BJP BRITISH JOURNAL OF PHARMACOLOGY
BPS BRITISH PHARMACOLOGICAL SOCIETY

BJP British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2010.01014.x
www.brjpharmacol.org

RESEARCH PAPER

# Cilostazol prevents amyloid β peptide$_{25-35}$-induced memory impairment and oxidative stress in mice

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**Keywords**
cilostazol; amyloid β peptide$_{25-35}$; oxidative stress; lipid peroxidation; malondialdehyde; memory

**Received**
25 January 2010
**Revised**
6 August 2010
**Accepted**
17 August 2010

## BACKGROUND AND PURPOSE
Cilostazol may be effective in dementia associated with a cerebral ischaemia. In this study, we examined whether it exerts beneficial effects on learning and/or memory impairment induced by Aβ$_{25-35}$ in mice, and compared its effects with those of aspirin.

## EXPERIMENTAL APPROACH
Aβ$_{25-35}$ (9 nmol) was administered to mice i.c.v. Learning and memory behaviour were evaluated by measuring spontaneous alternation in a Y-maze and a step-down type passive avoidance test, on the 5th and 8th days after injection respectively. Levels of lipid peroxidation (malondialdehyde) and cytokines in the frontal cortex and hippocampus were measured 2, 3, 5 and 7 days after the Aβ$_{25-35}$ injection. The effects of repeated administration of cilostazol and aspirin (both at 30 and 100 mg·kg$^{-1}$, p.o.) on any changes induced by Aβ$_{25-35}$ were evaluated.

## KEY RESULTS
Repeated administration of cilostazol significantly attenuated the impairment of spontaneous alternation and the shortened step-down latency induced by Aβ$_{25-35}$. Aspirin did not show any beneficial effect. A significant increase in the levels of malondialdehyde (MDA) and IL-1β (only measured in hippocampus) was observed 2, 3 and 5 days after the Aβ$_{25-35}$ injection in the frontal cortex and hippocampus. Repeated administration of cilostazol (100 mg·kg$^{-1}$) completely prevented the increase in MDA levels but failed to antagonize the increase in the expression of IL-1β induced by Aβ$_{25-35}$.

## CONCLUSIONS AND IMPLICATIONS
These results suggest that the protective effect of cilostazol on Aβ$_{25-35}$-induced memory impairment may be related to oxidative stress in the frontal cortex and the hippocampus.

## Abbreviations
Aβ, amyloid β; AD, Alzheimer's disease; APP, amyloid precursor protein; BACE, β-site of amyloid precursor protein cleaving enzyme; CMC, carboxymethyl cellulose sodium salt; CREB, cAMP-responsive element binding protein cilostazol:6-[4-(1-cyclohexyl-1H-tetrazol-5-yl)butoxy]-3,4- dihydro-2(1H)-quinolinone; GM-CSF, granulocyte macrophage colony-stimulating factor; IFN-γ, interferon-γ; IGF-1, insulin-like growth factor-1; IL, interleukin; IκB, inhibitor of nuclear factor-κB; LPS, lipopolysaccharide; LTP, long-term potentiation; MCA, middle cerebral artery; MDA, malondialdehyde; NF-κB, nuclear factor κB; NO, nitric oxide; NSAIDs, non-steroidal anti-inflammatory drugs; PDE III, phosphodiesterase III; ROS, reactive oxygen species; TBARS, thiobarbituric acid-reactive substance; TNF-α, tumour necrosis factor-α; i.h., intrahippocampal; iNOS, inducible nitric oxide synthase

## Introduction

It is well known that oxidative stress is involved in several diseases, such as neurodegenerative disorders and ischaemic encephalopathy (Pappolla *et al.*, 1998; Choi *et al.*, 2002). Alzheimer's disease (AD) is a progressive neurodegenerative disorder associated with global mental dysfunction and impairment of cognitive function (Palmer, 2002). Common pathological features of AD include senile plaques, neurofibrillary tangles, and neuronal loss in regions of the brain involved in learning and memory functions (Rowan *et al.*, 2007). In Alzheimer's type dementia, amyloid β (Aβ) protein such as $A\beta_{1\text{-}42}$, generates reactive oxygen species (ROS) and nitric oxide, which, by inducing hyperoxidation of neuronal proteins and lipids, may be associated with learning and memory impairment (Fang and Liu, 2006). The active fragment of Aβ protein, $A\beta_{25\text{-}35}$, has similar effects; therefore, learning and memory impairment induced by $A\beta_{25\text{-}35}$ is also mediated by ROS (Lu *et al.*, 2009). In animal experiments, intrahippocampal or i.c.v. injection of $A\beta_{25\text{-}35}$ has been shown to induce histological and biochemical changes; learning deficits (Maurice *et al.*, 1996; Meunier *et al.*, 2006; Alkam *et al.*, 2008); dysfunction of the cholinergic system, which plays an important role in the cognitive deficits associated with aging and neurodegenerative diseases (Tran *et al.*, 2001); and oxidative stress-mediated changes in hippocampal long-term potentiation (Trubetskaya *et al.*, 2003). Thus, $A\beta_{25\text{-}35}$-injected animals are one of the models often used to investigate the pathogenesis and progression of AD, and for evaluating new therapeutic agents for AD (Maurice *et al.*, 1996).

Cilostazol is an antagonist of PDE III and is used clinically as an antiplatelet drug. As cilostazol increases the cerebral blood flow (Kwon *et al.*, 2005) and decreases the size of cerebral infarcted area more strongly than low doses of aspirin (Lee *et al.*, 2005), it may be effective in treating the type of dementia associated with a decrease and stoppage of the cerebral blood flow in brain blood vessels. Interestingly, cilostazol has been shown to inhibit lipid peroxidation and apoptosis in a model of cerebral ischaemia (Watanabe *et al.*, 2006). Lipid peroxidation and apoptosis may be associated with the neuronal dysfunction caused by $A\beta_{25\text{-}35}$, including the altered transcription factors and the inhibition of inflammatory cytokine release from macrophages (Kim *et al.*, 2002). Furthermore, because cilostazol can trap ROS and inhibit the cell damage caused by oxidative stress (Choi *et al.*, 2002), it has an antioxidative effect and may be useful as a treatment for the dementia associated with AD. In addition, cilostazol has been shown to be more beneficial in the maintenance and improvement of memory function in Alzheimer's patients currently taking the medication donepezil (Arai and Takahashi, 2009). However, the ameliorative effect of cilostazol on the learning and memory impairment induced by Aβ has not been studied, and its mechanism is not yet clear.

Recent reports have indicated that patients suffering from inflammatory diseases (e.g. arthritis) who take anti-inflammatory medication have a reduced risk of developing AD (Montine *et al.*, 1999; Sugaya *et al.*, 2000). Clinical cohort studies have reported that the use of non-steroidal anti-inflammatory drugs reduces the risk of developing AD (Szekely *et al.*, 2008). Similar to cilostazol, aspirin, because of its anti-platelet action via inhibition of the cyclooxygenase enzyme, is used as a prophylactic treatment in patients at risk of embolic stroke (Muir and Lees, 1997).

In this study, we used the $A\beta_{25\text{-}35}$-injected mouse as a model of AD to examine the mechanism of action by which cilostazol exerts its effects on learning and memory impairment. Using both a spontaneous alternation task and a step-down type passive avoidance task, we compared the effects of cilostazol on learning and memory impairment induced by $A\beta_{25\text{-}35}$ with those of aspirin, and then examined the effect of cilostazol alone, using various dosing schedules, in more detail.

