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Blood-Brain Barrier Permeability of Novel [D-Arg^2]Dermorphin (1–4) Analogues: Transport Property Is Related to the Slow Onset of Antinociceptive Activity in the Central Nervous System

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Running title : BBB Transport of [D-Arg^2]Dermorphin (1-4) Analogues

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ABBREVIATIONS: ADAB, N^{α}-amidino-Tyr-D-Arg-Phe-βAla-OH; ADAMB, N^{α}-amidino-Tyr-D-Arg-Phe-MeβAla-OH (ADAMB) AME, adsorptive-mediated endocytosis; BBB, blood-brain barrier; B_{max}, maximum binding capacity, dermorphin, H- Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2; HPLC, high-performance liquid chromatography; K_d, half-saturation constant; $PS_{BBB,inf}$, BBB permeation influx rate; TAPA, H-Tyr-D-Arg-Phe-βAla-OH; TMPA, H-Tyr-D-MeO(RS)-Phe-MeβAla-OH; TM-BBB4, a conditionally immortalized mouse brain capillary endothelial cell line.

A recommended section

Absorption, Distribution, Metabolism, & Excretion

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Abstract

In order to clarify the pharmacological characteristics of N^{α}-amidino-Tyr-D-Arg- Phe-βAla-OH (ADAB) and N^{α}-amidino-Tyr-D-Arg-Phe-MeβAla-OH (ADAMB), μ_1-opioid receptor-selective [D-Arg^2]dermorphin tetrapeptide analogues, the plasma pharmacokinetics and the in vivo blood-brain barrier (BBB) transport of these peptides were quantitatively evaluated. The mechanism responsible for the BBB transport of these peptides was also examined. The in vivo BBB permeation influx rates of ^{125}I-ADAB and ^{125}I-ADAMB after an i.v. bolus injection into mice were determined to be 0.0515 ± 0.0284 μL/(min • g of brain) and 0.0290 ± 0.0059 μL/(min • g of brain), respectively, both rates being slower than that of ^{125}I-Tyr-D-Arg-Phe-βAla-OH (^{125}I-TAPA), a [D-Arg^2]dermorphin tetrapeptide analogue. In order to elucidate the BBB transport mechanism of ADAB and ADAMB, a conditionally immortalized mouse brain capillary endothelial cell line (TM-BBB4) was used as an in vitro model of the BBB. The internalization of both ^{125}I-ADAB and ^{125}I-ADAMB into cells was concentration-dependent with half-saturation constant (K_d) values of 3.76 ± 0.83 μM and 5.68 ± 1.75 μM, respectively. The acid-resistant binding of both ADAB and ADAMB was significantly inhibited by dansylcadaverine (an endocytosis inhibitor) and poly-L-lysine and protamine (polycations), but it was not inhibited by 2,4-dinitrophenol, or at 4 ºC.

These results suggest that ADAB and ADAMB are transported through the BBB with slower permeation rates than that of TAPA, and this is likely to be a factor in the slow onset of their antinociceptive activity in the CNS. The mechanism of the BBB transport of these drugs is considered to be adsorptive-mediated endocytosis.

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μ-Opioid receptor agonists are crucial for the control of pain in the clinical setting. However, the classical μ-opioid agonists such as morphine frequently produce undesirable side effects, including respiratory depression, constipation and physical and psychological dependence (Narita et al. 2001). Such undesirable effects are produced through binding to μ_2-receptor, one of the μ-receptor subtypes, whereas opioid analgesia is suggested to be mediated by the μ_1-receptor subtype (Pasternak, 2001). Therefore, a highly selective μ_1-opioid receptor agonist should be more effective as an analgesic or antinociceptive neuropharmaceutical than non-selective μ-opioid receptor agonists. Based on this concept, μ_1-receptor-selective [D-Arg^2]dermorphin tetrapeptide analogues, H-Tyr-D-Arg-Phe-Sar-OH (DAS-DER) and H-Tyr-D-Arg-Phe-β-Ala-OH (TAPA), have been developed (Sasaki et al. 1984; Chaki et al. 1988a). Indeed, these peptides show a potent antinociceptive activity with low physical and psychological dependence (Paakkari et al. 1993; Sakurada et al. 2000). In addition, it has been reported that pretreatment with naloxonazine, an irreversible μ_1-opioid receptor antagonist, produced a marked rightward displacement of the dose-response curve for TAPA antinociception after i.c.v. administration. This result suggests that TAPA acts through the μ_1-opioid receptor in the CNS (Sakurada et al. 2000).

Recently, N^{α}-amidino-[D-Arg^2]dermorphin analogues, N^{α}-amidino-Tyr-D-Arg-Phe-βAla-OH (ADAB) and N^{α}-amidino-Tyr-D-Arg-Phe-MeβAla-OH (ADAMB), were designed as μ_1-opioid receptor–selective agonists with more potent and longer-lasting antinociceptive activity than TAPA (Ogawa et al. 2002). The ED_{50} values for antinociceptive activity after s.c. administration of ADAB and ADAMB to rats were 0.13 μmol/kg and 0.45 μmol/kg, respectively, so that these compounds are 2-fold to 6.5-fold more potent than TAPA (Chaki et al. 1988b; Ogawa et al. 2002). The peak of antinociceptive activity after s.c. administration of ADAMB (0.28 μmol/kg) was at 2 hr (Ogawa et al. 2002), whereas that of TAPA (0.47 μmol/kg) was at 0.75 hr (Chaki et al. 1988b), suggesting delayed onset of the opioid activity of ADAMB. The duration of the opioid action of ADAMB after s.c. administration (0.28 mmol/kg) was over 10 hr (Ogawa et al. 2002), whereas that of TAPA (0.47 mmol/kg) was less than 2 hr (Chaki et al. 1988b). Moreover, the antinociceptive activity of ADAMB in mice after s.c. administration was markedly attenuated by pretreatment with naloxonazine (Ogawa et al. 2002). The above results suggest that the *N*-terminal modification of TAPA with an amidino group had indeed resulted in slow-onset, long-lasting antinociceptive activity through the μ_1-opioid receptor in the CNS.

