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Article

Monoamine Oxidase-A (MAO-A) Inhibitors Screened from the Autodisplayed Fv-Antibody Library

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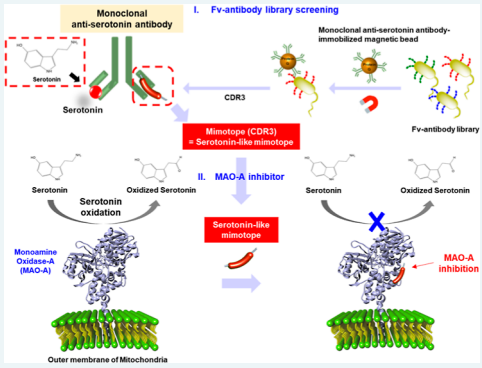
ABSTRACT: Serotonin-like mimotopes were screened from the Fv-antibody library to be used as inhibitors against monoamine oxidase A (MAO-A). The Fv-antibody [corresponding to the V_H region of immunoglobulin G (IgG)] consists of three complementarity-determining regions and four frame regions. The Fv-antibody library was prepared by site-directed mutagenesis of CDR3, which consists of 11 amino acid residues. Three target clones were screened from the Fv-antibody library, and the binding affinity of the screened clones to the monoclonal anti-serotonin antibody was analyzed using fluorescence-activated cell sorting. The screened Fv-antibodies were expressed as soluble proteins fused with green fluorescence protein. Additionally, the screened CDR3 regions (11 residues) of the selected Fv-antibodies were synthesized as peptides with linking amino acid residues. The binding constants (K_D) of the three serotonin-like mimotopes (Fv-antibodies and peptides) were estimated using a surface plasmon resonance biosensor. The inhibitory activity (IC_{50}) of the serotonin-like mimotopes (Fv-antibodies and peptides) was estimated separately for MAO-A and MAO-B enzymes and compared with that of conventional inhibitors. Finally, the screened serotonin-like mimotopes were used to treat a cell line (SH-SY5Y, ATCC code: CRL-2266) expressing serotonin receptors. This was done to confirm the following two aspects: (1) the binding of mimotopes to the serotonin receptors on the cell surface and (2) the inhibitory activity of mimotopes against MAO-A enzymes in the cell lysates.



KEYWORDS: *Fv-antibody library, serotonin-like mimotope, monoamine oxidase-A (MAO-A), peptide*

Monoamine oxidase A (MAO-A) and MAO-B are flavoenzymes located in the outer mitochondrial membrane. Both of these enzymes possess the cofactor flavin adenine dinucleotide (FAD) and exhibit over 70% homology in their amino acid sequence.[1,2] Although MAO-A and MAO-B share high amino acid sequence homology, they have different substrate specificities and inhibitor sensitivities.[3] MAO-A inhibitors are used to treat depression, anxiety, and migraines. MAO-B inhibitors have been used as dopamine-sparing agents for the treatment of Parkinson's disease.[4] Being a neurotransmitter, serotonin is released into the synapse and subsequently reuptaken into the presynapse. MAO-A has been reported to disrupt the reuptake of serotonin by oxidizing it through the coenzyme (FAD).[5] Inhibitors of MAO-A-like clorgiline have been used to maintain serotonin concentration.[6] In this study, serotonin-like mimotopes were screened as inhibitors of MAO-A from an Fv-antibody library using a monoclonal anti-serotonin antibody.

The structure of the Fv-antibody is based on the V_H of immunoglobulin G (IgG), which is composed of three complementarity-determining regions (CDRs) and four frame regions (FRs).[7−10] CDRs are known to be hypervariable regions located in the heavy and light chains of IgGs, which interact specifically with the target antigen. As shown in Figure 1a, the Fv-antibody library was prepared with a randomized amino acid sequence of CDR3 through site-directed mutagenesis of 11 amino acid residues.[11,12] The prepared Fv-antibody library was expressed on the outer membrane of *Escherichia coli* using autodisplay technology.[13] As shown in Figure 1b, the autodisplay of target proteins was reported to achieve a high expression yield of $>10^5$ Fv-antibodies/*E. coli*; additionally, >90% of the *E. coli* cells within the total *E. coli* population in the Fv-antibody library autodisplayed the Fv-antibodies. The Fv-antibody library has been reported to exhibit a high diversity of $>10^5$ clones/library.[14] It has been used to screen Fv-antibodies with binding affinity to biotin, fluorescent dyes (fluorescein and rhodamine B), biocrystals (monosodium urate and calcium pyrophosphate dehydrate), and viral proteins, among others.[12,15−19]

In the previous work, the inhibitors of MAO-B (Fv-antibodies and peptides) were screened using the Fv-antibody library, and