## Methods

### *Animals*

Seven – 9-week-old male ddY mice (31–41 g, Japan SLC, Hamamatsu, Japan) were kept in a controlled environment for at least 5–7 days, with a 12-h light/12-h dark cycle (lights on; 7:45–19:45), and given food and tap water *ad libitum*. Experimental protocols concerning the use of laboratory animals were approved by the committee of Meijo University and were performed in accordance with the guidelines of the Japanese Pharmacological Society (Folia Pharmacol. japon, 1992, 99: 35A) and the interministerial decree of 25 May 25 1987 (The Ministry of Education).

### *Drugs*

Amyloid β-protein fragment 25-35 ($A\beta_{25\text{-}35}$) was purchased from NeoMPS (NeoMPS, San Diego, CA, USA) and dissolved in distilled water at a concentration of 1.8 mM. The $A\beta_{25\text{-}35}$ was incubated, or 'aged', by incubation in distilled water at 37°C for 4 days. Aged $A\beta_{25\text{-}35}$ (9 nmol 5 $\mu L^{-1}$) or distilled water (5 μL) was injected i.c.v. as described previously (Maurice *et al.*, 1996) according to the methods of Haley and

McCormick (1957). The mice were lightly anaesthetized with ether, after which a 28-gauge stainless-steel needle was inserted into the lateral ventricle, and $A\beta_{25\text{-}35}$ was gradually injected over 30 s using a microinfusion pump (KDS210, KD Scientific, Holliston, MA, USA). The needle was left in place for 10 s following the injection. The injection site was verified in preliminary experiments by injecting blue dye solution. Neither insertion of the needle nor injection of the distilled water had a significant influence on behavioural responses or cognitive functions.

6-[4-(1-Cyclohexyl-1H-tetrazol-5-yl)butoxy]-3,4-dihydro-2(1H)-quinolinone (cilostazol) was obtained from Otsuka Pharmaceuticals, Inc. (Tokyo, Japan), and acetylsalicyclic acid (aspirin) was obtained from Sigma (St. Louis, MO, USA). Drugs were suspended in 0.5% carboxymethyl cellulose sodium salt (CMC; Nacalai tesque, Kyoto, Japan) dissolved in distilled water. Cilostazol and aspirin were administered p.o. 60 min before the behavioural tests. Sham control mice were administered CMC, p.o. at a volume of 0.1 mL 10 $g^{-1}$ body weight.

## *Experimental schedules*

The experimental schedules are shown in Figure 1. In Schedule #1, cilostazol and aspirin (30 and 100 mg·$kg^{-1}$, p.o.) were administered repeatedly for 8 days following the injection of $A\beta_{25\text{-}35}$ (9 nmol per mouse, i.c.v.). Spontaneous alternation performance and the passive avoidance test were conducted on the 5th and 7th days after $A\beta_{25\text{-}35}$ injection respectively. On these days, cilostazol and aspirin were administered 60 min before the behavioural tests. In acute experiments, mice were only administered cilostazol or aspirin 60 min before these behavioural tests on the 5th and 7th days. On the remaining days, these mice were injected with 0.5% CMC solution as reported in Schedule #1 (Schedule #2).

As the results of Schedule #1 revealed that only cilostazol ameliorated the learning and/or memory impairments induced by $A\beta_{25\text{-}35}$, in the following experiments only the effects of cilostazol, using various drug administration schedules, were studied. The schedules were as follows: repeated doses of cilostazol (100 mg·$kg^{-1}$, p.o.) were administered from 8 days to 1 day before $A\beta_{25\text{-}35}$ injection (Schedule #3), from day 2 to 9 after $A\beta_{25\text{-}35}$ injection (Schedule #4), and from day 5 to 12 after $A\beta_{25\text{-}35}$ injection (Schedule #5). In these schedules, the spontaneous alternation performance was completed on the 5th day, and the training trial of the passive avoidance test was conducted on the 7th day after $A\beta_{25\text{-}35}$ injection (Schedules #1–3) or the first injection of cilostazol (Schedule #4). Each mouse received eight injections of cilostazol in the repeated experiments. On the 5th and 7th days, cilostazol and aspirin were administered 60 min prior to the behavioural experiments. Sham control mice received 0.5% CMC solution using these same schedules.

Lipid peroxidation and cytokine levels were measured 2, 3, 5 and/or 7 days post-Aβ peptide (25-35) (9 nmol per mouse, i.c.v.) injection for both the acute and repeated cilostazol administration schedules.

## *Spontaneous alternation behaviour*

Immediate working memory performance was assessed by recording spontaneous alternation behaviour during a single session in a Y-maze as described previously (Itoh *et al.*, 1993; Hiramatsu and Inoue, 1999), with minor modifications of the original study performed in rats (Sarter *et al.*, 1988). This paradigm includes exploratory, locomotor, motivational and systematic behaviours; the selectivity involving memory and especially learning processes is weak. However, this was used as a first-intent test because it is pharmacologically predictive and is not constraining for animals. Furthermore, unlike the passive avoidance task, mice do not receive electric shocks during the test periods. Our previous data indicated that there are certain correlations between the spontaneous alternation task in the Y-maze and the passive avoidance task (Hiramatsu and Inoue, 2000a,b).

Each mouse, new to the maze, was placed at the end of one arm and allowed to move freely through the maze during an 8 min session. The series of arm entries was recorded visually. Alternation was defined as the mouse entering different arms of the maze three times in succession as a result of consecutive arm entering. The number of overlapping entrance sequences (e.g. ABC, BCA) was defined as the number of alternations. The effect was calculated as % alternation according to the following formula:

$$\%\ \text{alternation} = (\text{number of alternations}/\text{total number of arm entries} - 2) \times 100$$

In this test, the animals must memorize the arm entered immediately prior to the arm they are currently occupying. Mice have a tendency to enter a newer environment, which in this case would be the arm not entered just before. Therefore, the % alternation is used as an index of short-term memory. The total number of arm entries is measured as an index of locomotor activity, although it is not direct evidence. Locomotor activity was also measured for 10 min in control mice with Schedule #1 using digital counters with infrared sensors (MDC-W01,