The antinociceptive activity of μ_1-opioid agonists is thought to be regulated by the

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concentration in the brain interstitial fluid around the μ_1-opioid receptors. Therefore, the transport characteristics at the blood-brain barrier (BBB), as well as the plasma pharmacokinetics, should govern the pharmacological characteristics of μ_1-opioid agonists after systemic administration. The BBB is formed by complex tight junctions of the brain capillary endothelial cells and restricts the movement of hydrophilic peptides and proteins between the blood and the brain interstitial fluid. A considerable variety of transporters and receptors is expressed in the brain capillary endothelial cells (Terasaki et al. 2003) and some are involved in regulating the BBB transport of opioid peptides (Banks and Kastin, 1990; King et al., 2001; Kastin et al, 1999; Dagenais et al, 2001; Fiori et al. 1997). We have already clarified that TAPA is transported through the BBB via the adsorptive-mediated endocytosis (AME) system, which is triggered by binding of the peptides to negatively charged sites on the surface of brain capillary endothelial cells (Deguchi et al. 2003). On the other hand, DALDA (Tyr-D-Arg-Phe-Lys-NH_2), a dermorphin analog, has been reported to cross the BBB via a non-saturable mechanism, probably simple diffusion (Samii et al. 1994), although this peptide has higher basicity than TAPA. The C-terminal structure of DALDA is different from that of TAPA. These findings indicated that *N*-terminal modification of TAPA with an amidino group might change the transport characteristics at the BBB, thereby

altering the pharmacological characteristics to afford slow-onset, long-lasting antinociceptive activity in the CNS.

Therefore, the purpose of this study was to evaluate quantitatively the plasma pharmacokinetics and the BBB transport of ADAB and ADAMB in vivo. Moreover, we examined the mechanism responsible for the BBB transport by using a conditionally immortalized mouse brain capillary endothelial cell line, TM-BBB4, as an in vitro model of the BBB (Hosoya et al. 2000). Clarification of the relationship between structure and BBB transport mechanism would be helpful in identifying promising candidates for novel μ_1-selective opioid peptides.

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Materials and Methods

Materials

TAPA (MW: 663.7), ADAB (MW: 705.7), ADAMB (MW: 719.7) and H-Tyr-D-MetO(RS)-Phe-MeβAla-OH (TMPA, MW: 560.7) were kindly supplied by Daiichi Fine Chemical Co., Ltd., Toyama, Japan. $Na^{125}I$ (37 GBq/mL, Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, UK) and [^{14}C(U)]sucrose (^{14}C-Sucrose, 14.8 GBq/mmol) were purchased from Perkin Elmer Life Sciences (Boston, MA, USA). All other chemicals were of analytical grade and were used without further purification.

Animals

Male ddY mice weighing 35–40 g were purchased from Japan SLC (Shizuoka, Japan). They were housed, four or five to a cage, in an animal room with a controlled environment (12-hr dark/12-hr light cycle; temperature of 23 ± 1 ˚C), with free access to food and water. All of the animal experiments were done in accordance with generally accepted animal care guidelines and with the approval of the Laboratory Animal Committee of Hokkaido College of Pharmacy.

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Cell Culture

TM-BBB4 cells were subcultured at a density of 1 x 10^4 cells/cm^2 and grown routinely in collagen type 1-coated 75 cm^2 tissue flasks (BD, Biosciences, Bedford, MA, USA) at 33 ˚C for 3 ~ 4 days under 5% CO_2 in air. The permissive temperature for TM-BBB4 cells culture is 33 ˚C, because the expression of large T-antigen is temperature-sensitive (Hosoya *et al*. 2000). The culture medium was Dulbecco's modified Eagle's medium (DMEM, Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 1.5 mg/mL sodium bicarbonate, 15 μg/mL bovine endothelial cell growth factor (bECGF, Roche Applied Science, Mannheim, Germany), 70 μg/mL benzylpenicillin potassium, 100 μg/mL streptomycin sulfate, and 10% fetal bovine serum (FBS, Moregate, Bulimba, Australia). For the binding experiments, TM-BBB4 cells were seeded at a density of 3 ~ 5 x 10^4 cells/cm^2 on collagen type 1-coated 24-well plates (BD Biosciences).

Radiolabeling of peptides

TAPA, ADAB and ADAMB were labeled with $Na^{125}I$ by the chloramine T method. The reaction mixture of ^{125}I-labeled peptides was purified by HPLC as described below. The specific activity of ^{125}I-TAPA, ^{125}I-ADAB and ^{125}I-ADAMB was approximately 1.2

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MBq/mg (purity >99%).

In Vivo Transport Studies in Mice

^{125}I-ADAB and ^{125}I-ADAMB were each intravenously administered into the right jugular vein of mice at a dose of 5.7 kBq/g body weight, which corresponds to 7.3 ~ 18.4 pmol/g body weight. At the times designated after administration, the mice were sacrificed, and the plasma and brain were removed. The total radioactivity was counted with a γ-counter (WIZARD 1480, PerkinElmer Life Sciences). To quantify unchanged ^{125}I-ADAB and ^{125}I-ADAMB in plasma and brain, the labeled peptides were intravenously administered into the right jugular vein of mice at a dose of 170 ~ 240 kBq/g body weight. The plasma and the brain homogenate were each mixed with 2 volumes of acetonitrile (for deproteinization) and an excess amount of unlabeled ADAB or ADAMB (in order to suppress co-precipitation with protein). The supernatant fluid was evaporated to dryness and then reconstituted in HPLC mobile phase (12.5% acetonitrile in 0.1% trifluoroacetic acid).

In Vitro Binding Studies with TM-BBB4 cells

TM-BBB4 cells do not form rigid tight junctions. Indeed, the net transendothelial

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electric resistance of TM-BBB4 cells grown on a filter was about 30 ohm·cm^2. Although TM-BBB4 cells express the genes of complex tight junction proteins, including claudin-5, occludins and junctional adhesion molecules, the expression of these proteins might be down-regulated (Terasaki et al. 2003). Therefore, the transport mechanism of ADAB and ADAMB was evaluated by means of an uptake/binding study rather than a transcellular transport study. The binding of ^{125}I-ADAB or ^{125}I-ADAMB to TM-BBB4 cells was examined by using a method reported previously (Deguchi et al., 2003). Briefly, TM-BBB4 cells cultured in 24-well dishes were washed three times with 1 mL of incubation buffer (138 mM NaCl, 5.0 mM KCl, 1.3 mM $CaCl_2$, 0.8 mM $MgCl_2$, 0.3 mM KH_2PO_4, 0.3 mM Na_2HPO_4, 5.6 mM D-glucose, 10 mM HEPES, pH 7.4, containing 0.1 % bovine serum albumin) and preincubated in 230 μL of incubation buffer at 37 °C or 4 °C. The binding studies were initiated by adding either 20 μL of a solution of ^{125}I-ADAB or ^{125}I-ADAMB, to give a final concentration of 160 kBq/mL (240 nM) in the presence or absence of the unlabeled ADAB or ADAMB. After the predetermined time period, the incubation buffer was removed, and the cells were washed three times with 1 mL of ice-cold incubation buffer. The acid-resistant binding, which represents the amount internalized in the TM-BBB4 cells, was evaluated by removing the labeled peptide bound to the cell surface (Deguchi et al., 2003). Briefly,

