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Fenobam promoted the neuroprotective effect of PEP-1-FK506BP following oxidative stress by increasing its transduction efficiency

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We examined the ways in which fenobam could promote not only the transduction of PEP-1-FK506BP into cells and tissues but also the neuroprotective effect of PEP-1-FK506BP against ischemic damage. Fenobam strongly enhanced the protective effect of PEP-1-FK506BP against H_2O_2-induced toxicity and DNA fragmentation in C6 cells. In addition, combinational treatment of fenobam with PEP-1-FK506BP significantly inhibited the activation of Akt and MAPK induced by H_2O_2, compared to treatment with PEP-1-FK506BP alone. Interestingly, our results showed that fenobam significantly increased the transduction of PEP-1-FK506BP into both C6 cells and the hippocampus of gerbil brains. Subsequently, a transient ischemic gerbil model study demonstrated that fenobam pretreatment led to the increased neuroprotection of PEP-1-FK506BP in the CA1 region of the hippocampus. Therefore, these results suggest that fenobam can be a useful agent to enhance the transduction of therapeutic PEP-1-fusion proteins into cells and tissues, thereby promoting their neuroprotective effects. [BMB Reports 2013; 46(11): 561-566]

INTRODUCTION

FK506-binding proteins (FK506BPs) are the immunophilin proteins that bind with high affinity to various immunosuppressive drugs such as FK506, cyclosporine A, and rapamycin (1, 2). The family of FK506BPs has been classified according to their molecular size ranging from 12 to 59 kDa, and are known to have multiple functions in cells. FK506BPs exhibit peptidylprolyl cis/trans isomerase (PPIase) activity that is associated with the protein folding process (1). Also, FK506BP12, which has a molecular weight of 12 kDa, can form complexes with FK506 or rapamycin and the resulting complex of FK506 and FK506BP12 interacts with the regulatory B subunit of the calcium-dependent phosphatase calcineurin (CaN), inhibiting the activity of CaN, thereby inhibiting the activation of interleukin-2 (IL-2) and T-cells (3). In the absence of FK506, FK506BP12 interacts with the TGF beta (TGFβ) receptor, calcium channels or the inositol-(1,4,5)- triphosphatae (IP_3) receptor (4-6). Furthermore, previous studies have shown that FK506BP12 knockout mice have cardiac defects due to calcium channel dysfunction and cell cycle deregulation (7, 8).

An anxiolytic drug, fenobam (1-(3-chlorophenyl)-3-(3-methyl-5-oxo-4H-imidazol-2-yl) urea), is known to be a potent antagonist for the metabotropic glutamate (mGlu) 5 receptor, which is abundantly expressed in brain tissues, particularly in the CA1 and CA3 regions of the hippocampus, and plays an important role in emotional processes including depression and anxiety (9, 10).

Here we demonstrate that, aside from its anxiolytic function, fenobam has a positive influence on not only the neuroprotective effect of PEP-1-FK506BP against oxidative stress in gerbil brains but also on the inhibitory effect of PEP-1-FK506BP in the oxidative-stress-induced activation of Akt and mitogen activating protein kinase (MAPK) in C6 cells. Also, we provide evidence that fenobam mediates the transduction increase of PEP-1-FK506BP into C6 cells and brain tissues, although the action mechanism of fenobam that elevate the cellular level of PEP-1-FK506BP is not exactly understood.

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RESULTS

Pretreatment with fenobam significantly increased the protective effect of PEP-1-FK506BP on hydrogen peroxide (H_2O_2)-induced cell toxicity

We investigated the effect of fenobam and PEP-1-FK506BP on the survival of C6 cells against oxidative stress. The chemical structure of fenobam and schematic structures of PEP-1-FK506BP are shown in Fig. 1A and B, respectively. Fenobam rarely exhibits cytotoxic effects in C6 cells at concentrations of 50-150 ng/ml (Fig. 1C). Next, we examined whether PEP-1-FK506BP could decrease H_2O_2-induced death of C6 cells and in what way the protective effect of PEP-1-FK506BP against H_2O_2 is influenced by fenobam. H_2O_2 strongly decreased C6 cell viability by up to 40% compared to the control. However, PEP-1-FK506BP suppressed H_2O_2-induced toxicity in a dose-dependent manner (Fig. 1D). In addition, pretreatment with fenobam further increased the protective effect of PEP-1-FK506BP against H_2O_2 toxicity (Fig. 1D). Also, in the presence of fenobam and/or PEP-1-FK506BP, DNA damage in H_2O_2-treated cells was assessed using terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling (TUNEL) assay. As shown in Fig. 1E, PEP-1-FK506BP inhibited H_2O_2-induced DNA damage and, furthermore, combinational treatment with PEP-1-FK506BP and fenobam significantly enhanced the suppression of H_2O_2-induced DNA damage by PEP-1-FK506BP. By contrast, single treatment with fenobam rarely produced significant protection against DNA damage. From these results, it is suggested that fenobam promotes the protective effect of PEP-1-FK506BP against H_2O_2-inducedoxidative stress in C6 cells.