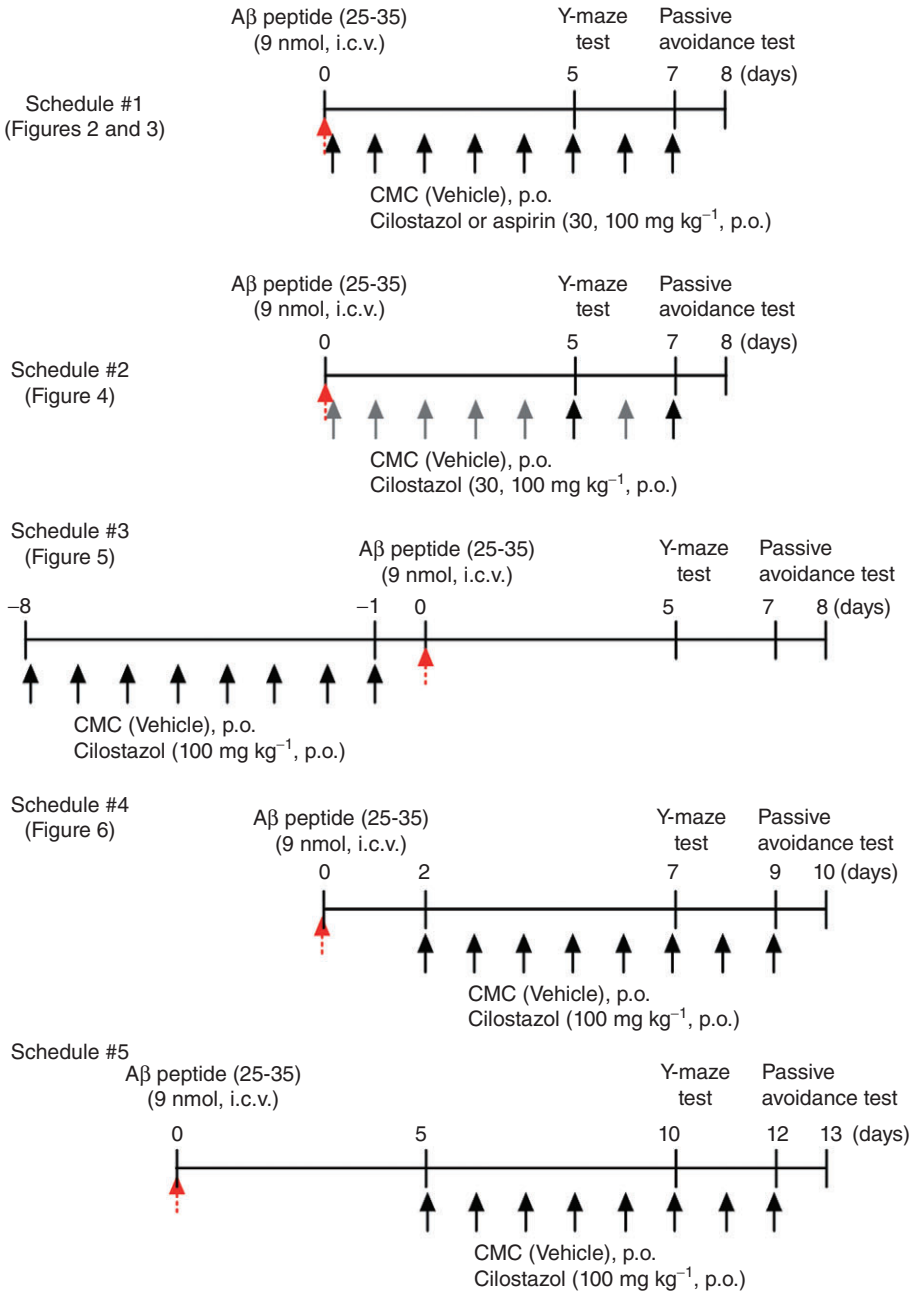



**Figure 1**

Experimental protocols used in this study. CMC, carboxymethyl cellulose sodium salt.

Brain Science Idea, Co., Ltd., Osaka, Japan) in a black Plexiglas box.

### *Step-down type passive avoidance task*

A step-down type of passive avoidance task was performed as described previously (Hiramatsu and Inoue, 2000a) with minor modification. The apparatus consisted of a transparent acrylic rectangular cage (30 × 30 × 40 cm high) comprised of a grid floor with a wooden platform (4 × 4 × 4 cm) in the centre set in a semi-soundproof wooden outer box (35 × 35 × 90 cm high). Illumination was provided by a 15 W lamp above the apparatus. An electric current (1 Hz, 500 ms, 60 V, DC) was delivered to the grid floor by an isolated stimulator (SEN-3201, Nihon Koden, Tokyo, Japan). The electrical resistance varied between 100 and 250 kΩ when mice were placed in the test cage. Therefore, each mouse received an electric shock varying between 0.24 and 0.60 mA.

Each mouse was placed on the wooden platform for the training test. When the mouse stepped down from the platform onto the grid floor, an electric shock was delivered for 15 s. A retention test was carried out 24 h after the training session in a manner similar to the training, except that no electric shock was delivered to the grid floor. Each mouse was placed on the platform and the step-down latency was recorded. An upper cut-off time of 300 s was set.

### *Responses to electric shock*

Responses to electric shock during the training sessions were recorded. The following scores were given based on the responses to electric shock: 3 = jumping, 2 = vocalization, 1 = flinching, 0 = no response. Shock sensitivity was shown as the total score, which was determined as the sum of each score following 15 s of recording.

### *Determination of lipid peroxidation level*

Mice were decapitated, and the hippocampus and frontal cortex were removed. Brain samples were frozen immediately by liquid $N_2$ and stored at –80°C in a deep freeze until assay.

Malondialdehyde (MDA) was measured with a thiobarbituric acid-reactive substance assay kit (Cayman, Ann Arbor, MI, USA). Briefly, the isolated brain samples were homogenized in cooled RIPA buffer (50 mM Tris-HCl buffer pH 7.4, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS,1 mM PMSF, 1 mM $Na_3VO_4$, 1 mM NaF as protease inhibitor cocktail) with an ultrasonic homogenizer (Microson Ultrasonic Cell Disruptor, Misonix, Farmingdale, NY, USA) for 15–20 s and centrifuged at 4500× *g* for 10 min at 4°C. The homogenate of brain samples was incubated with 8.1% sodium dodecylsulphate for 10 min followed by the addition of 20% acetic acid (pH 3.5). The reaction mixture was incubated with 0.6% TBA in boiling water for 2 h. After a 10 min cooling period in the ice bath, the mixture was centrifuged at 1600× *g* for 10 min at 4°C. The absorbance was determined by a plate reader (Wallac 1420 ARVOsx, Perkin Elmer, Waltham, MA, USA) at 550 nm. MDA content was expressed as μmol·mg$^{-1}$ protein.

### *Determination of cytokine concentration*

The same tissue homogenates as those used for the lipid peroxidation assay were used for the cytokine assays (IL-1β, IL-2, IL-4, IL-5, IL-10, GM-CSF, IFN-γ and TNF-α) using a multiplex bead-based immunoassay kit (Mouse Cytokine 8-Plex A Assay kit, Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. In brief, 50 μL of homogenate was plated into a 96-well filter plate, coated with antibody-coupled beads, and incubated for 1 h in a shaded room using a platform shaker at ambient temperature. The wells were then vacuum-filtered and washed. Next, 50 μL of fluorescent solution was added, and the wells were incubated for 30 min. The wells were again vacuum-filtered and washed, 125 μL of cytokine assay buffer was added, and the wells were allowed to stand for 30 s before the intensity of fluorescence was measured (Bio-Plex 200, Bio-Rad).

### *Protein assay*

The protein content in diluted samples (×50 and ×100) was measured based on a standard BSA using the Bio-Rad DC protein assay (Bio-Rad). Samples were read on a photometer (iMark Microplate Reader, Bio-Rad) set at 595 nm.

### *Data analysis*

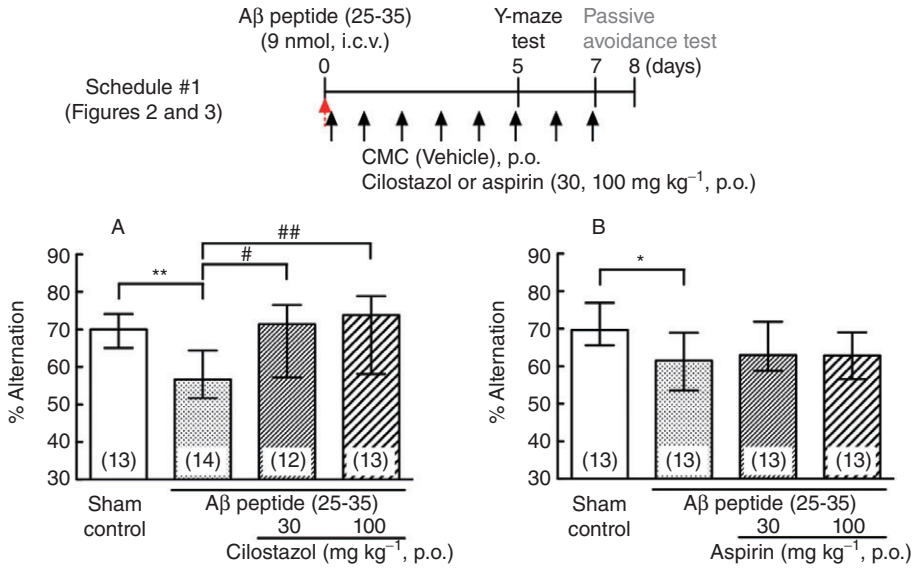
The behavioural data are expressed in terms of median (vertical column) and interquartile ranges from the first to the third quartile (vertical line) for the Y-maze test or box-plot (median and interquartile ranges) for the passive avoidance test. The data for these memory tests were analysed using non-parametric type statistical methods because a Gaussian distribution was not always evident. Thus the significance of the differences was evaluated using the Mann–Whitney *U*-test for two group comparisons or the Kruskal–Wallis test followed by the Bonferroni's test for multiple comparisons.