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the TM-BBB4 cells were incubated with 1 mL of ice-cold acetate-barbital buffer (28 mM CH_3COONa, 120 mM NaCl, 20 mM barbital, pH 3.0, 320 mOsm/kg) for 10 min. The cells were then washed three times with the acetate-barbital buffer, followed by solubilization with 500 μL of 1 M NaOH overnight at 4 °C. To quantify unchanged ^{125}I-ADAMB, the TM-BBB4 cells washed with the acetate-barbital buffer were solubilized with 0.5% Triton X-100 for 60 min on ice. The solubilized cells were evaporated to dryness, and then reconstituted in HPLC mobile phase as described above. The protein in the cells was measured using BCA Protein Assay Reagent (Pierce, Rochford, IL, USA).

Effects of Metabolic and Endocytosis Inhibitors and Selected Compounds on the Acid-Resistant Binding in TM-BBB4 Cells

The effects of a metabolic inhibitor, an endocytosis inhibitor and selected compounds on the acid-resistant binding of ^{125}I-ADAB and ^{125}I-ADAMB to TM-BBB4 cells were examined by the addition of 1 mM 2,4-dinitrophenol (DNP), 500 μM dansylcadaverine, 300 μM poly-L-lysine, 300 μM protamine, or 300 μM poly-L-glutamic acid.

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HPLC Analysis

An aliquot of the sample was subjected to HPLC using a Mightysil RP-18 GP, ODS column, 150 x 4.6 mm Ø (Kanto Kagaku, Tokyo Japan) and an LC-6A gradient pump system (Shimadzu, Kyoto, Japan) with an SCL-6A controller (Shimadzu). The mobile phase was 7% - 70% acetonitrile in 0.1% TFA (linear gradient: 0 – 45 min).

Data Analysis

In Vivo

The plasma concentrations at time t (C_p (t)) (total radioactivity), normalized by the injected dose, were analyzed by the non-compartmental method to estimate the area under the plasma concentration – time curve (AUC), the mean residence time (MRT), the total body clearance (CL_{tot}) and the volume of distribution at the steady state (Vd_{ss}).

The BBB permeation influx rate of ^{125}I-labeled peptides from the circulating blood to the brain was calculated by integration plot analysis using the plasma and brain concentrations after administration of ^{125}I-labeled peptides (Blasberg *et al.* 1983, Patlak *et al*. 1983).

$$K_{p,app}(t) = PS_{BBB,inf} \times \frac{AUC(t)}{} + V_i \qquad (2)$$

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$C_p(t)$

where $K_{p,app}(t)$ is the apparent brain-to-plasma concentration ratio at time t. AUC(t) is the AUC value from time 0 to t, V_i is the rapidly equilibrated distribution volume and $PS_{BBB,inf}$ is the BBB permeation influx rate from the circulating blood to the brain.

In Vitro

The data for the acid-resistant binding were expressed as the cell-to-medium concentration ratio. This value was corrected for the volume of adhering medium, estimated by the use of ^{14}C-sucrose.

$$\text{Cell / medium } (\mu L / \text{mg protein}) \text{ of } ^{125}\text{I-labeled peptide} = \frac{^{125}\text{I counts in the cell (cpm/mg protein)}}{^{125}\text{I counts in the incubation medium (cpm/}\mu L)} - \frac{^{14}\text{C counts in the cell (dpm/mg protein)}}{^{14}\text{C counts in the incubation medium (dpm/}\mu L)} \quad (3)$$

The maximal binding capacity ($B_{max,}$ pmol/mg protein), the half-saturation constant ($K_{d,}$ μM) and the nonspecific binding (α, μL/mg protein) for the acid-resistant binding were calculated by the homologous one-site competition model included in the

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nonlinear least-squares regression program PRISM-3 (GraphPad Software, CA, USA).

Cell/medium (μL/mg protein) of ^{125}I-labeled peptide =

$$\frac{B_{max}}{C + K_d} + \alpha \qquad (4)$$

where C is the peptide concentration in the medium (μM).

The ability of ADAB and ADAMB to inhibit the acid-resistant binding of ^{125}I-TAPA was estimated in terms of IC_{50} value, which is the molar concentration of unlabeled ADAB or ADAMB necessary to displace 50% of the acid-resistant binding of ^{125}I-TAPA to TM-BBB4 cells. The value of K_i was calculated from the following equation.

$$K_i = \frac{IC_{50}}{(1 + L / K_d)} \qquad (5)$$

where L equals the concentration of ^{125}I-TAPA.

Unless otherwise indicated, all data represent the mean ± S.E. Student's *t* test was used to compare individual means, and one-way analysis of variance followed by the modified Fisher's least-squares difference method was used for comparisons among more than two groups.

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Results

In Vivo Pharmacokinetics and BBB Permeability

Figures 1 A and 1 B show the time-courses of the plasma and brain concentrations after i.v. administration of ^{125}I-ADAB and ^{125}I-ADAMB at a dose of 5.7 kBq/g body weight (7.3 ~ 18.4 pmol/g body weight). Data were subjected to a pharmacokinetic analysis and these parameters are shown in Table 2. The total body clearance (CL_{tot}) and the volume of distribution at the steady state (Vd_{ss}) of ^{125}I-ADAMB were 1.8-fold and 2.5-fold smaller, respectively, than those of ^{125}I-ADAB. Therefore, the plasma AUC of ^{125}I-ADAMB was approximately 2-fold greater than that of ^{125}I-ADAB. From the integration plot analysis of the brain uptake of these peptides, $PS_{BBB,inf}$ was estimated to be 0.0515 $\pm$ 0.0284 μL/(min • g of brain) for ^{125}I-ADAB and 0.0290 $\pm$ 0.0059 μL/(min • g of brain) for ^{125}I-ADAMB, which were smaller than that of ^{125}I-TAPA (dotted line in Fig. 1 (C); data cited from Deguchi et al. 2003). The value of V_i for ^{125}I-ADAB or ^{125}I-ADAMB approximates the plasma volume of the mouse brain (Table 2). The HPLC chromatograms of plasma after i.v. administration of ^{125}I-ADAB and ^{125}I-ADAMB showed that more than 70% of the total radioactivity was recovered at the retention times of the intact peptides (Fig. 2 (B) and (D)). The supernatant prepared from the brain homogenate was also subjected to HPLC, but recovery of radioactivity was poor,

probably because separation of the peptides from proteins was not effective with the present procedure (data not shown). The opioid receptors in the brain parenchymal fraction may be included in the binding proteins.

