive and memory deficits in AD patients, a number of therapeutic strategies have been developed to enhance the effectiveness of the remaining functional cholinergic neurons [5], including the use of cholinergic agonists to directly activate the target neurons [27,28]; administration of drugs that act on the presynaptic synapse to facilitate acetylcholine release [29]; use of biosynthetic precursors to increase the availability of acetylcholine for release [30]; and use of acetylcholinesterase inhibitors to prolong the effects of the released acetylcholine [9].

Tacrine as an acetylcholinesterase inhibitor is currently in use for the treatment of AD [9]. However, peripheral cholinergic effects and toxicity have limited its therapeutic benefits [11]. Comparative studies of Hup A and tacrine showed that Hup A with more selective inhibition on acetylcholinesterase activity in the cortex and hippocampus, was ten times as potent as tacrine in reversing AF64A-induced working memory deficits in rats whilst causing minimal peripheral effects [31]. MEP, an analogue of arginine-vasopressin of which the active fragment contributing to the peripheral effects was removed, is also superior in that it could recover memory impairments in rats without the peripheral side effects [21,26].

In the current study, the therapeutic effects of MEP, Hup A, tacrine and the combination of the former two against memory impairments induced by scopolamine were monitored in two behavioral models. An enhancement in the cognitive ability was indicated by an increased step-through latency and a decreased number of electric shocks received in the passive avoidance test, or less escaping time and fewer errors made in the Y-water maze test.

The results verified the therapeutic effects of MEP, Hup A and tacrine on the cognitive abilities of brain-lesioned mice, in both acquisition (Figures 1 and 3) and retention (Figures 2 and 4) trials of the passive avoidance and Y-water maze tests. The effective doses of tested drugs were related to the particular trial as well as to the particular test of memory, which had been noted in previous reports [7]. It was found that in a particular test, the statistical significance for the changes between the drug-treated group and control *varied* when calculated using different parameters. However, the results were highly consistent regarding the respective changes of different drug treatments, and of each drug at different doses. In addition, for subgroups treated with the same drug, the performances of mice in the two behavioral tasks were consistent regarding the optimal tested dose of each drug. Indeed, the optimal therapeutic effects of MEP were induced at $4.0\ \mu g\ kg^{-1}$, the medium test dose, in both the acquisition and retention trials. Similarly, at the tested dose regime, the optimal doses were $0.3\ mg\ kg^{-1}$ and $1.5\ mg\ kg^{-1}$ for Hup A and tacrine, respectively.

For the therapeutic effects of different drugs, it was observed that mice administered with $4.0\ \mu g\ kg^{-1}$ MEP displayed a higher average step-through latency (Figure 1A) and spent significantly less time in escaping than those treated with $1.5\ mg\ kg^{-1}$ tacrine (Figure 3A), indicating a better effect of MEP in memory acquisition. However, for memory retention, the same dose of tacrine showed higher efficacy than MEP in increasing the step-through latency in mice measured 2 days after memory acquisition (Figure 2A). Such a difference was not observed in the Y-water maze test carried out 3 days after the acquisition trial, which suggests that the relative efficacy between MEP and tacrine may vary with the retention period, or between the different test models. In other situations, MEP at $4.0\ \mu g\ kg^{-1}$ elicited similar therapeutic effects on mice compared with Hup A at $0.3\ mg\ kg^{-1}$ and tacrine at $1.5\ mg\ kg^{-1}$. These results suggest that MEP was highly potent and efficient in correcting cognition deficits. In particular, MEP was effective at $\mu g\ kg^{-1}$ doses, about two orders of magnitude more potent than tacrine (effective at $mg\ kg^{-1}$ doses) in overcoming scopolamine-induced memory impairments in mice.

The brain-lesioned mice co-administered with $2.0\ \mu g\ kg^{-1}$ of MEP and $0.1\ mg\ kg^{-1}$ of Hup A showed obvious improvements in cognitive abilities in both the acquisition (Figures 1 and 3) and retention (Figures 2 and 4) trials. The improvements were comparable or better than that achieved upon treatment with $1.5\ mg\ kg^{-1}$ tacrine, demonstrating the co-administration of Hup A and MEP to be as effective as, if not better than, tacrine treatment. Successful multidrug therapy often takes advantage of different mechanisms of action [32]. In this regard, Hup A was reported to prolong acetylcholine activity by inhibiting acetylcholinesterase [15], whilst MEP was suggested to ameliorate memory disability by promoting nerve growth factor gene expression in the brain [26]. Indeed, the co-administration of MEP ($2.0\ \mu g\ kg^{-1}$) and Hup A ($0.1\ mg\ kg^{-1}$) significantly enhanced step-through latency in brain-lesioned mice, whereas either drug alone at the same dosage did not show such effect (Figures 1A and 2A). It is noteworthy that the co-administration of MEP and Hup A was less effective at higher doses, which was also observed in treatment with MEP alone ($6.0\ \mu g\ kg^{-1}$). More in-depth studies on the mechanism of action of MEP are called for to explain the observed reduction in effectiveness at higher doses.

In conclusion, the present study represents a comparative study of the effects of MEP, Hup A, tacrine, and a combination of MEP and Hup A on the cognitive abilities of brain lesioned-mice. Results in the passive avoidance and Y-water maze tests suggest that MEP was highly efficient in recovering scopolamine-induced cognition deficits in mice, for both acquisition and retention abilities. In particular, the effective doses of MEP were about two orders of magnitude lower than that of tacrine. The clinical value of tacrine is known to be limited by side effects such as dose-dependent liver-toxicity, whereas memory enhancing peptides have been shown to improve cognitive abilities in animals without the peripheral side effects [21,26]. The potency of MEP observed in the present study, in comparison with tacrine, further suggest MEP as a promising drug candidate. Most prominently, the co-administration of MEP and Hup A was able to produce an additive effect of the individual drugs, resulting in considerable therapeutic effects against memory deficits in mice, therefore providing a rationale for their combined usage in the treatment of cognitive deficits.

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