The biochemical data are expressed as the means ± SEM. Statistical differences among the experimental groups were tested using two-way analysis of variance (ANOVA). Student's *t*-test was used for comparisons between two groups, and one-way ANOVA followed by the Dunnett's *post hoc* test was used for multiple comparisons. The criterion for significance was set at $P < 0.05$ for all statistical evaluations. All statistical analyses were performed using the Prism 5 Stat program (GraphPad Software, Inc., San Diego, CA, USA).

## Results

### *Effects of repeated administration of cilostazol and aspirin on Aβ$_{25-35}$-induced impairment of spontaneous alternation performance in mice*

The administration of $A\beta_{25-35}$ (9 nmol per mouse, i.c.v.) significantly decreased the % alternation in the Y-maze when animals were tested 5 days later (Figure 2A,B). Repeated administration of cilostazol (30 and 100 mg·kg$^{-1}$, p.o.) significantly and dose-dependently attenuated the impairment of spontaneous alternation induced by $A\beta_{25-35}$ (Figure 2A).




## Figure 2

Effects of repeated administration of cilostazol (A) and aspirin (B) on $A\beta_{25\text{-}35}$-induced impairment of spontaneous alternation in the Y-maze test. $A\beta_{25\text{-}35}$ (9 nmol per mouse, i.c.v.) was injected 5 days before the Y-maze test. Mice were treated with cilostazol (30 and 100 mg·kg$^{-1}$, p.o.) or aspirin (30 and 100 mg·kg$^{-1}$, p.o.) once a day for 5 days. On the 5th day, these drugs were injected again 60 min before testing. Data are shown as median (vertical column) and first and third quartiles (vertical line). The number of mice used is shown in parentheses. Significant levels; *$P$ < 0.05, **$P$ < 0.01 versus sham control (Mann–Whitney's $U$-test). #$P$ < 0.05, ##$P$ < 0.01 versus $A\beta_{25\text{-}35}$ alone (Bonferroni's test). CMC, carboxymethyl cellulose sodium salt.

Repeated administration of aspirin (30 and 100 mg·kg$^{-1}$, p.o.) did not alter the impairment of spontaneous alternation induced by $A\beta_{25\text{-}35}$ (Figure 2B). Cilostazol and aspirin did not affect the total number of arm entries at these dosages (data not shown). Repeated administration of cilostazol (100 mg·kg$^{-1}$, p.o.) did not affect % alternation [control: 69.3 (64.5–75.4), cilostazol: 66.2 (58.1–70.2)] or locomotor activity (control: 302.0 (274.8–318.5), cilostazol: 255.0 (219.8–296.8)] in control mice.

### *Effects of repeated administration of cilostazol and aspirin on $A\beta_{25\text{-}35}$-induced impairment of learning and memory in mice using the passive avoidance test*

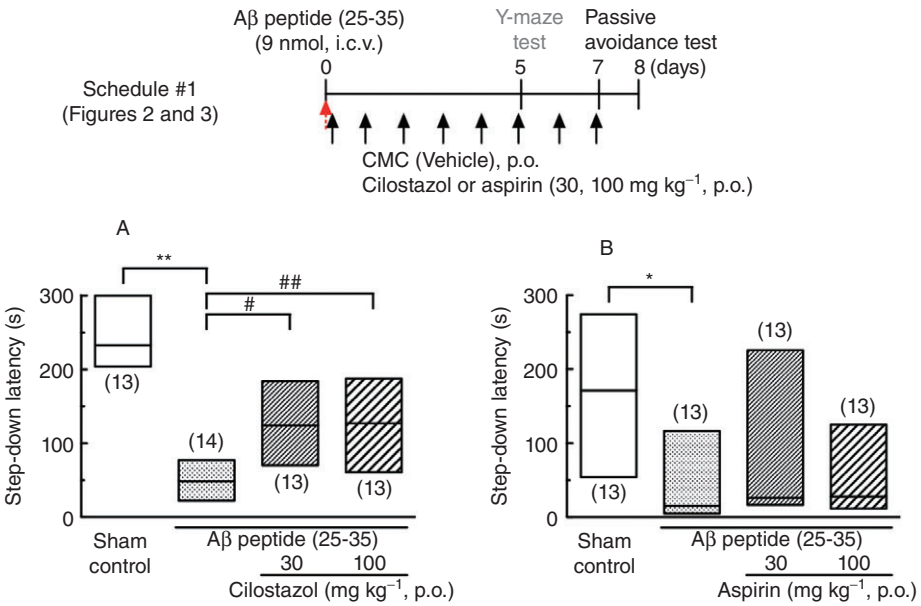
The step-down type passive avoidance test was conducted to test the long-term memory of animals administered cilostazol. In the passive avoidance test, $A\beta_{25\text{-}35}$ (9 nmol per mouse, i.c.v.) administration significantly shortened the step-down latency when animals were tested 8 days after injection (Figure 3A,B). Repeated administration of cilostazol (30 and 100 mg·kg$^{-1}$, p.o.) significantly improved the shortened step-down latency induced by $A\beta_{25\text{-}35}$ (Figure 3A). Compared with cilostazol, repeated administration of aspirin (30 and 100 mg·kg$^{-1}$, p.o.) did not improve the learning and memory of $A\beta_{25\text{-}35}$-injected mice (Figure 3B). Cilostazol and aspirin did not affect the response to electric shocks at these dosages (data not shown). Repeated administration of cilostazol (100 mg·kg$^{-1}$, p.o.) did not prolong the step-down latency in control mice [control: 82.3 (47.7–228.2), cilostazol: 119.0 (30.5–252.5)].

### *Effects of acute administration of cilostazol on $A\beta_{25\text{-}35}$-induced behavioural impairment*

$A\beta_{25\text{-}35}$ (9 nmol per mouse, i.c.v.) significantly decreased the % alternation and the step-down latency 5 and 8 days after injections respectively. Acute administration of cilostazol (30 and 100 mg·kg$^{-1}$) did not improve these $A\beta_{25\text{-}35}$-induced impairments in learning and memory assessed in the Y-maze and step-down type passive avoidance tests (Figure 4A,B).