In Vitro BBB Transport of ^{125}I-ADAB and ^{125}I-ADAMB using TM-BBB4 Cells

The values of cell/medium ratio of acid-resistant binding of ^{125}I-ADAB and ^{125}I-ADAMB at equilibrium were 4.33 ± 0.37 μL/mg protein (n = 18) and 5.94 ± 0.18 μL/mg protein (n = 4), respectively. The HPLC chromatogram showed that more than 75% of the radioactivity of acid-resistant bound ^{125}I-ADAMB was eluted at the position of intact ADAMB, suggesting that the intact form of the labeled peptide is taken up by TM-BBB4 cells (data not shown). As shown in Fig. 3, the acid-resistant binding of both ^{125}I-ADAB and ^{125}I-ADAMB to TM-BBB4 cells was concentration-dependent over the range of 0.01~1000 μM. The data were subjected to a nonlinear least-squares regression analysis, and the estimated binding parameters (B_{max}, K_d and α) are shown in Table 3. The value of B_{max} of ^{125}I-ADAMB was approximately 2-fold greater than that of ^{125}I-ADAB, whereas the values of K_d and α were almost identical between these peptides. In addition, B_{max}/K_d, the binding potency in the low concentration range, was identical for ^{125}I-ADAB and ^{125}I-ADAMB. These values were 7-fold to 8-fold greater than that of

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^{125}I-TAPA (Deguchi et al. 2003).

Effects of an Endocytosis Inhibitor, Selected Compounds, Temperature and a Metabolic Inhibitor on the Acid-Resistant Binding of ^{125}I-TAPA

As shown in Table 4, the acid-resistant binding of ^{125}I-ADAB and ^{125}I-ADAMB was inhibited by about 40 % by 500 μM dansylcadaverine (an endocytosis inhibitor), and by up to 65 % by polycations (300 μM poly-L-lysine and 300 μM protamine). These results are consistent with those of the previous report which suggested the AME mechanism for uptake of the peptide (Terasaki et al. 1992). On the other hand, a polyanion (300 μM poly-L-glutamic acid) had no significant effect. The acid-resistant binding of ^{125}I-ADAB and ^{125}I-ADAMB to TM-BBB4 cells at 4 °C was not reduced compared with the control. In addition, no reduction of the acid-resistant binding of these peptides was observed upon treatment of TM-BBB4 cells with 1 mM DNP (a metabolic inhibitor).

Inhibitory effects of ADAB and ADAMB on the acid-resistant binding of ^{125}I-TAPA to TM-BBB4 cells

As shown in Fig.4, ADAB and ADAMB competed with ^{125}I-TAPA for binding to TM-

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BBB4 in a concentration-dependent manner, whereas TMPA (a peptide without D-Arg at position 2) had no inhibitory effect. Nonlinear least-squares regression analysis of the competition curves gave K_i values of 92.1 μM for ADAB and 27.3 μM for ADAMB, suggesting that ADAMB inhibited the acid-resistant binding of ^{125}I-TAPA in TM-BBB4 cells with 3.3 times more potency than ADAB.

Discussion

The present study demonstrates that ^{125}I-ADAB and ^{125}I-ADAMB cross the BBB at slower rate than ^{125}I-TAPA (Deguchi et al. 2003), and this may account for the slow onset of antinociceptive activity after systemic administration. The mechanism responsible for their BBB transport is likely to be AME.

ADAB and ADAMB both show slow-onset, prolonged-duration antinociceptive activity after systemic administration, compared with TAPA. These pharmacological characteristics of ADAB and ADAMB are considered to be related to the pharmacokinetic properties, such as the BBB permeability and the plasma pharmacokinetics, which govern the brain interstitial concentration around the μ_1-opioid receptor. Thus, firstly, we examined the plasma pharmacokinetics of ADAB and ADAMB in order to elucidate the underlying mechanism(s) responsible for the long duration of the pharmacological effect. As shown in Table 2, the plasma AUC values of ^{125}I-ADAB (146 (% dose • min)/mL) and ^{125}I-ADAMB (265 (% dose • min)/mL) were only 1.1-fold and 2.0-fold greater than that of ^{125}I-TAPA. In addition, the MRT values of ^{125}I-ADAB (27.1 min) and ^{125}I-ADAMB (18.5 min) are both smaller than that of ^{125}I-TAPA (36.7 min). Therefore, the long duration of antinociceptive activity of ADAB and ADAMB is not directly related to the plasma pharmacokinetic properties. One possible

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explanation may be sustained occupancy in vivo of μ_1-opioid receptors in the CNS by these peptides.

Next, the BBB transport of ADAB and ADAMB was quantitatively evaluated by the intravenous injection method to elucidate the mechanism responsible for the slow onset of the antinociceptive activity after systemic administration. The values of $PS_{BBB,inf}$ for ^{125}I-ADAB (0.0515 μL/(min • g brain)) and ^{125}I-ADAMB (0.0290 μL/(min • g brain)) were slower than those of ^{125}I-TAPA (0.265 μL/(min • g brain)) (Deguchi et al. 2003) and ^{14}C-sucrose (0.28 μL /(min • g brain)) (Urayama et al. 2003). The same methodology was used in those papers. In addition, the $PS_{BBB,inf}$ of these peptides was significantly greater than that of ^{125}I-albumin (Tanabe et al. 1999) or ^{99m}Tc-albumin (Kastin et al. 2003), the vascular control. These results suggest that BBB permeation of ADAB and ADAMB is significantly slower than that of TAPA. Therefore, the slow onset of the antinociceptive effect after the systemic administration of ADAB and ADAMB appears to be related to the slow permeation influx rate across the BBB.