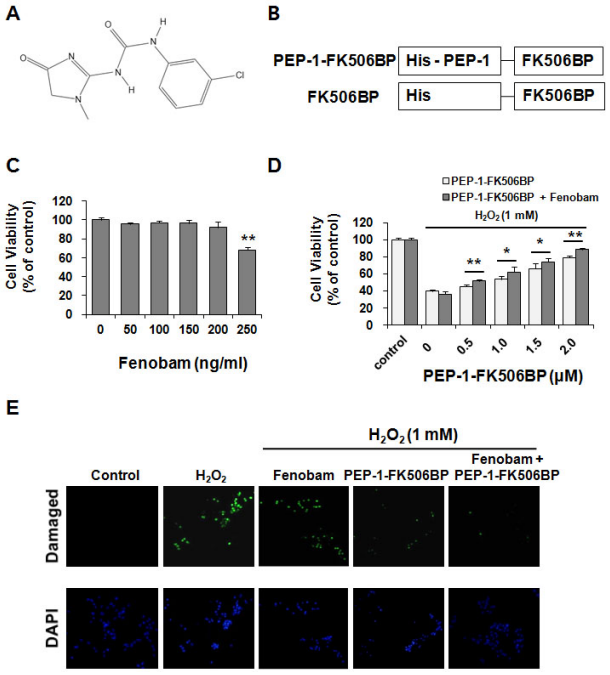

Fig. 1. Effect of fenobam and PEP-1-FK506BP on H_2O_2-induced cell toxicity and DNA damage in C6 glioma cells. (A) Chemical structure of fenobam, 1-(3-chlorophenyl)-3-(3-methyl-5-oxo-4H-imidazol-2-yl) urea. (B) Schematic structures of PEP-1-FK506BP. The His sequence is used for purification and detection of both PEP-1-FK506BP and Tat-GFP. (C) Effect of fenobam on survival of C6 cells. After, C6 cells were treated with various concentrations of fenobam (50-250 ng/ml) for 18 h, cell viability was evaluated by MTT assay. (D) Effect of PEP-1-FK506BP on H_2O_2-induced cell toxicity under pretreatment with fenobam. C6 cells were sequentially treated with fenobam (150 ng/ml) 1 h prior to treatment with PEP-1-FK506BP, and then exposed to H_2O_2 (1 mM) for 18 h. Cell viability was assessed using MTT assay. (E) Effect of PEP-1-FK506BP on H_2O_2-induced DNA damage in the presence of fenobam. C6 cells were pretreated with fenobam (150 ng/ml) for 1 h and then treated with PEP-1-FK506BP (2.0 μM). After the cells were exposed to H_2O_2 (1 mM) for 6.5 h, DNA damage of all samples was compared after TUNEL staining. Data was analyzed by Student's t test. (C) $^{**}P < 0.01$ between control and fenobam-treated groups. (D) $^{*}P < 0.05$ and $^{**}P < 0.01$ between PEP-1-FK506BP-treated and PEP-1-FK506BP + fenobam-treated groups.

Activation of H_2O_2-induced MAPK and Akt can be inhibited by co-treatment with fenobam and PEP-1-FK506BP more significantly than by treatment with PEP-1-FK506BP alone

Survival signaling kinase, that is Akt, and MAPKs including p38 and Erk1/2, are activated in response to various stimuli such as cytokines and ultraviolet irradiation (11, 12). The phosphorylations of Akt, p38, and Erk1/2 were increased at intervals of 8, 35, and 25 min, respectively, after exposure to H_2O_2.