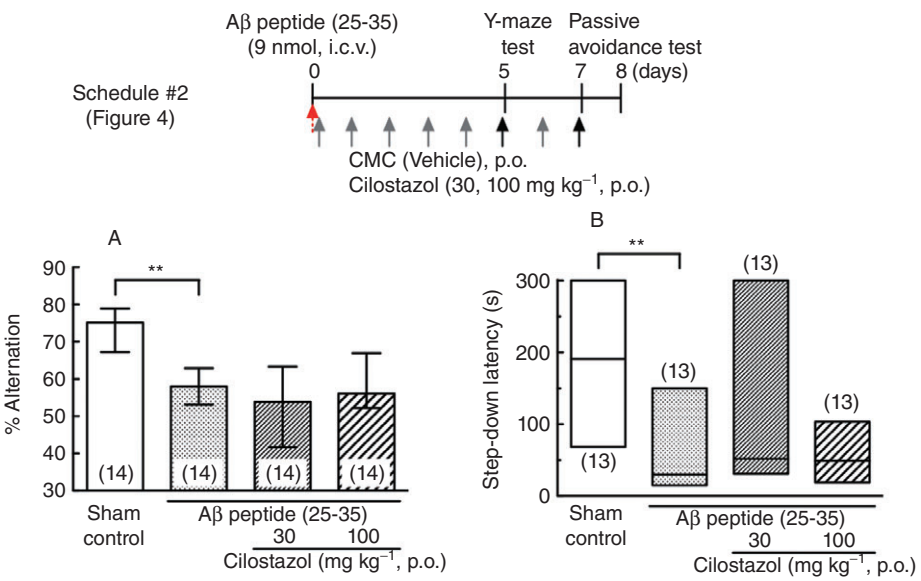
### *Effects of pre-administration of cilostazol on $A\beta_{25\text{-}35}$-induced behavioural impairment*

Cilostazol (100 mg·kg$^{-1}$) was administered repeatedly beginning 8 days before $A\beta_{25\text{-}35}$ injection to test whether cilostazol can protect mice from $A\beta_{25\text{-}35}$ (9 nmol per mouse)-induced impairments in learning and memory. $A\beta_{25\text{-}35}$ (9 nmol per mouse) significantly decreased the % alternation and the step-down latency 5 and 8 days after injection respectively. However, pre-administration of cilostazol did not appear to protect mice from these



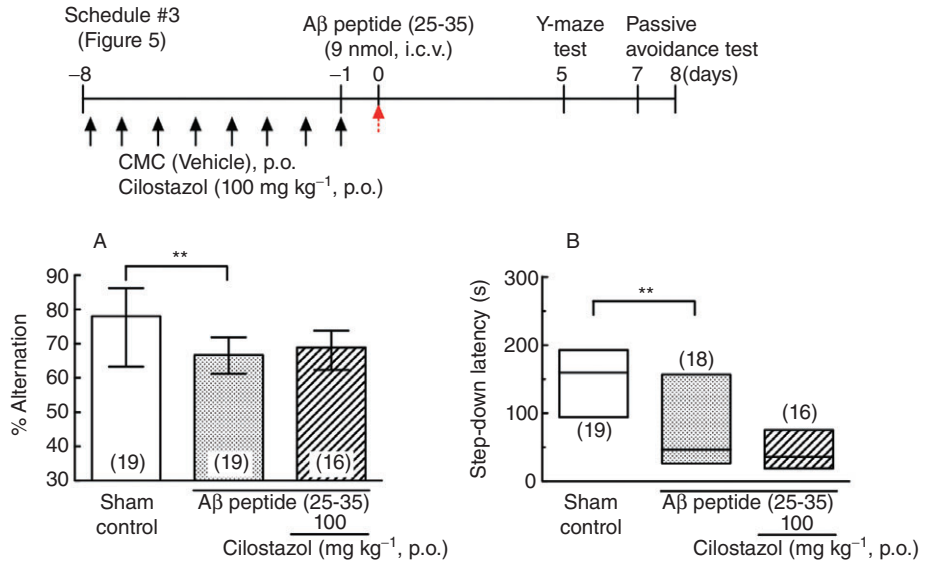

## Figure 3

Effects of repeated administration of cilostazol (A) and aspirin (B) on $A\beta_{25\text{-}35}$-induced impairment of learning and memory in a passive avoidance test. $A\beta_{25\text{-}35}$ (9 nmol per mouse, i.c.v.) was injected 7 days before the training trial. Mice were treated with cilostazol (30 and 100 mg·kg$^{-1}$, p.o.) or aspirin (30 and 100 mg·kg$^{-1}$, p.o.) once a day for 7 days. On the 7th day, these drugs were injected again 60 min before retention testing. A retention trial was carried out 24 h after the training trial. Data are shown as median (horizontal bar) and first and third quartiles (vertical column). The number of mice used is shown in parentheses. Significant levels; *$P < 0.05$, **$P < 0.01$ versus sham control (Mann–Whitney's *U*-test), #$P < 0.05$, ##$P < 0.01$ versus $A\beta_{25\text{-}35}$ alone (Bonferroni's test). CMC, carboxymethyl cellulose sodium salt.




## Figure 4

Effects of acute administration of cilostazol on $A\beta_{25\text{-}35}$-induced impairment of spontaneous alternation (A) and learning and memory in a passive avoidance test (B). Mice were injected with $A\beta_{25\text{-}35}$ (9 nmol per mouse, i.c.v.) 5 days before the training trial for the Y-maze test. Cilostazol (30 and 100 mg·kg$^{-1}$, p.o.) was injected 60 min before the Y-maze test and the training trial of the passive avoidance test. The retention trial was carried out 24 h after the training trial. Data are shown as the median (vertical column) and first and third quartiles (vertical line) for the Y-maze and median (horizontal bar), and first and third quartiles (vertical column) for the passive avoidance tests. The number of mice in each experiment is shown in parentheses. Significant levels; **$P < 0.01$ versus sham control (Mann–Whitney's *U*-test). CMC, carboxymethyl cellulose sodium salt.




## Figure 5

Effects of repeated pre-administration of cilostazol on $A\beta_{25\text{-}35}$-induced impairment of spontaneous alternation (A) and learning and memory in a passive avoidance test (B). Mice were treated with cilostazol (100 mg·kg$^{-1}$, p.o.) repeatedly for 8 days beginning 8 days before $A\beta_{25\text{-}35}$ (9 nmol per mouse, i.c.v.) injection. The Y-maze test was carried out 5 days after (A) $A\beta_{25\text{-}35}$ injection, and the passive avoidance test was carried out 7–8 days after $A\beta_{25\text{-}35}$ injection (B). The retention trial was carried out 24 h after the training trial. Data are shown as the median (vertical column) and first and third quartiles (vertical line) for the Y-maze, and median (horizontal bar) and first and third quartiles (vertical column) for the passive avoidance tests. The number of mice used is shown in parentheses. Significant levels; $^{**}P < 0.01$ versus sham control (Mann–Whitney's *U*-test). CMC, carboxymethyl cellulose sodium salt.

deleterious effects of $A\beta_{25\text{-}35}$ on performance in the Y-maze test and step-down type passive avoidance test (Figure 5A,B).

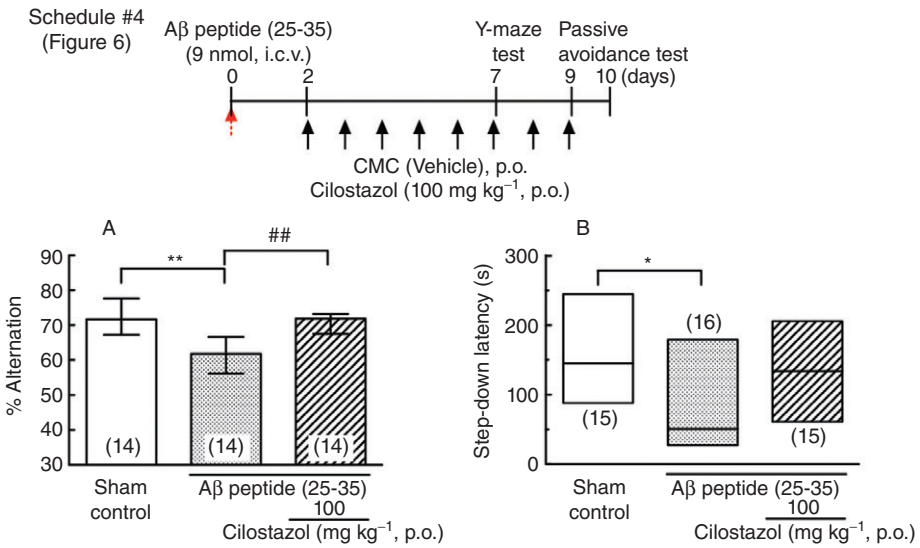
### *Effects of repeated administration of cilostazol on $A\beta_{25\text{-}35}$-induced behavioural impairments*