The mechanism(s) responsible for the BBB transport of ADAB and ADAMB may be passive diffusion and/or fluid phase endocytosis, because ADAB and ADAMB have lower BBB permeability than sucrose. Alternatively, P-glycoprotein-mediated efflux transport would also explain the slow BBB permeation of ADAB and ADAMB, as has

been suggested for some endogenous opioid peptides (King et al. 2001, Elferink and Zadina, 2001). On the other hand, TAPA crosses the BBB via the AME mechanism (Deguchi et al. 2003). Therefore, ADAB and ADAMB might also be transported through the BBB via the AME mechanism. To test this hypothesis, we carried out a transport study using TM-BBB4 cells, an in vitro model of the BBB. The acid-resistant binding of ^{125}I-ADAB and ^{125}I-ADAMB, which represents the internalization of peptides in TM-BBB4 cells, after 60 min of incubation was 4.3 μL/mg protein and 5.9 μL/mg protein, respectively. These values were significantly greater than the intracellular space of TM-BBB4 cells (1.51 μL/mg protein, as measured with 3-O-methyl-D-glucose), indicating that both ^{125}I-ADAB and ^{125}I-ADAMB are substantially internalized into TM-BBB4 cells. The acid-resistant binding of these peptides to TM-BBB4 cells was saturable (Fig. 3). Kinetic analysis of the data showed that the B_{max} values of ^{125}I-ADAB and ^{125}I-ADAMB are 12.8 pmol/mg protein and 22.5 pmol/mg protein, respectively. The K_d values of ^{125}I-ADAB and ^{125}I-ADAMB are 3.76 μM and 5.68 μM, respectively. Thus, the binding potency at the low concentration range (B_{max} / K_d) was 6.8-fold ~ 8.0-fold greater than that of ^{125}I-TAPA. These results suggest that *N*-terminal amidination of TAPA increases the potential for internalization in the BBB. On the other hand, the isoelectric points of ADAB and ADAMB (both 11.3) as calculated from

the acid-base dissociation constants of ionizable functional groups were greater than that of TAPA (9.6). Therefore, increase in the internalization of both ADAB and ADAMB appears to parallel increase in the basicity of the peptide structure, brought about by the *N*-terminal amidination of TAPA. These results are consistent with a previous report indicating that the basicity of peptide molecules is a determinant factor for transport via the AME system in primary cultured brain capillary endothelial cells (Tamai et al. 1997).

We then carried out an inhibition study to clarify the mechanism of the internalization of ADAB and ADAMB in TM-BBB4 cells. As shown in Table 4, the acid-resistant bindings of ^{125}I-ADAB and ^{125}I-ADAMB were significantly inhibited by 500 μM dansylcadaverine, which is reported to be a potent inhibitor of transglutaminase and to prevent the internalization of many proteins and hormones into cells (Ray and Samanta, 1996). In addition, poly-L-lysine and protamine significantly inhibited the acid-resistant binding of both ^{125}I-ADAB and ^{125}I-ADAMB by up to 68 %. Moreover, ADAB and ADAMB inhibited the acid-resistant binding of ^{125}I-TAPA to TM-BBB4 cells in a concentration-dependent manner (Fig. 4), whereas TMPA did not. These results suggest that ADAB and ADAMB are recognized by a common transport system with TAPA. Accordingly, ADAB and ADAMB appear to be internalized into the cells via the

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AME mechanism.

In contrast to these results, the acid-resistant binding of both ^{125}I-ADAB and ^{125}I-ADAMB was not influenced by low temperature (4 ºC) or by treatment of TM-BBB4 cells with 1 mM DNP, suggesting that the internalization of ^{125}I-ADAB and ^{125}I-ADAMB occurs in a temperature- and energy-independent manner. This transport system appears to be distinct from the AME system, which is utilized by other cationic peptides such as cationic AVP analogue (Tanabe et al. 1999) and cationic cell-penetrating peptides (Richard et al. 2003). The same type of endocytosis, that is a temperature-independent translocating process, has been seen with model amphipathic peptide (MAP) (Oehlke et al. 1998).

The potent opioid effect of ADAB and ADAMB could also be attributable to their enzymatic stability. The Tyr^1-D-Arg^2 and Phe^3-β-Ala^4 bonds increase the stability of the molecule to aminopeptidase and decarboxypeptidase, respectively (Chaki et al., 1990). However, 25 ~ 30 % of ^{125}I-ADAB and ^{125}I-ADAMB was metabolized in the periphery 30 min after the intravenous administration (Fig. 2). Therefore, we cannot rule out the possibility that ^{125}I-tyrosine was taken up by the brain via the large neutral amino acids transporter (LAT1) at the BBB (Boado et al. 1999). If this were correct, the values of $PS_{BBB,inf}$ should be similar to that of tyrosine 65 μL/(min • g of brain) (Smith and Stoll,

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1999). However, the $PS_{BBB,inf}$ values of ^{125}I-ADAB and ^{125}I-ADAMB were less than 0.1 μL/(min • g of brain), suggesting that the radioactivity detected in the brain is mainly due to the intact ^{125}I-ADAB and ^{125}I-ADMB. These low $PS_{BBB,inf}$ values may suggest the possible involvement of ABC efflux transporter at the BBB.

From the pharmacological point of view, as mentioned above, the slow BBB permeation influx rates of ADAB and ADAMB may be responsible for the slow onset of antinociceptive effect after systemic administration. However, the extent of internalization of ^{125}I-ADAB and ^{125}I-ADAMB in the TM-BBB4 cells was greater than that of ^{125}I-TAPA. These results were not parallel with the extent of the BBB permeability in vivo. In general, the amounts of peptide that are accumulated into the cells would be determined by the net balance between endocytosis and exocytosis. Therefore, one reason why ADAB and ADAMB are avidly accumulated in the cells, compared with TAPA, could be that the endocytosis rate is much greater than the exocytosis rate. If this hypothesis is correct, the in vivo exocytosis rate from the brain capillary endothelial cells to the brain interstitial fluid would limit the overall rate of the transcytosis process of ADAB and ADAMB through the BBB, leading to the slow BBB permeation influx rate of these peptides. The validity of this hypothesis should be verified by transcellular transport studies using an in vitro BBB model with tight-

junctions.

In conclusion, the present study suggests that the slow BBB permeability of ADAB and ADAMB contributes the slow onset of the antinociceptive effect after systemic administration. The BBB transport of these peptides may occur via the AME mechanism. The N-terminal modification of TAPA with an amidino group afforded desirable opioid activity characteristics in the CNS. Such pharmacological characteristics were, in part, brought about by the changes in the transport characteristics at the BBB. Today, it is a mainstream approach to develop drugs which exhibit a strong effect at low dose. However, this strategy is not necessarily appropriate for a CNS drug, because a drug with a strong effect could readily manifest side effects. The utilization of the transport system demonstrated in the present study would be useful for controlling the delivery of μ_1-selective opioid peptides into the CNS, and thereby controlling their pharmacological effects.