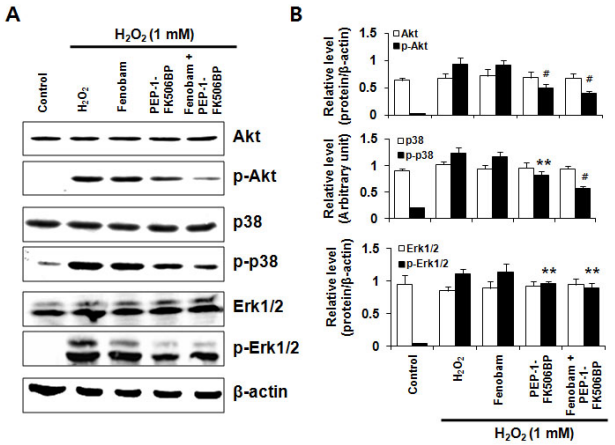

Fig. 2. Effect of co-treatment with fenobam and PEP-1-FK506BP on H_2O_2-induced activation of Akt and MAPK. C6 cells were incubated with fenobam (150 ng/ml) for 1 h, followed by a treatment with PEP-1-FK506BP (2.0 μM). Then, to evaluate cellular levels of Akt/p-Akt, p38/p-p38, and Erk1/2/p-Erk1/2, cells were harvested at 8, 35, and 25 min, respectively, after H_2O_2 treatment (1 mM). (A) Representative Western blotting data showing levels of p-p38/p38, p-Akt/Akt, and p-Erk1/2/Erk1/2. (B) Their levels are quantified using a densitometer. The bar graph represents means ± SD from three independent experiments. Data was analyzed by Student's t test. $^{**}P < 0.01$ and $^{\#}P < 0.001$ between H_2O_2 and other groups.

Fenobam very slightly reduced H_2O_2-induced activation of Akt and p38, whereas PEP-1-FK506BP highly suppressed the activation of Akt, p38, and Erk1/2 (Fig. 2A and 2B). In addition, a combinational treatment with fenobam and PEP-1-FK506BP led to a very significant reduction in the levels of p-Akt and p-p38 and a slight decrease in the level of p-Erk1/2, compared to the PEP-1-FK506BP-treated sample (Fig. 2A and 2B). Together with Fig. 1D and 1E, these results demonstrate that fenobam contributes to the protective effect of PEP-1-FK506BP by suppressing H_2O_2-induced activation of Akt and MAPK rather than directly protecting C6 cells from H_2O_2.

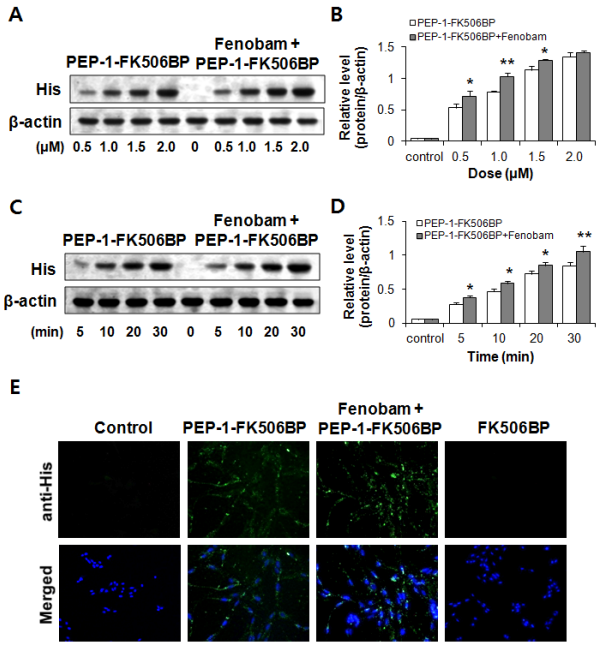

Fig. 3. Effect of fenobam on transduction of PEP-1-FK506BP into C6 glioma cells. C6 glioma cells were preincubated with or without fenobam (150 ng/ml) for 1 h, followed by exposure to PEP-1-FK506BP. (A) and (B) To investigate the dose-dependency of PEP-1-FK506BP transduction into cells, the treated concentrations of two proteins were as follows; 0.5, 1.0, 1.5, and 2.0 μM. (C) and (D) Also, to investigate the time-dependency of transduction of PEP-1-FK506BP, treatment times were chosen as follows; 5-30 min. After incubation with PEP-1-FK506BP, the cells were trypsinized and washed with PBS three times. Cell lysates were analyzed by Western blot analysis using a His antibody and a secondary antibody. Relative band density in the Western blot was measured using a densitometer. The bar graph represents means ± SD from three independent experiments. Data was analyzed by Student's t test. *P < 0.05 and **P < 0.01 between PEP-1-FK506BP-treated and PEP-1-FK506BP + fenobam-treated groups. (E) Distribution of PEP-1-FK506BP in C6 cells in the presence of fenobam. Cells were pre-treated with fenobam (150 ng/ml) for 1 h and then treated with FK-506BP and PEP-1-FK506BP (2.0 μM) for 1 h. The cells were immunostained with a His and Alexa fluor 488-conjugated secondary antibody.