To test whether cilostazol has protective and/or improving effects on $A\beta_{25\text{-}35}$ (9 nmol per mouse)-induced impairments in behaviour, cilostazol (100 mg·kg$^{-1}$) was administered 2 or 5 days after the $A\beta_{25\text{-}35}$. $A\beta_{25\text{-}35}$ (9 nmol per mouse) significantly decreased the % alternation 7 days after injection and the step-down latency 10 days after injection (Figure 6A,B). Cilostazol significantly improved $A\beta_{25\text{-}35}$-induced impairment of spontaneous alternation (Figure 6A) and tended to protect the mice from the learning and memory impairment in the step-down type passive avoidance test (Figure 6B, $P = 0.063$).

As described previously, an impairment of spontaneous alternation was observed 5 days after $A\beta_{25\text{-}35}$ injection. Cilostazol administration was started 5 days after $A\beta_{25\text{-}35}$ injection and continued for 6 days in order to test whether cilostazol can have an effect after the development of the impairment. The results indicated that cilostazol did not have an ameliorating effect after repeated administration under this dosing schedule (Schedule #5) (data not shown).

### *Effect of cilostazol on MDA levels in the frontal cortex and hippocampus of $A\beta_{25\text{-}35}$-injected mice*

The effect of cilostazol on the levels of MDA in the frontal cortex and hippocampus 2, 3, 5 and 7 days after $A\beta_{25\text{-}35}$ (9 nmol per mouse) injection was examined to determine whether lipid peroxidation is involved in its ameliorating effect in these mice. A significant increase in the levels of MDA was observed in both brain areas of $A\beta_{25\text{-}35}$-injected mice compared with the control group ($F(1,41) = 38.75$, $P < 0.01$ and $F(1,44) = 16.55$, $P < 0.01$, respectively, Figure 7). The level of MDA increase was dependent upon the numbers of days that had passed since the $A\beta_{25\text{-}35}$. The levels of MDA were significantly higher at days 2, 3 and 5 post-$A\beta_{25\text{-}35}$ injection in the frontal cortex and at 3 and 5 days after $A\beta_{25\text{-}35}$ injection in the hippocampus compared with the control group. However, at 7 days after $A\beta_{25\text{-}35}$ injection, the increase in MDA levels in both brain regions returned to the control levels. Repeated treatment with cilostazol (100 mg·kg$^{-1}$) prevented the increase in MDA levels in the frontal




## Figure 6

Effects of repeated administration of cilostazol on $A\beta_{25\text{-}35}$-induced impairment of spontaneous alternation (A) and learning and memory in a passive avoidance test (B). Mice were injected with $A\beta_{25\text{-}35}$ (9 nmol per mouse, i.c.v.) 7 days before the Y-maze test or 9 days before the passive avoidance test. Mice were treated with cilostazol (100 mg·kg$^{-1}$, p.o.) repeatedly for 5 days beginning 2 days after $A\beta_{25\text{-}35}$ (9 nmol per mouse, i.c.v.) injection. Cilostazol was administered 60 min before testing on the Y-maze test day. On the next day, cilostazol was injected. On the day of the training trial for the passive avoidance test, cilostazol was administered 60 min before testing. A retention trial was carried out 24 h after the training trial. Data are shown as the median (vertical column) and first and third quartiles (vertical line) for the Y-maze, and median (horizontal bar) and first and third quartiles (vertical column) for the passive avoidance tests. The number of mice in each experiment is shown in parentheses. Significant levels; *$P$ < 0.05, **$P$ < 0.01 versus sham control, #$P$ < 0.05 versus $A\beta_{25\text{-}35}$ alone (Mann–Whitney's $U$-test). CMC, carboxymethyl cellulose sodium salt.

cortex and hippocampus of $A\beta_{25\text{-}35}$-treated mice (Figure 7A,B). In contrast, acute treatment with cilostazol (100 mg·kg$^{-1}$) had no effect on the changes in MDA levels induced by $A\beta_{25\text{-}35}$ injection (Figure 7A,B). In control mice, repeated administration of cilostazol (100 mg·kg$^{-1}$, p.o.) for 5 days slightly increased and decreased MDA levels in the frontal cortex (control: 1.23 ± 0.05, cilostazol: 1.41 ± 0.04 nmol·mg$^{-1}$ protein) and hippocampus (control: 1.03 ± 0.04, cilostazol: 0.91 ± 0.03 nmol·mg$^{-1}$ protein) respectively.

### *Effects of cilostazol on cytokine levels in the hippocampus of $A\beta_{25\text{-}35}$-injected mice*

To determine whether pro-inflammatory cytokines are involved in the lipid peroxidation and/or the ameliorating effect of cilostazol in $A\beta_{25\text{-}35}$-treated mice, we examined the effect of cilostazol on the levels of cytokines [IL-1β, IL-2, IL-4 and granulocyte macrophage colony-stimulating factor (GM-CSF)] in the hippocampus 2, 3 and 5 days after administration of $A\beta_{25\text{-}35}$ (9 nmol per mouse). A significant increase in the levels of IL-1β was observed in the hippocampus of $A\beta_{25\text{-}35}$-injected mice ($F(1,38)$ = 9.447, $P$ < 0.01, Figure 8). In the group of 5 days post-injection, the levels of IL-1β significantly increased compared with the control group ($P$ < 0.05). Repeated treatment with cilostazol (100 mg·kg$^{-1}$) did not prevent this increase in IL-1β levels in the hippocampus of $A\beta_{25\text{-}35}$-treated mice (Figure 8). Further, cilostazol had no effect on the levels of other cytokines, including IL-2, IL-4 and GM-CSF. Acute treatment with cilostazol also had no effect on IL-1β levels after $A\beta_{25\text{-}35}$ injection (Figure 8).

## Discussion

In the present study, $A\beta_{25\text{-}35}$ induced memory impairment in both the Y-maze and the step-down type passive avoidance tests. These results are consistent with previous reports that $A\beta_{25\text{-}35}$ induces cognitive impairment in mice (Maurice *et al.*, 1996; Hiramatsu *et al.*, 2000; Alkam *et al.*, 2007; 2008). Using this model, the effects of cilostazol on memory impairment were examined. Cilostazol prevented $A\beta_{25\text{-}35}$-induced short-term and long-term memory impairment in the Y-maze and the step-down type passive avoidance tests respectively. Cilostazol had no effect on locomotor activity, assessed as the number of arm entries during the Y-maze test and ambulation in the locomotor test, or on shock sensitivity, assessed as the total score in the passive avoidance test. These results suggest that cilostazol attenuates cognitive impairments in the $A\beta_{25\text{-}35}$-injected mice without affecting motor function,