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Footnotes

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Legends to figures

Fig. 1. Plasma (A) and brain (B) concentrations after i.v. administration of either ^{125}I-ADAB (○) or ^{125}I-ADAMB (●)to mice at a dose of 5.7 kBq/g body weight (7.3 ~ 18.4 pmol/g of body weight). The plasma and brain concentrations were normalized by the injected dose. The error bar is smaller than the size of the symbol in each case. (C) Integration plot of the uptake of ^{125}I-ADAB (○) and ADAMB (●) by the brain over 1 to 60 min after i.v. administration of labeled peptide to mice. Each point represents the mean ± S. E. of three animals. The solid lines represent the values obtained by linear least-squares regression of the data. The dotted line is the result for ^{125}I-TAPA reported previously (Deguchi et al., 2003).

Fig. 2. HPLC chromatograms of authentic ^{125}I-ADAB (A) and ^{125}I-ADAMB (C), and typical chromatograms of plasma samples 30 min after intravenous administration of ^{125}I-ADAB (B) and ^{125}I-ADAMB (D) (170 ~ 240 kBq/g body weight).

Fig. 3. Concentration-dependence of the acid-resistant binding of ^{125}I-ADAB (○) and ^{125}I-ADAMB (●) to TM-BBB4 cells. The TM-BBB4 cells were incubated with medium containing ^{125}I-ADAB and ^{125}I-ADAMB (240 ~ 720 nM) and the respective unlabeled peptide at various concentrations (0.1 – 1000 μM) at 37 °C for 60 min. The cells were washed three times with 1 mL of ice-cold incubation buffer, and then washed

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with acetate-barbital buffer at 4 °C for 10 min. The cells were lysed with 1 M NaOH and the radioactivity was counted. Each point represents the mean ± S.E. of four determinations. The solid lines represent the theoretical lines, eq. (4), and the estimated parameters. The error bar is smaller than the size of the symbol in each case.

Fig. 4. Inhibition of the acid-resistant binding of ^{125}I-TAPA to TM-BBB4 by ADAB (○), ADAMB (●) and TMPA (△). The inhibition of the acid-resistant binding of ^{125}I-TAPA by these peptides was determined by incubating ^{125}I-TAPA (240 nM) with various concentrations of ADAB (2 nM ~ 200 μM), ADAMB (2 nM ~ 200 μM) and TMPA (0.2 ~ 200 μM). Ordinate, percentage of acid-resistant binding of ^{125}I-TAPA in the absence of each peptide. Each point represents the mean ± S. E. of four determinations. The solid lines represent the theoretical lines with the one-site competition model and the estimated parameters. The error bar is smaller than the size of the symbol in each case.

Table 1 Structure, molecular weight (MW), lipophilicity and isoelectric point (pI) of [D-Arg^2]-dermorphin (1-4) analogues

Peptides	Amino acid sequence	MW	Lipophilicity[1)] (t_R, min)	pI[2)]
TAPA	H-Tyr-D-Arg-Phe-β-Ala-OH	663.7	11.4	9.6
ADAB	*N*$^{\alpha}$-Amidino-Tyr-D-Arg-Phe-β-Ala-OH	705.7	12.2	11.3
ADAMB	*N*$^{\alpha}$-Amidino-Tyr-D-Arg-Phe-Me-β-Ala-OH	719.7	14.0	11.3
TMPA	H-Tyr-D-MetO(RS)-Phe-Me-β-Ala-OH	560.7	14.3	5.7

1) Lipophilicity of peptides was evaluated in terms of retention time on an ODS column.

2) pI values were calculated from the acid-base dissociation constants of ionizable functional groups in each peptide. t_R : retention time.

Table 2 Plasma pharmacokinetics and BBB permeability of ^{125}I-ADAB and ^{125}I-ADAMB after intravenous administration to mice.

Parameters		^{125}I-ADAB	^{125}I-ADAMB	^{125}I-TAPA [2)]
Plasma pharmacokinetics				
AUC	((% dose · min) / mL)	146	264	129.2
MRT	(min)	27.1	18.5	36.7
CL_{tot}	(μL / (min · g of BW)	14.7	8.37	14.2
Vd_{ss}	(μL / (min · g of BW)	399	155	869
BBB permeability [1)]				
$PS_{BBB,inf}$	(μL / min · g of brain)	0.0515 ± 0.0284	0.0290 ± 0.0059	0.265 ± 0.025
V_i	(μL / g of brain)	9.18 ± 3.08	8.47 ± 0.92	8.69 ± 0.84

(To be continued)

AUC, MRT, CL_{tot} and Vd_{ss} were calculated using the mean plasma concentration at each time. BW. body weight

1) $PS_{BBB,inf}$ and V_i were estimated by fitting the data of Fig. 1 (C) to eq. (2). Values are the calculation results and represent the mean ± calculated S.D.

2) Values are cited from our previous report (Deguchi et al. 2003).

Table 3 Kinetic parameters for the acid-resistant binding of ^{125}I-ADAB and ^{125}I-ADAMB to TM-BBB4 cells

Peptides	B_{max} (pmol / mg protein)	K_d (μM)	α (μL / mg protein)	B_{max} / K_d (μL / mg protein)
^{125}I-ADAB	12.8 ± 2.6	3.76 ± 0.83	1.43 ± 0.09	3.4
^{125}I-ADAMB	22.5 ± 11.9	5.68 ± 1.75	1.98 ± 0.29	4.0

The maximal binding capacity (B_{max}), the half-saturation constant (K_d) and the non-specific binding (α) were estimated by nonlinear least-squares analysis using Prism 3 software from the data in Figure 2. Each value represents the mean ± calculated S.D.