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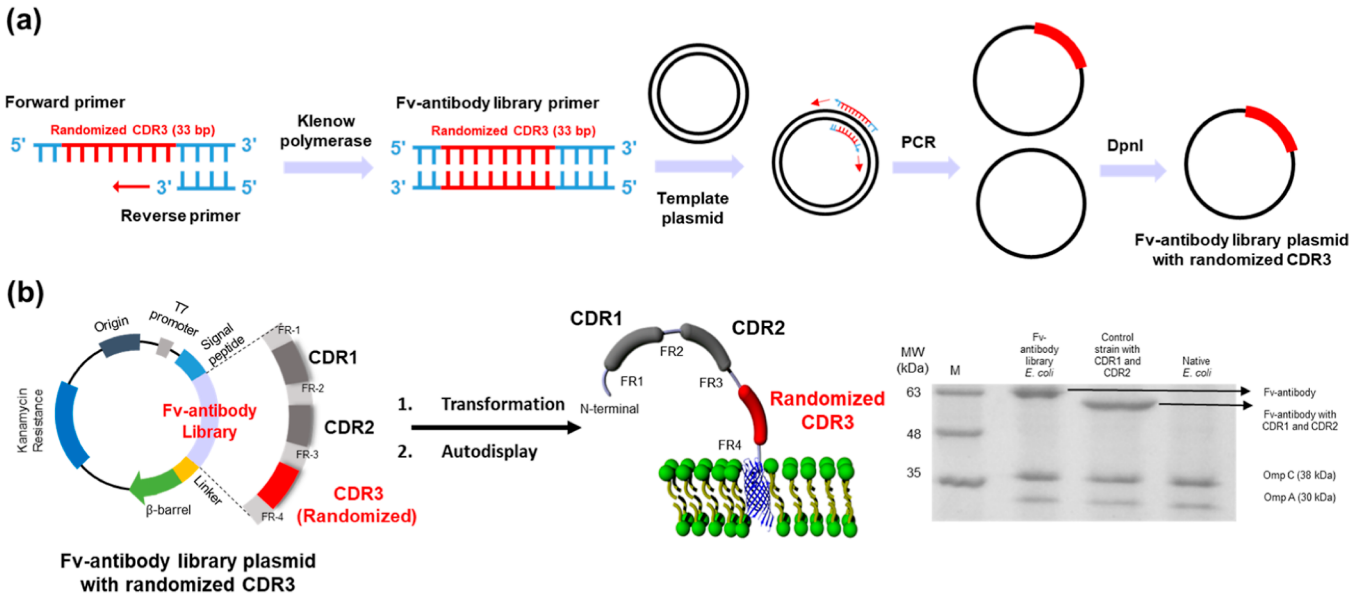

Figure 1. Preparation of the Fv-antibody library with a randomized amino acid sequence of CDR3 using autodisplay technology. (a) Construction of the Fv-antibody library plasmid by site-directed mutagenesis. (b) Autodisplay of the Fv-antibodies on the outer membrane of *E. coli* and SDS-PAGE of the autodisplayed Fv-antibodies on the outer membrane of *E. coli* and two control strains (control strain with CDR1 and CDR2 and native *E. coli*).

Table 1. Oligonucleotide and Amino Acid Sequences of the Expressed Fv-Antibodies Composed of CDRs and FRs (N Terminal-FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4-C Terminal)

region	oligonucleotide sequence (5′ → 3′)	amino acid sequence (N-term → C-term)
frame-1 (FR1)	GAA GTG CAG CTC GTG GAA AGC GCT GCC GAA GTT CGG CGT CCT GGG GCT AGC GTG AAG ATC ACC TGC AAA GCG TCC GGC TAT TCA TTC AGC	EVQLVESAAEVRRPGASVKITCKASGYSFS
CDR1	ACC TAT GGG ATT CAG	TYGIQ
frame-2 (FR2)	TGG ATG CGC CAA GCG CCA GGC CAG CGT CCG GAA TGG CTT GGG	WMRQAPGQRPEWLG
CDR2	TGG ATA CAT GCA GGC ACA GGT GGG ACT AAG TAC TCG CGC AAA TTT CAG GGT	WIHAGTGGTKYSRKFQG
frame-3 (FR3)	CGC ATT ACT ATC ACC CGT GAT ACC AGC GCG AAT ACC GTC TAT CTG GAT CTG AAC TCT CTG ACA TCG GAG GAT ACG GCC GTC TAT TGC GCT CGT	RITITRDTSANTVYLDLNSLTSEDTAVYYCAR
CDR3 (template)	GAC AAA GTT ACA GTC TGG GCT TGT CAG GAT AAT	DKVTVWACQDN
frame-4 (FR4)	TGG GGT CAA GGT ACT ACG GTT ACG GTC AGC AGT	WGQGTTVTVSS