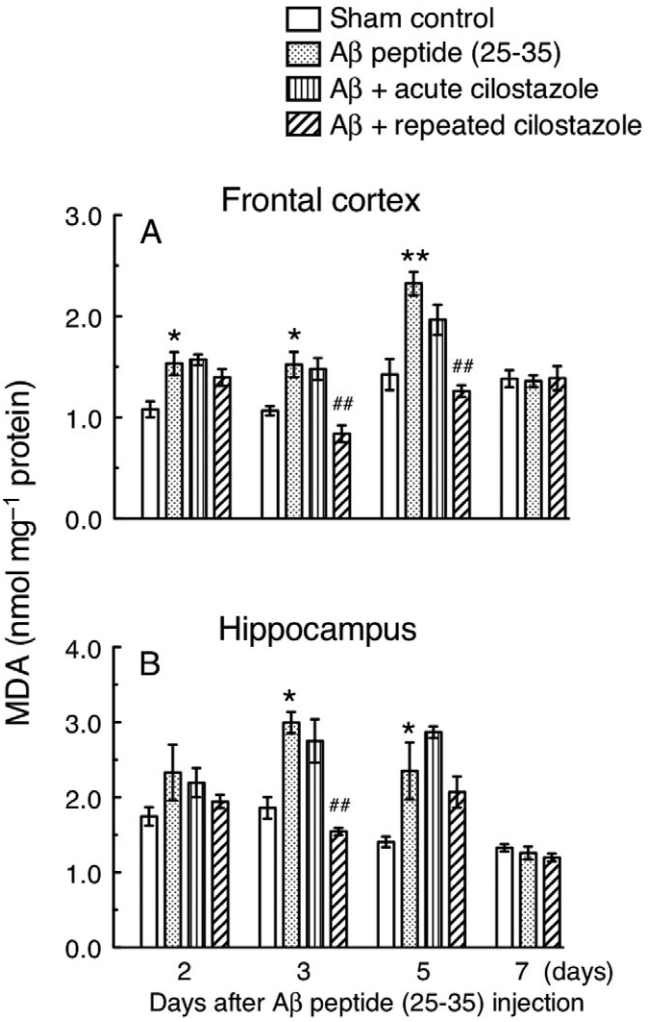
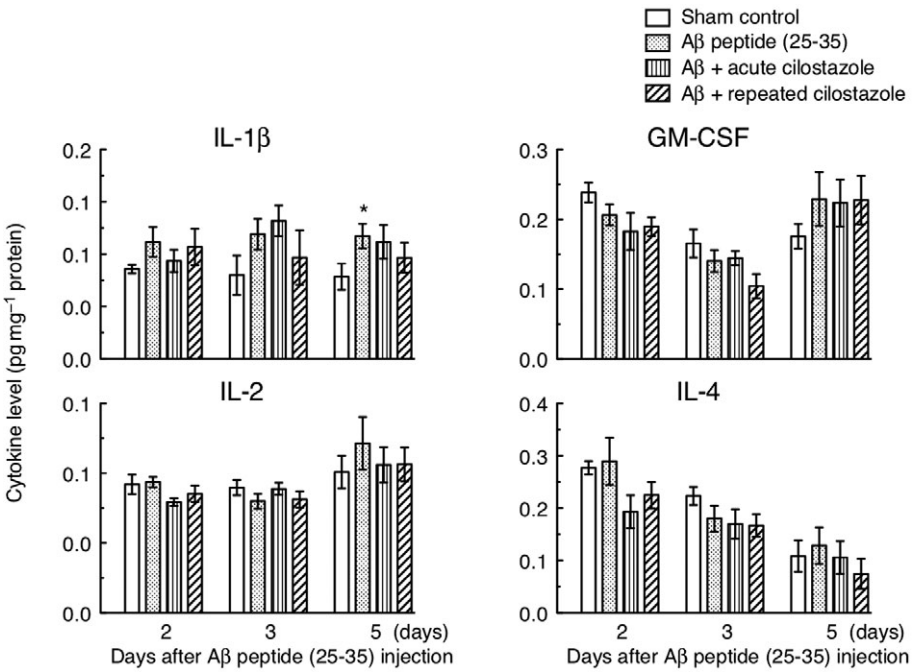



**Figure 7**

Effects of cilostazol on $A\beta_{25\text{-}35}$-induced increase in lipid peroxidation in the mouse frontal cortex (A) and hippocampus (B). $A\beta_{25\text{-}35}$ (9 nmol per mouse) was injected (i.c.v.) and the mice were killed 2, 3, 5 and/or 7 days later. Mice were treated with cilostazol (100 mg·kg$^{-1}$, p.o.) 60 min before being killed (acute) or repeatedly (3, 4, 6 or 8 times). On the 1st day, cilostazol was administered 60 min before $A\beta_{25\text{-}35}$. Lipid peroxidation levels were assessed by the thiobarbituric acid method. Each lipid peroxidation level was normalized per unit protein. Values represent means ± SEM ($n$ = 6–9). Significant levels; *$P$ < 0.05, **$P$ < 0.01 versus sham control (Student's $t$-test), ##$P$ < 0.01 versus $A\beta_{25\text{-}35}$ alone (Dunnett's test). MDA, malondialdehyde.

motivation, exploratory activity and/or shock sensitivity. Furthermore, cilostazol prevented the accumulation of lipid peroxide (MDA level) in the frontal cortex and hippocampus in the early period after $A\beta_{25\text{-}35}$ treatment, as MDA levels in both regions returned to control levels by 7 days after $A\beta_{25\text{-}35}$ treatment. To our knowledge, this is the first report stating that cilostazol protects against $A\beta_{25\text{-}35}$-induced neurotoxicity, maybe involving oxidative stress in the brain. This finding is interesting because cilostazol has been shown to have beneficial effects in clinical situations, improving the memory function of Alzheimer patients taking donepezil (Arai and Takahashi, 2009). In a recent study, it was shown that donepezil (5 mg·kg$^{-1}$, p.o.) taken for 13 days following okadaic acid injection improved memory performance and also significantly restored MDA and glutathione levels in rats (Kamat *et al.*, 2010). Lee *et al.* (2007) reported that concurrent administration of cilostazol (30 mg·kg$^{-1}$, p.o.) with donepezil (0.3 mg·kg$^{-1}$, i.p.) effectively attenuates cognitive dysfunction and increases neuroprotection after chronic cerebral hypoperfusion in rats. Further, Okajima and his colleagues have recently reported that donepezil and cilostazol improve cognitive function in mice by increasing the production of insulin-like growth factor-1 (IGF-1) (Narimatsu *et al.*, 2009; Zhao *et al.*, 2010). Taken together, these findings indicate that cilostazol can have a beneficial effect not only on vascular dementia, but also in AD.

It has been reported that oxidative stress plays a critical role in the development of AD and mild cognitive impairment (Markesbery, 1997; Mecocci, 2004). Lipid peroxidation is one of the major outcomes of free radical-mediated injury; it directly damages membranes and generates a number of secondary products including aldehydes, such as MDA (Slater, 1984). Further, analysis of brains of AD patients has demonstrated that they have an increase in lipid peroxidation products in the hippocampus and amygdala compared with age-matched controls (Markesbery and Lovell, 1998). Amyloid precursor protein transgenic mice, a genetic mouse model of AD, display a systemic increase in lipid peroxidation (Praticò *et al.*, 2001). In addition, lipid peroxidation up-regulates the β-site of amyloid precursor protein (APP) cleaving enzyme 1 (BACE1) expression *in vivo* (Chen *et al.*, 2008), suggesting that prevention of lipid peroxidation is a very important early event for amyloidogenesis in AD. Because MDA is the most abundant individual aldehyde resulting from lipid peroxidation, it is a useful oxidative marker for AD and mild cognitive impairment (Greilberger *et al.*, 2008). To confirm the effect of cilostazol on $A\beta_{25\text{-}35}$-induced oxidative stress, we measured the levels of MDA (a marker of lipid peroxidation) in the frontal cortex and hippocampus. $A\beta_{25\text{-}35}$ significantly increased the levels of MDA in the frontal cortex and hippocampus 2, 3 and/or 5 days after injection. Interestingly, there was no significant increase in MDA levels in the $A\beta_{25\text{-}35}$-treated group on day 7. This suggests that it is very important to prevent oxidative stress in the early period after $A\beta_{25\text{-}35}$ treatment. After $A\beta_{25\text{-}35}$ treatment, oxidative stress increases during days 1–5 (in the case of hippocampus, days 3–5) and this may