Effect of fenobam on transduction of PEP-1-FK506BP into C6 cells

We previously reported that PEP-1-FK506BP could significantly transduce into HaCaT cells and ameliorate atopic dermatitis in mice (13). The manner in which the cellular level of PEP-1- FK506BP is increased could lead to an enhanced protective effect from PEP-FK506BP, as previously described (14, 15). Therefore, we evaluated whether fenobam affects the transduction ability of PEP-1-FK506BP into C6 cells. PEP-1-FK506BP was transduced into C6 cells in a dose- (Fig. 3A and 3B) and a time-dependent manner (Fig. 3C and 3D). Pretreatment with fenobam significantly enhanced the transduction level of PEP-1-FK506BP into C6 cells (Fig. 3A-D). Also, consistent with the result from PEP-1-FK506BP, preincubation with fenobam produced increased transduction of the Tat-green fluorescence protein (Tat-GFP) into the cells (data not shown). Our fluorescence microscopy data showed that PEP-1-FK506BP was found insignificant amounts in C6 cells and fenobam promoted the transduction of PEP-1-FK506BP into the cells, compared with a single treatment of PEP-1-FK506BP (Fig. 3E). Next, we examined whether fenobam could promote *in vivo* transduction of PEP-1-FK506BP into gerbil brain tissues. Fenobam was intraperitoneally (i.p.) injected into gerbils at a dose of 150 μg/kg 1 h prior to an injection of PEP-1-FK506BP

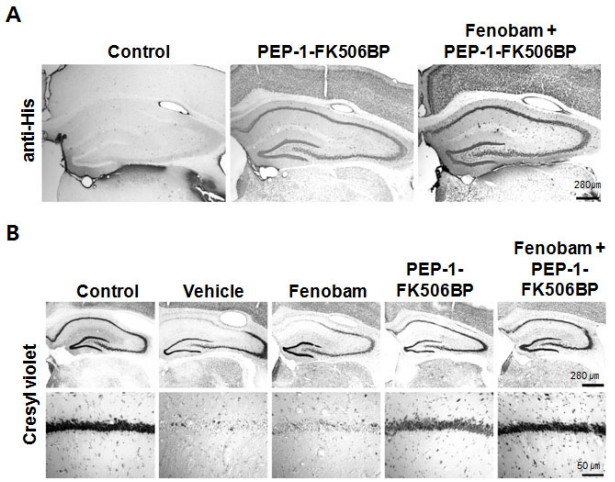

Fig. 4. Fenobam increased neuroprotection of PEP-1-FK-506BP in the CA1 regions by increasing *in vivo* transduction of PEP-1-FK506BP into the brain tissues (A) Effect of fenobam on transduction of PEP-1-FK-506BP into brain tissues. Fenobam (150 μg/kg) was i.p. injected into gerbils (n=7/group) 1 h prior to an injection of PEP-1-FK506BP (200 μg/kg). Six hour later gerbil brains from all groups were collected. Histological analysis of the hippocampus was carried out using a His antibody and a secondary antibody. (B) Neuroprotection of PEP-1-FK506BP on the CA1 regions in a transient cerebral forebrain ischemic model. Fenobam (150 μg/kg) was i.p. injected into gerbils (n=7/group) 1 h prior to an injection of PEP-1-FK506BP (200 μg/kg). Gerbil brains were collected 6 h after transient forebrain ischemic damage was induced in the gerbils. Brain sections of the gerbils were stained with cresyl violet and neuron survival in the CA1 of the hippocampus was evaluated.

(200 μg/kg). Six hours later the brains were collected and brain sections were probed with a His and a secondary antibody. Immunohistological analysis demonstrated that PEP-1-FK506BP was much more strongly observed in the presence of fenobam than in the absence of fenobam (Fig. 4A). This data demonstrates that fenobam significantly enhanced the blood-brain barrier (BBB) penetration of PEP-1-FK506BP.