these inhibitors were proved to have a high specificity to MAO-B in comparison with MAO-A.[17] In this work, the inhibitors of MAO-A (Fv-antibodies and peptides) were screened using the same Fv-antibody library, and these inhibitors were proved to have a high specificity to MAO-A in comparison with MAO-B. As the first step, target clones with binding affinity to monoclonal anti-serotonin antibody were screened from the Fv-antibody library. The binding affinity of the screened clones to the monoclonal anti-serotonin antibody was analyzed using fluorescence-activated cell sorting (FACS). The screened Fv-antibodies were expressed as soluble fusion proteins, while the identified CDR3 regions (consisting of 11 residues) were synthesized into peptides. The binding constants (K_D) of the three serotonin-like mimotopes (Fv-antibodies and peptides) were estimated using a surface plasmon resonance (SPR) biosensor. The inhibitory activity (IC_{50}) of serotonin-like mimotopes (Fv-antibodies and peptides) was estimated separately for MAO-A and MAO-B enzymes and compared with those of conventional inhibitors. After the screening process, the serotonin-like mimotopes were used to treat a cell line (SH-SY5Y, ATCC code: CRL-2266) expressing serotonin receptors, thus confirming their ability to inhibit MAO-A enzymes within the cell lysate.

MATERIALS AND METHODS

Materials. Mercaptoundecanoic acid (MUA), ethanolamine, and other chemicals were purchased from Sigma-Aldrich (Seoul, Korea). The monoclonal anti-serotonin antibody (NB 100-65037) was purchased from Novus Biologicals (Littleton, CO, USA). For MAO-A and MAO-B activity assays, a MAO assay kit (ab241031) and a MAO-A inhibitor screening kit (Fluorometric; ab284510) were purchased from Abcam (Cambridge, UK). NEBuffer 2 and Klenow DNA polymerase were purchased from New England Biolabs (Ipswich, MA, USA). Isopropyl β-D-1-thiogalactopyranoside (IPTG) and kanamycin were obtained from Kisan Bio (Seoul, Korea). Dynabeads protein G, FastDigest DpnI, Phusion High-Fidelity DNA polymerase, trypsin, penicillin/streptomycin (P/S), and Super-Signal ELISA Femto Maximum Sensitivity Substrate were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). For PCR product purification and plasmid extraction, a PCR purification mini kit and plasmid extraction kits were purchased from Favorgen Biotech Corp. (Pingtung, Taiwan).

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Table 2. Screened Oligonucleotide and Amino Acid Sequences of CDR3 Region for Serotonin-like Mimotopes

screened CDR3		sequences	$K_{D,\ SPR}$ [M] (peptide)	$K_{D,\ SPR}$ [M] (Fv)	IC_{50} [M] (peptide)	IC_{50} [M] (Fv)
Peptide-7 (clone no. 7)	oligo-nucleotide	5′-^{1}TGCCC^{6}GACTC^{11}TGCCG ^{16}GGGGT^{21}TCTTG^{26}ATGAT^{31}GT^{33}T-3′	6.81×10^{-7}	2.00×10^{-8}	6.16×10^{-8}	7.37×10^{-8}
	amino acid	^{1}CSLPM ^{6}TAGDD ^{11}V				
Peptide-33 (clone no. 33)	oligo-nucleotide	5′-^{1}GGTAG^{6}TGTTC^{11}TTCTT ^{16}GCGGT^{21}GATCG^{26}TAGAT^{31}TT^{33}-3′	6.93×10^{-7}	2.70×10^{-8}	2.42×10^{-7}	1.62×10^{-7}
	amino acid	^{1}GLTGP ^{6}PTTQD ^{11}F				
Peptide-40 (clone no. 40)	oligo-nucleotide	5′-^{1}TGTGG^{6}TCCTC^{11}CGGTT ^{16}GTTGC^{21}ACTAC^{26}ATGAT^{31}GA^{33}T-3′	7.33 × 10−7	1.24×10^{-8}	1.14×10^{-7}	1.10×10^{-7}
	amino acid	^{1}GPDET ^{6}INLGD ^{11}F				