Table 4 Effects of an endocytosis inhibitor, selected inhibitors, a metabolic inhibitor and temperature on the acid-resistant binding of ^{125}I-ADAB and ^{125}I-ADAMB to TM-BBB4 cells

Inhibitors	Concentration	Acid-resistant binding (% of control)	
		^{125}I-ADAB	^{125}I-ADAMB
Dansylcadaverine [1)]	500 μM	57.7 ± 3.6 *	44.7 ± 2.0 **
Poly-L-lysine	300 μM	23.6 ± 3.7 **	31.9 ± 0.9 **
Protamine	300 μM	26.1 ± 4.1 **	27.7 ± 0.9 **
Poly-L-glutamic acid	300 μM	72.8 ± 5.6	90.1 ± 5.0
2,4-dinitrophenol	1 mM	91.1 ± 7.4	109 ± 7.9
4 ºC		100 ± 19	129 ± 23

(To be continued)

^{125}I-Labeled peptides were incubated with TM-BBB4 at 37℃ or 4℃ for 60 min in the presence of 2,4-dinitrophenol, dansylcadaverine and selected inhibitors. After the incubation, the cells were washed with PBS, and then further washed with cold acetate-barbital buffer (pH 3.0) at 4℃ for 10 min. After solubilization of cells with 1 N NaOH, radioactivity was counted as a measure of acid-resistant binding. Each value represents the mean ± S.E. of four or more experiments. *p<0.05, **p<0.01, significantly different from the control. 1) After preincubation, the medium was replaced with incubation buffer without inhibitor.