Fenobam positively affected the protective effect of PEP-1-FK506BP on the CA1 region in a transient cerebral forebrain ischemic model

We investigated whether the transduction increase of PEP-1-FK506BP, which was caused by fenobam, has an influence on the neuroprotective effect of PEP-1-FK506BP against oxidative stress by assessing the neuroprotection of the CA1 in a gerbil model of ischemic damage. Histolgical analysis revealed that the fenobam group showed very weak protection of neurons in the CA 1 region against ischemic damage (Fig. 4B, the 3rd panel) and, by contrast, PEP-1-FK506BP exhibited a strong protective effect on neurons in the same region (Fig. 4B, the 4th panel). Most importantly, pretreatment with fenobam significantly enhanced the neuroprotection of PEP-1-FK506BP on the CA1 region from ischemic damage (Fig. 4B, the 5th panel). Taken together, it can be concluded that fenobam could serve as an agent to significantly enhance the neuroprotective effect of PEP-1-FK506BP on neurons against oxidative stress.

DISCUSSION

In this study, we demonstrated that fenobam enhanced the neuroprotective activity of PEP-1-FK506BP12 against oxidative stress and its functions are associated with significant increases of *in vitro* and *in vivo* transduction of PEP-1-FK506BP. This shows that fenobam is useful as an agent to enhance the delivery of therapeutic protein transduction domains (PTDs) fusion proteins to cells and tissues.

The ischemia/reperfusion condition, which refers to a return of oxygenated blood to the ischemic injury site after a restriction of blood supply, causes depletion of ATP, ionic pump failure, ionic imbalance (including Ca^{2+}), and also highly elevated levels of reactive oxygen species (ROS) (16, 17). ROS may cause lipid peroxidation, protein denaturation, DNA damage, and finally apoptosis and necrosis of cells (18). Astrocytes, the predominant cell type of the brain, provide energy substrates to neurons and support the BBB (19). In addition, following brain injury, reactive astrocytes protect neurons by producing neurotrophic factors and activating various cellular signals such as inflammatory responses, Akt, and MAPK (12, 19). Accordingly, suppressing the activation of astrocytes in response to oxidative stress is generally recognized as a therapeutic target for the protection of neurons. Our data showed that PEP-1-FK506BP12 significantly suppressed not only H_2O_2-induced toxicity and DNA damage (Fig. 1D and 1E) but also H_2O_2-induced phosphorylation of Akt and MAPK including p38 and Erk1/2 (Fig. 2A and 2B). On the other hand, interestingly, treatment with fenobam prior to treatment with PEP-1-FK506BP provided much higher suppression in DNA damage, cell toxicity, and the phosphorylation of Akt and MAPK in response to H_2O_2, compared to the sample treated with PEP-1-FK506BP alone.

PTDs are small size peptide fragments such as Tat and PEP-1. Their conjugations with other proteins, having low delivery to various cells and tissues, have been reported to confer enhanced penetration, although the cellular entry mechanisms of PTD fusion proteins have not been clearly revealed (20). Subsequently, it has been suggested that an increase in cellular delivery of therapeutic biomolecules using PTD tools could lead to the possibility of using various macromolecules with low permeability but high potency as drugs. Also, we demonstrated that PEP-1-FK506BP, which showed highly potent delivery to cells and tissues, inhibits the inflammatory reaction in Raw264.7 cells and mice ears and alleviates atopic dermatitis (13, 21). Therefore, as shown in Fig. 3 and 4, it is very noteworthy that the presence of fenobam, which does not show a toxic effect, could increase the transduction of PEP-1-FK506BP into astrocyte cells and brain tissues, subsequently leading to cell protection.

Fenobam was known to be an antagonist of the G-protein-coupled mGlu 5 receptor activated by glutamate (10). The mGlu5 receptor is highly expressed in the limbic brain regions, including hippocampus, which is involved in emotional processes. Activation of mGlu5 receptor in pre- and postsynaptic neurons hydrolyzes phosphoinositide phospholipids into inositol 1,4,5-triphosphate and diacyl glycerol by phospholipase C via interaction with G proteins, subsequently leading to diverse biological effects, including modulation of various ion channels and regulatory and signaling molecules (9, 22, 23). Therefore, several antagonists of the mGlu5 receptor, including fenobam, which are considered as therapeutic targets for anxiety and depression, Parkinson's disease, pain, addiction, and fragile X syndrome, have been developed for modulating the activation of the mGlu5 receptor (24). For example, a previous study suggested that fenobam may be effective for enhancement of procedural memory formation and avoidance behavior in *Fmr*1 knockout mice (25). In addition, fenobam alleviates both inflammatory and non-inflammatory bladder nociception (26). In fact, previous studies on fenobam have mainly focused on its therapeutic potential for various central nervous system disorders including memory and pains. By contrast, our results are very interesting in that fenobam is likely to be involved in *in vitro* and *in vivo* transduction of PTD-fusion proteins, although the precise mechanism is unknown.