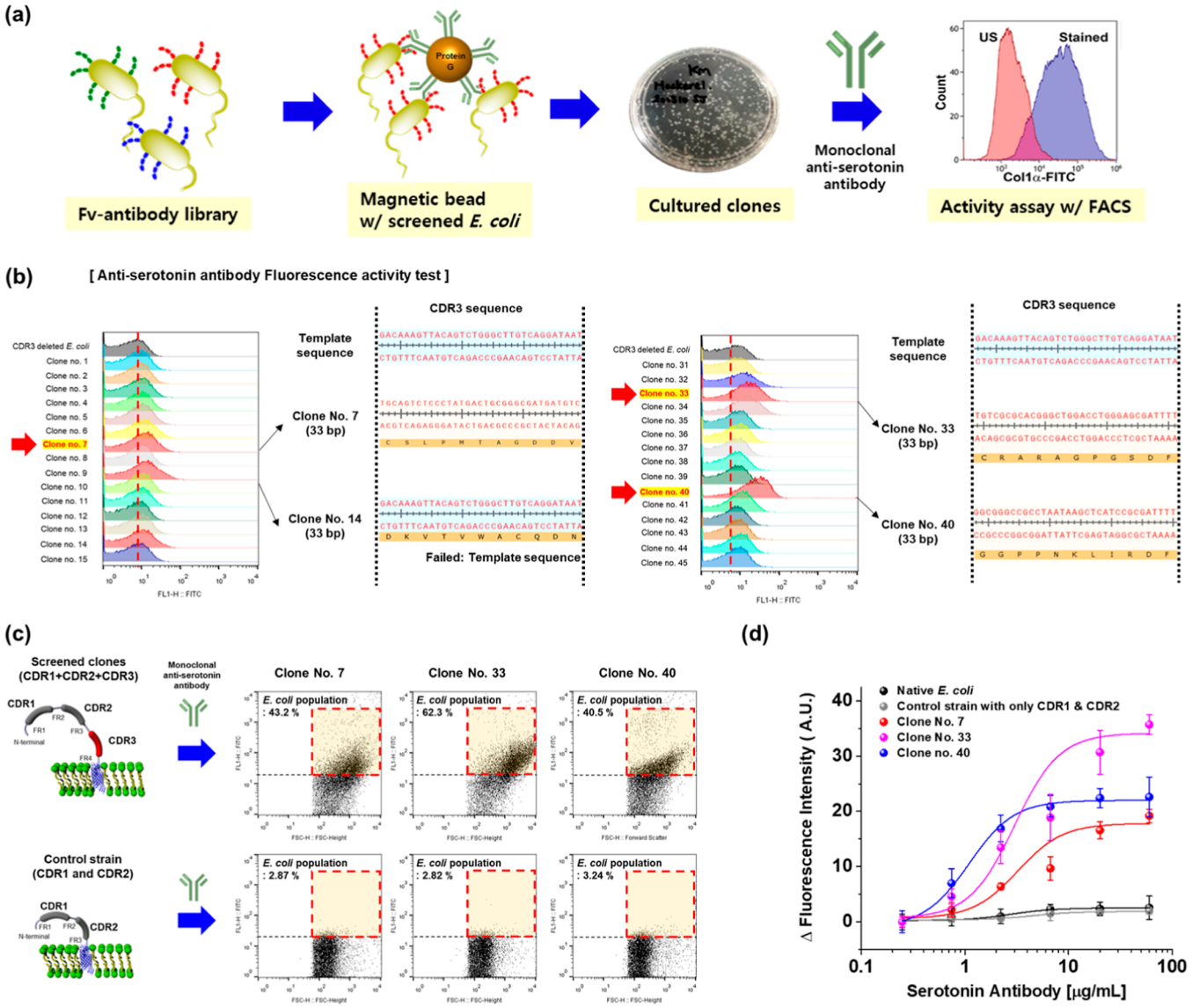

Figure 2. Screening of serotonin-like mimotopes by the Fv-antibody library. (a) General screening progress of serotonin-like mimotope by the monoclonal anti-serotonin antibody-immobilized magnetic beads. (b) Genetic analysis of the CDR3 region of candidates for serotonin-like mimotopes and selection of the three valid clones (clone nos. 7, 33, and 40). (c) Flow cytometric binding affinity analysis of the selected clones and the control strain against the monoclonal anti-serotonin antibody. (d). Quantitative binding analysis of the selected clones (clones no. 7, 33, and 40) by flow cytometry.

Peptides listed in Table 2 were synthesized by Peptron (Daejeon, Korea) with a purity of more than 90%. SH-SY5Y cells (ATCC: CRL-2266) were obtained from the Korean Cell Line Bank (Seoul, Korea). Fetal bovine serum (FBS) and minimum essential medium (MEM) media were purchased from Biowest (Nuaillé, France).