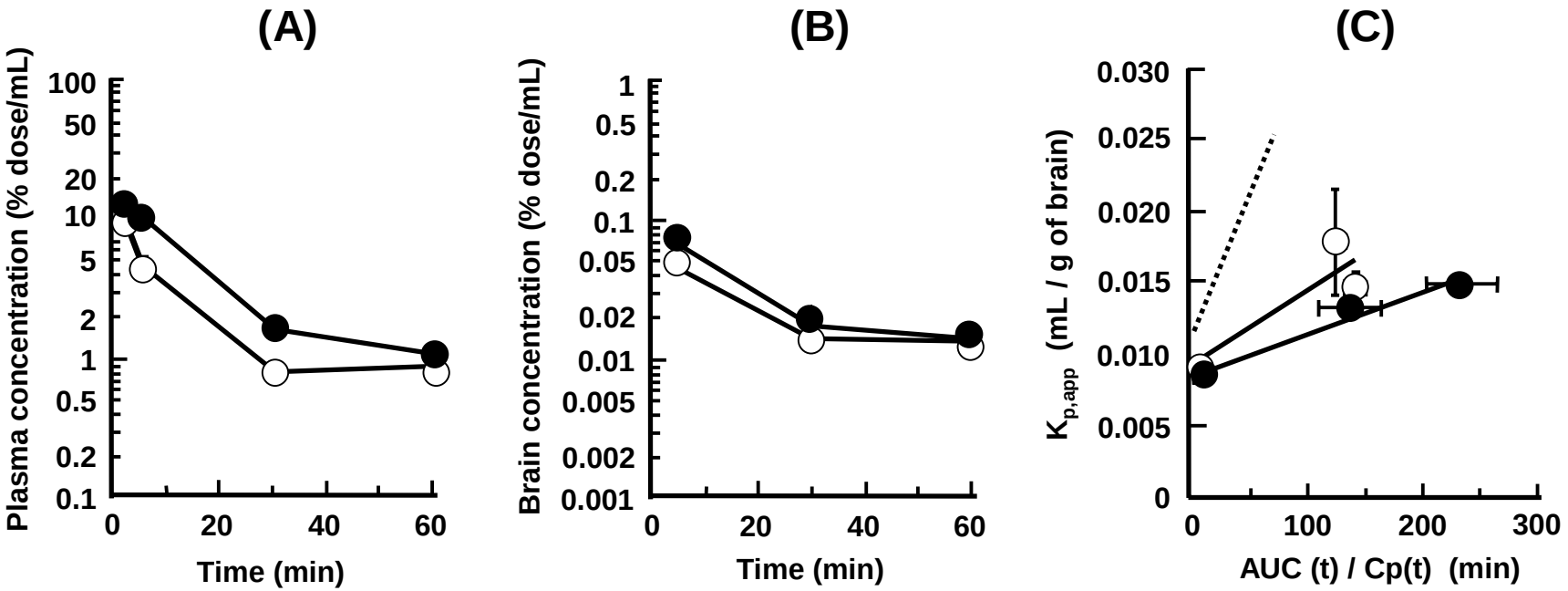

Fig. 1

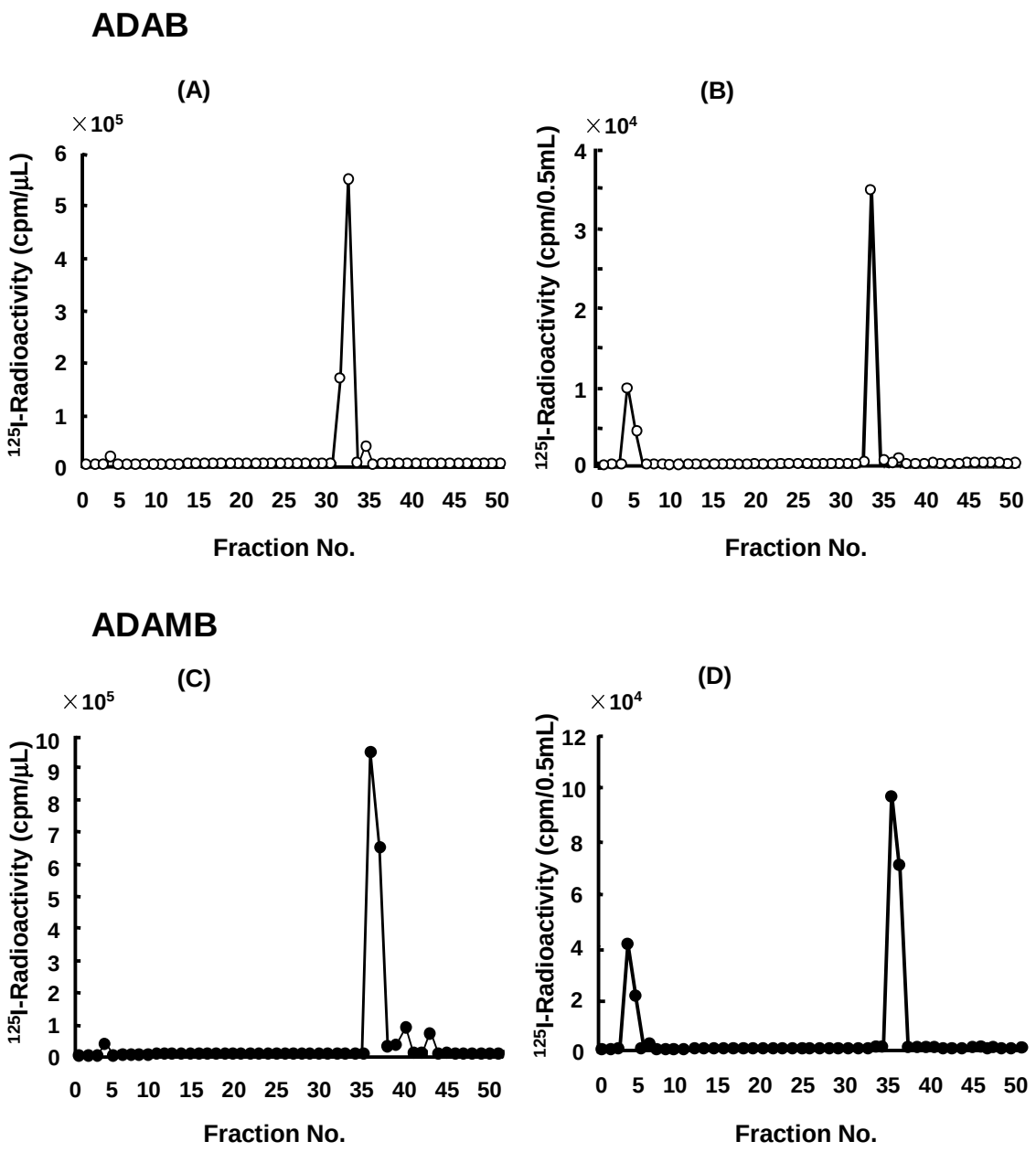

Fig. 2

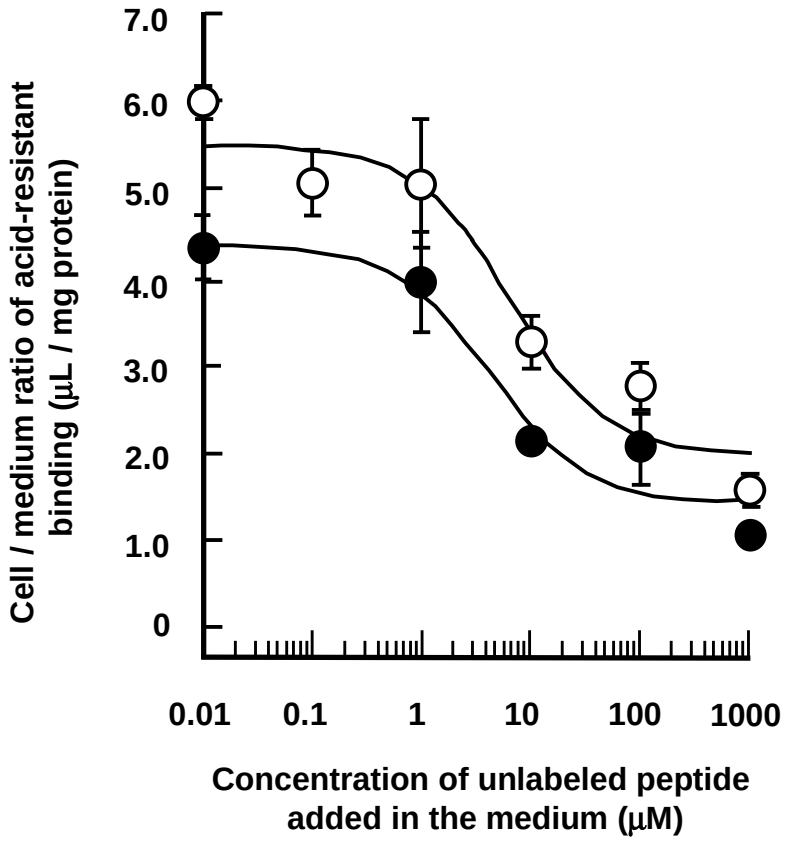

Fig. 3

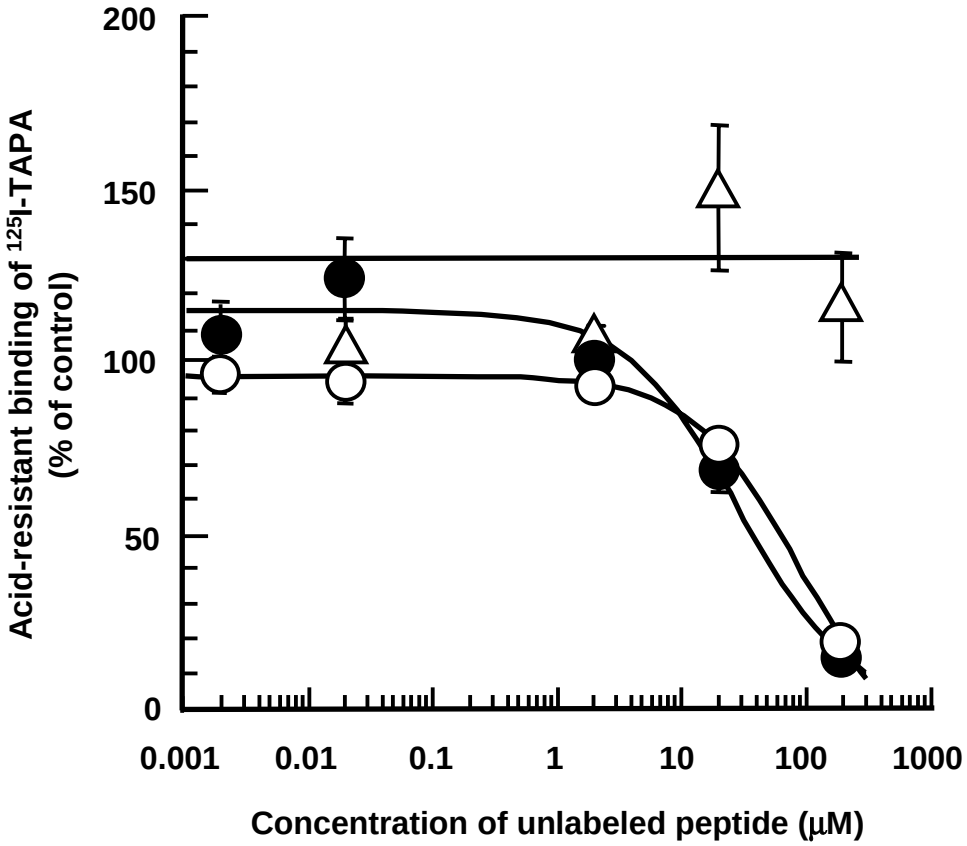

Fig. 4