Taken together, we have demonstrated that fenobam has the potential to highly improve transduction of PEP-1-FK506BP protein into cells or brain tissues and enhance the inhibitory effect of PEP-1-FK506BP against oxidative stress-induced cell toxicity and signal activation, and thereby contributes to the neuroprotective potential of PEP-1-FK506BP. Therefore, fenobam may be very useful as an agent to increase the levels of

therapeutic PTD fusion proteins in various cells and tissues.

MATERIALS AND METHODS

Materials

PEP-1-FK506BP and FK506BP proteins were purified as described previously (13). All other chemicals and reagents, unless otherwise stated, were obtained from Sigma-Aldrich (St. Louis, USA) and were of the highest analytical grade available.

Measurement of cell viability

C6 rat astrocytoma cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% heat-inactivated fetal bovine serum (Gibco BRL, USA) and antibiotics (100 μg/ml streptomycin, 100 U/ml penicillin; Gibco BRL) at 37°C under humidified conditions of 95% air and 5% CO_2. To determine cell viability, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed (14). After cells were incubated with fenobam (150 ng/ml) and/or PEP-1-FK506BP, cell toxicity was induced by H_2O_2 (1 mM) for 4 h. Then, a MTT solution was added to each well for 4 h and the supernatant in each well was removed. Formazan crystal was dissolved in dimethyl sulfoxide and the observance was measured at 570 nm. Cell viability was expressed as a percentage of the H_2O_2-untreated control.

TUNEL assay

DNA damage of C6 cells was evaluated by TUNEL assay. Briefly, C6 cells treated with fenobam and/or PEP-1-FK506BP were additionally maintained in DMEM for 6.5 h after treatment with H_2O_2 (1 mM). TUNEL staining was performed using a Cell Death Detection kit (Roche Applied Science, Basel, Switzerland) according to the manufacturer's instructions. Fluorescence was detected using an Eclipse 80i fluorescence microscope (Nikon, Tokyo, Japan).

Western blot analysis

Equal amounts of cell lysates were separated using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrically transferred to a nitrocellulose membrane. The membrane was blocked with 5% skim milk and target proteins were probed with specific antibodies and horseradish peroxidase-conjugated secondary antibodies. The bands were detected using enhanced chemiluminescence reagents according to the manufacturer's instructions (Amersham, USA).

Fluorescence microscopy

Cells were seeded on glass coverslips and incubated with fenobam and/or PEP-1-FK506BP. After incubation, cells were fixed with 4% paraformaldehyde for 10 min and incubated sequentially with an anti-His and Alexa Fluor 488-conjugated secondary antibodies. Nuclei were stained with 1 μg/ml 4′6-diamidino-2-phenylindole (DAPI) (Roche Applied Science) for 30 min. Fluorescence was analyzed using an Olympus FV-300 confocal fluorescent microscope (Olympus, Japan).

Animal experiments

Male Mongolian gerbils were maintained under standard animal care conditions. All experimental procedures involving animals and their care conformed to the Guide for the Care and Use of Laboratory Animals of the National Veterinary Research & Quarantine Service of Korea and were approved by the Hallym Medical Center Institutional Animal Care and Use Committee.

To assess the ability of PEP-1-FK506BP to penetrate the BBB, fenobam (150 μg/kg) and PEP-1-FK506BP (200 μg/kg) were i.p. injected into gerbils (n=7/group), respectively and the brains were then collected. To examine the survival of neurons in the CA1 region, fenobam, PEP-1-FK506BP, and fenobam+PEP-1-FK506BP were administered i.p. 30 min prior to an induction of ischemic injury. Cerebral forebrain ischemia damage was induced as previously described (27). For the histological analysis, the brains sections were incubated with an anti-His antibody (1 : 1,000) for 48 h at 4°C or stained with cresyl violet.

Acknowledgements

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