Screening of the Fv-Antibody Library. Construction of the Fv-antibody library and expression of the autodisplayed Fv-

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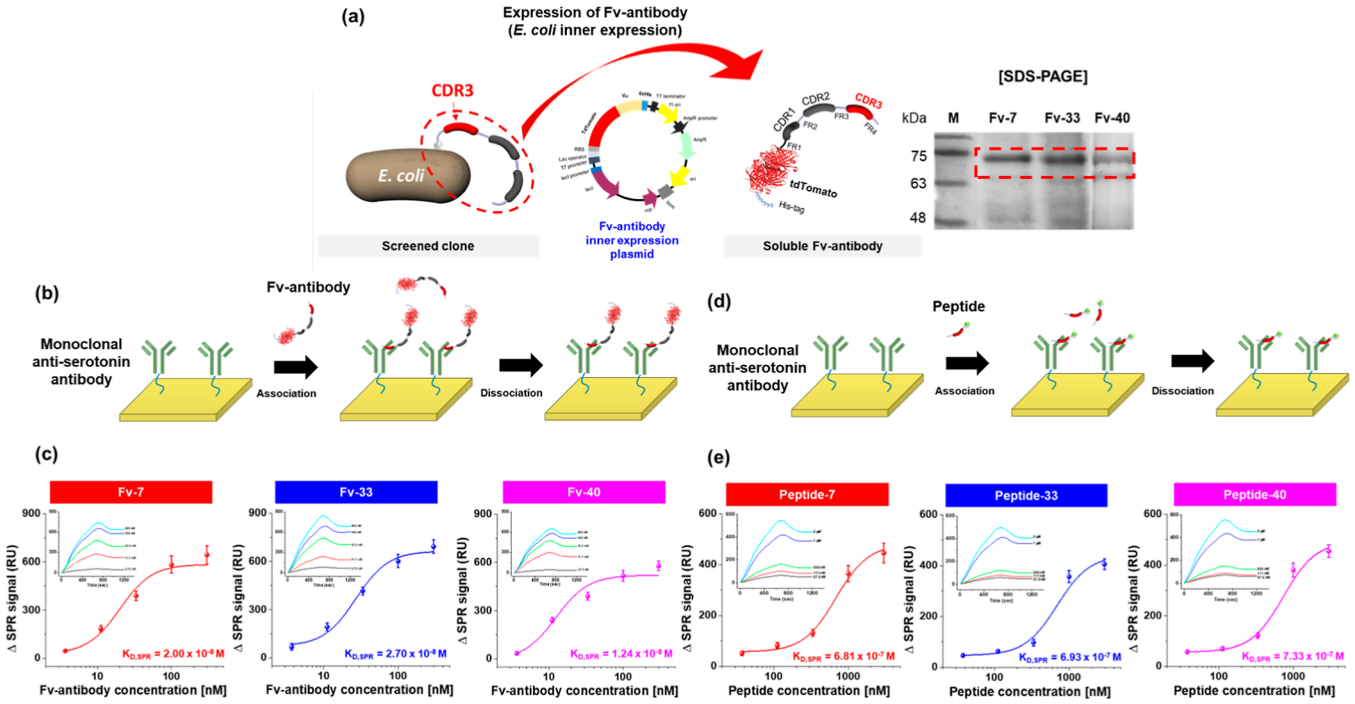

Figure 3. Expression of the Fv-antibodies and SPR analysis of the Fv-antibodies and peptides against the monoclonal anti-serotonin antibody. (a) Expression and SDS-PAGE of the soluble Fv-antibodies (Fv-7, Fv-33, and Fv-40). (b) Scheme for the SPR analysis of the Fv-antibodies against the anti-serotonin antibody. (c) Binding constants (K_D) estimation of the Fv-antibodies against the antibody by SPR biosensor. (d). Scheme for the SPR analysis of the synthesized peptides (Peptide-7, Peptide-33, and Peptide-40) against the antibody. (e) Binding constant (K_D) estimation of the peptides against the antibody.

antibody library on the outer membrane of *E. coli* was conducted as previously described.[12,15] Target clones with binding affinity for the monoclonal anti-serotonin antibody were screened using the magnetic bead-activated cell sorting (MACS) screening method. MACS screening begins with the immobilization of monoclonal antibodies onto protein-G-coated magnetic beads. The monoclonal anti-serotonin antibody (1 mg/mL, 5 μL) and Dynabeads Protein G (5 μL) were mixed with 0.1% PBST buffer (0.1% Tween20 in PBS) for 2 h, and the monoclonal antibody-immobilized magnetic beads were filtered using an external magnet. The monoclonal antibody-immobilized magnetic beads were then mixed with the Fv-antibody library ($OD_{600\ nm}$ = 1.0, 100 μL) for 1 h. Screened *E. coli* with binding affinity to the monoclonal antibody-immobilized magnetic beads were filtered by using a magnet and washed 15 times with 0.1% PBST and PBS buffer. The sorted *E. coli*-bound-monoclonal antibody-immobilized magnetic beads were resuspended in a Luria–Bertani (LB) broth medium and spread on an LB agar plate to obtain screened clones with binding affinity against the monoclonal anti-serotonin antibody.[19−21]