## Figure 8

Effects of cilostazol on the levels of pro-inflammatory cytokines in mouse hippocampus after $A\beta_{25\text{-}35}$ injection. $A\beta_{25\text{-}35}$ (9 nmol per mouse) was injected (i.c.v.) and the mice were killed 2, 3 and/or 5 days later. Mice were treated with cilostazol (100 mg·kg$^{-1}$, p.o.) 60 min prior to death (acute) or repeatedly (3, 4 or 6 times). On the 1st day, cilostazol was administered 60 min before $A\beta_{25\text{-}35}$. Pro-inflammatory cytokine levels were assessed by the suspension array method. Each cytokine level was normalized per unit protein. Values represent means ± SEM ($n$ = 4–6). Significant levels; *$P$ < 0.05 versus corresponding sham control (Student's $t$-test). GM-CSF, granulocyte macrophage colony-stimulating factor.

affect subsequent behavioural events. Thereafter, oxidative stress may decrease defensive systems, at the next step contributing to behavioural impairment or, simply, a single injection of $A\beta_{25\text{-}35}$ does not have a long-lasting effect on lipid peroxidation. This could explain why cilostazol did not have a significant ameliorating effect when it was administered 5 days after the $A\beta_{25\text{-}35}$ injection. Repeated treatment with cilostazol completely prevented the accumulation of MDA induced by $A\beta_{25\text{-}35}$, while acute treatment did not show any effect. The level of MDA in the hippocampus 2 days after $A\beta_{25\text{-}35}$ injection was not significantly different from control levels. Behaviourally, only the schedule in which cilostazol was injected repeatedly, starting from 1 or 2 days, but not 5 days, after $A\beta_{25\text{-}35}$ administration, significantly prevented the memory impairments induced by this peptide. These results suggest that the protective effect of cilostazol on $A\beta_{25\text{-}35}$-induced memory impairment is related to the accumulation of oxidative stress in the early period after $A\beta_{25\text{-}35}$ injection, in part in the frontal cortex and hippocampus. This indicates that not only oxidative stress, but also other mechanisms, may be involved in the ameliorative effect of cilostazol. In fact, as described previously, it has been reported that cilostazol improved cognitive function by increasing the production of IGF-1 in the hippocampus (Zhao *et al.*, 2010). Therefore, we need to check whether IGF-1 production in the hippocampus and/or frontal cortex is involved in this ameliorating effect of cilostazol.

It has been reported that $A\beta_{25\text{-}35}$ increases the mRNA levels of APP and IL-1β. Consistent with these changes in mRNA levels, immunoblotting analysis revealed that $A\beta_{25\text{-}35}$ also increases APP, BACE and IL-1β in the rat hippocampus (Lin *et al.*, 2009). Previous research in our laboratory has shown that lipopolysaccharide (LPS) administration produces learning and/or memory deficits in a variety of paradigms along with an increase in IL-1β. ROS are also generated following LPS administration, indicating that there are some common mechanisms between these models. ROS release by activated microglia, which are present near amyloid fibres, has been reported in the brains of patients with AD (Tarkowski *et al.*, 2001). The mechanism by which ROS are generated by $A\beta_{1\text{-}40}$ involves the production of cytokines by activated microglia that then act on nuclear factor κB, inhibitor of NF-κB or other transcription factors leading to the induction of iNOS (Medeiros *et al.*, 2007). The production of cytokines by these microglia, as well as lipid peroxidation, may be associated with the onset and pro-

gression of the dementia associated with AD. However, in the present study, repeated administration of cilostazol not only failed to antagonize the increase in the expression of IL-1β, but there was also no significant difference in the expression of IL-2, IL-4 or GM-CSF after $A\beta_{25-35}$ injection. Therefore, there are some differences between these animal models, and cilostazol may only provide protection against oxidative stress.

Although the mechanism by which cilostazol regulates $A\beta_{25-35}$-induced oxidative stress remains to be determined in detail, there are several possible explanations. DNA fragmentation in the cortical tissue of rats with focal ischaemia has been shown to be significantly suppressed by cilostazol, but not by aspirin (Lee *et al.*, 2005). This suggests that cilostazol, but not aspirin, has an anti-apoptotic effect. Lee *et al.* (2004) elucidated the signalling pathway by which cilostazol suppresses increased phosphatase and tensin homolog deleted from chromosome 10 (PTEN) phosphorylation, which is evoked following 2 h of middle cerebral artery occlusion/24 h reperfusion, as being in association with reduced apoptotic cell death in rats. Cilostazol has a broad spectrum of activities, including antagonism of PDE III. The mode of action of cilostazol is different from that of aspirin in that cilostazol inhibits not only secondary platelet aggregation but also primary platelet aggregation induced by aggregating agents such as ADP. Cilostazol is potent at preventing death due to pulmonary thrombosis by platelet aggregates in mice *in vivo* (Kimura *et al.*, 1985). Cilostazol also increases intracellular cyclic AMP levels by inhibiting PDE III, scavenging hydroxyl radicals, and reducing intracellular ROS and TNF-α production induced by LPS (Kim *et al.*, 2002) and chronic cerebral hypoperfusion (Lee *et al.*, 2006). Furthermore, it has been reported that cilostazol exerts a protective effect in the brain through the cAMP-responsive element binding protein (CREB) phosphorylation pathway leading to the up-regulation of Bcl-2 and COX-2 expression (Kim *et al.*, 2006) after chronic cerebral hypoperfusion. These data suggest that cilostazol is potentially useful for the treatment of cognitive impairment in post-stroke patients (Watanabe *et al.*, 2006). Recently, Miyamoto *et al.* (2009) reported that phosphorylated CREB within neuroblasts is markedly decreased in the subventricular zone and olfactory bulb of vehicle-treated rats under conditions of chronic cerebral hypoperfusion. However, treatment with cilostazol resulted in recovery of the expression of this CREB throughout the hypoperfusion period, leading to enhanced neurogenesis. Further, cilostazol improved cognitive function by increasing the production of IGF-1 in the hippocampus (Zhao *et al.*, 2010). In line with these findings, it is possible that cilostazol not only prevents oxidative damage directly by scavenging free radicals but may also work via other mechanisms such as CREB signalling, which may be a key mediator of neurogenesis. While we have used acute treatment of mice with the $A\beta_{25-35}$ as a model for AD in the present study, it may not be the best model for AD; therefore, the effects of cilostazol need to be replicated in more physiological models of AD, and the involvement of additional molecular correlates needs to be clarified, such as CREB signalling, DNA fragmentation, as an antiapoptotic effect and IGF-1 production.

In conclusion, the results from the present study confirm, for the first time, that cilostazol ameliorates the memory deficits induced by $A\beta_{25-35}$ in mice. The effect of cilostazol may be attributed to the prevention of oxidative damage in the hippocampus as measured in terms of the amount of peroxidized lipid. Cilostazol is well tolerated as a therapeutic agent, and is itself, or in combination with other drugs such as donepezil, a potential candidate for the treatment of cognitive deficits in AD.

## Acknowledgements

This study was supported by Otsuka Pharmaceutical Co., Ltd. and in part by the Academic Frontier Project for Private Universities (2007-11) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

## Conflict of interest

None.

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