Expression and Purification of the Fv-Antibody. To express the Fv-antibody, which can act as a serotonin-like mimotope, three plasmids (pJS004, pJS005, and pJS006) containing an open reading frame of Fv antibodies with high binding affinity to the monoclonal antibody against serotonin were custom-synthesized by Cosmogenetech (Seoul, Korea), as shown in Figure 3a. The oligonucleotide and amino acid sequences of the CDRs and FRs of the three Fv-antibodies are summarized in Table 1. Fv-antibodies were intracellularly expressed in *E. coli*. For the expression, three custom-synthesized plasmids (pJS004, pJS005, and pJS006) were transformed into competent cells using electroporation, and the transformed *E. coli* were cultured in 20 mL of LB medium with 1 mM IPTG and 30 μg/mL carbenicillin for 16 h at 30 °C. The incubated *E. coli* cells were centrifuged (3000 × *g*, 3 min) and then resuspended in 30 mL of binding buffer (5 mM Tris–HCl, 0.5 M NaCl, and 3 M urea). The resuspended *E. coli* cells were sonicated (Vibra cell VCX-130, Sonics, USA), and the lysate was centrifuged (25 000 × *g*, 10 min); subsequently, the supernatant containing the expressed Fv-antibody was collected. Fv antibodies were purified using a His-tag purification column (Roche, Basel, Switzerland). A binding buffer containing 100 mM imidazole was used as the elution buffer.[22] After elution, dialysis was performed for 16 h at 4 °C and 150 rpm to remove imidazole and urea from the eluted sample. Subsequently, purified Fv-antibodies were obtained.

SPR Analysis. To estimate the binding affinity (K_D) of the synthesized peptides and expressed Fv-antibodies against the monoclonal anti-serotonin antibody, SPR analysis was performed using an SPR biosensor from i-Cluebio (Seongnam, Korea).[23] The binding assay of the synthesized peptide and expressed Fv-antibodies was carried out by immobilizing the monoclonal anti-serotonin antibody on the gold surface of the SPR chip (BK-7 glass coated with 2 nm Ti film and 48 nm Au layer) used in the SPR biosensor. The SPR chip was modified to incorporate carboxylic acid using MUA (1 mM). Subsequently, the monoclonal anti-serotonin antibody (10 μg/mL) was immobilized through a covalent bond using the {1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide/*N*-hydroxysuccinimid} EDC/NHS reagent.[24]

After blocking with ethanolamine (1 M), the synthesized peptides or expressed Fv-antibodies were injected into the monoclonal antibody-immobilized SPR chip at a flow rate of 20 μL/min for 10 min to facilitate association. After the association step, PBS was subsequently injected into the system at a flow

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rate of 20 μL/min for 10 min to induce dissociation. For the analysis, the result of the SPR signal was fitted to Hill's equation

$$y = \frac{a - d}{1 + (x/c)^b} + d$$

where a and d represent the maximum and minimum signals, respectively, c represents the concentration of the soluble protein or peptides, and b represents the Hill's slope of the curve.[25−27]

Blood−Brain Barrier Permeability Test. The blood−brain barrier (BBB) permeability test of the three synthesized peptides (Peptide-7, Peptide-33, and Peptide-40) was carried out using an artificial membrane permeation test kit from BioAssay Systems (Hayward, CA, USA).[28−30] The kit was used to estimate the BBB permeability of test compounds through an artificial membrane, which was produced using brain lipids, as shown in Figure 5a. The permeability of the test compounds was evaluated by comparing them with the reference standards in the kit, such as promazine (representing high permeability), clonidine (representing medium permeability), and diclofenac (representing low permeability).[30,31] Calculation of the BBB permeability rate (P_e) was performed by the following equation

$$C = \frac{V_B \times V_A}{(V_B + V_A) \times \text{area} \times \text{time}} \text{cm/s}$$

where OD_B (or OD_A) represented absorbance or fluorescence intensity of analyte solution before (and after) permeation of membrane, respectively. The parameter C was determined to be 7.72×10^{-6} (based on 18 h of incubation) and V_B (or V_A) indicated plate well volume of the analyte solution before (and after) the membrane permeation, respectively. The BBB permeation test was performed according to the instructions of the manufacturer. To calculate the P_e value of the three synthesized peptides (Peptide-7, Peptide-33, and Peptide-40), the fluorescence intensity was measured before and after the membrane permeation using a fluorescence spectrometer (GloMax Discover) from Promega (Madison, WI, USA). The permeability rate (P_e) for the three reference standards with high (promazine), medium (clonidine), and low (diclofenac) permeability was determined by measuring the absorbance at 250, 250, and 270 nm, respectively, using a Nanodrop spectrophotometer (DS-11 Spectrophotometer) from DeNovix Inc. (Wilmington, DE, USA).

MAO-A and MAO-B Activity Inhibition Assays. SH-SY5Y cells (ATCC: CRL-2266) from the Korean Cell Line Bank (Seoul, Korea) were cultured in MEM supplemented with 10% heat-inactivated FBS (Biowest, Nuaillé, France) and 1% P/S.[32]

For MAO-A and MAO-B activity inhibition assays, three synthesized serotonin-like mimotopes (Peptide-7, Peptide-33, and Peptide-40) and Fv-antibodies (Fv-7, Fv-33, and Fv-40) were employed. The MAO assay kit (ab241031) and MAO-A inhibitor screening kit (fluorometric) (ab284510) from Abcam (Cambridge, UK) were used according to the manufacturer's instructions.

The three synthesized peptides were mixed with either MAO-A or MAO-B enzyme from the assay kits in the concentration range of 100 pM to 10 μM, and the fluorescence (λ_{ex} = 535 nm and λ_{em} = 587 nm) was measured using the fluorescence spectrometer (Victor5) from PerkinElmer Co (Waltham, MA, USA). For the MAO-A and MAO-B activity inhibition assays using the three expressed Fv-antibodies, the luminol reaction was used and luminescence was measured using a Glomax

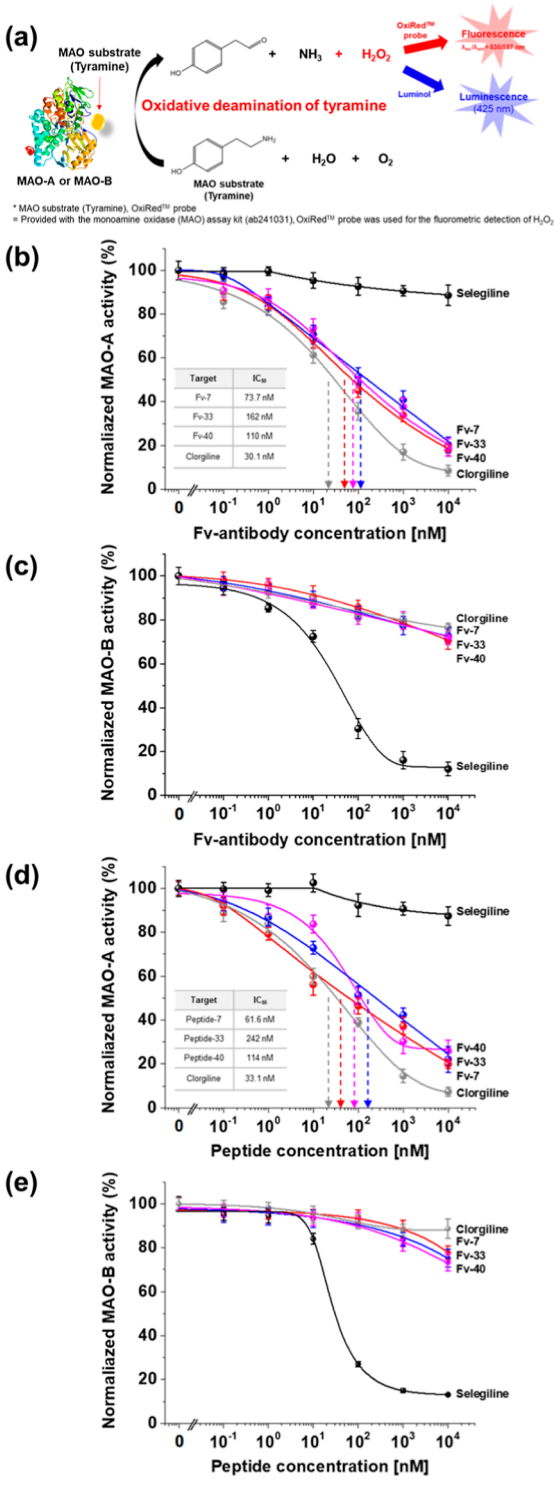

Figure 4. Analysis of the inhibitory activity of MAO-A and MAO-B. (a) Principle of the MAO-A and MAO-B inhibition activity assay. (b) MAO-A inhibitory activity and (c) MAO-B inhibitory activity of the Fv-antibodies (Fv-7, Fv-33, and Fv-40) and MAO-A inhibitor (clorgiline) and MAO-B inhibitor (selegiline). (d). MAO-A inhibitory activity and (e) MAO-B inhibitory activity of peptides (Peptide-7, Peptide-33, and Peptide-40) and two reference compounds (clorgiline and selegiline).

Discover microplate reader from Promega (Sunnyvale, CA, USA). For fluorescence imaging, SH-SY5Y cells (1×10^5 cells/mL) were incubated until they reached 70−80% confluence. Subsequently, the cells were further incubated with 20 μM of

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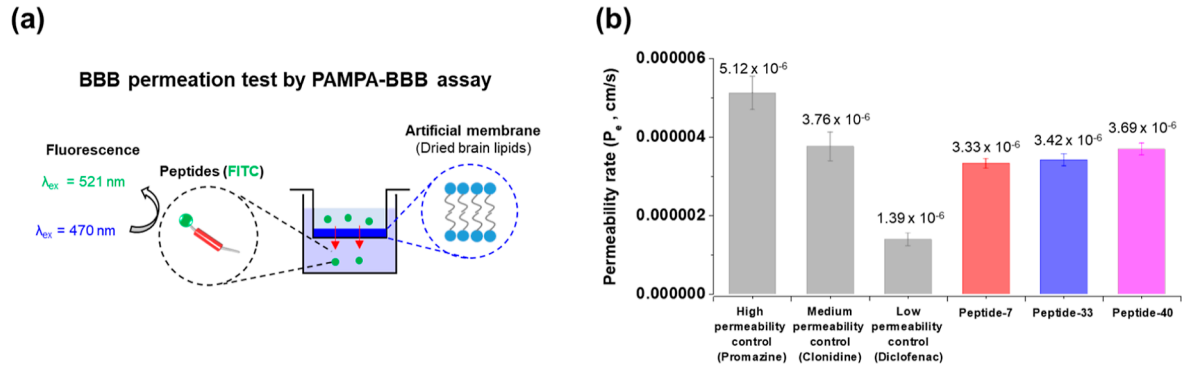

Figure 5. BBB permeation test of the three synthesized peptides (Peptide-7, Peptide-33, and Peptide-40) using a commercial test kit. (a) Principle of permeability rate (P_e) using a fluorescence-labeled test compound. (b) Comparison of permeability rate (P_e) of the three synthesized peptides (Peptide-7, Peptide-33, and Peptide-40) and three reference standards, such as promazine (high permeability), clonidine (medium permeability), and diclofenac (low permeability).

serotonin-like mimotopes (Peptide-7, Peptide-33, and Peptide-40) for 1 h in PBS buffer. After incubation with the peptides, the cells were washed three times with PBS buffer.

For the immunocytochemistry, the cells were fixed with 4% paraformaldehyde for 40 min, following the previously described protocol.[17,33]

After washing the cells in PBS, the nuclei and plasma membrane of the cells were stained with (4′,6-diamidino-2-phenylindole) DAPI and PlasMem Red, respectively. Images were acquired using an Olympus BX53 microscope equipped with the Olympus Cell Sens software (Carl Zeiss, Jena, Germany). For FACS analysis, SH-SY5Y cells (1×10^5 cells/mL) were incubated until they reached a 70−80% confluence, after which they were incubated with the three synthesized serotonin-like mimotopes in the concentration range of 0.1−10 μM for 1 h in PBS buffer containing 2% FBS. The cells were washed three times with 2% FBS containing PBS buffer, and the FACS analysis was performed using a FACSCalibur flow cytometer at the fluorescence channel of FL1 (λ_{ex} = 488 nm and λ_{em} = 525 nm) for measuring the fluorescein-labeled serotonin-like mimotopes.

A cell-based MAO-A activity inhibition assay was performed by incubating SH-SY5Y cells (ATCC: CRL-2266) with the three peptides in a concentration range of 0.1−10 μM for 24 h. After incubation, peptide-treated cells were trypsinized and homogenized in the MAO assay buffer (0.1 mg/μL) provided in the MAO assay kit. The MAO-A activity of the cell lysates was subsequently analyzed using an MAO-A inhibitor screening kit, following the manufacturer's instructions. Fluorescence (λ_{ex} = 535 nm and λ_{em} = 587 nm) was measured using the fluorescence spectrometer (Victor5) from PerkinElmer Co (Waltham, MA, USA).

RESULTS AND DISCUSSION

Screening of the Serotonin-like Mimotopes. Serotonin-like mimotopes were screened from the Fv-antibody library using a monoclonal anti-serotonin antibody. In the screening process, the monoclonal antibody was immobilized on magnetic beads and mixed with the Fv-antibody library, as shown in Figure 2a. The magnetic beads were isolated by using an external magnet and cultured on an agar plate. Cultured colonies were randomly selected, and genetic analysis was performed. After analyzing the oligonucleotide sequences, three clones (clone nos. 7, 33, and 40) were selected as valid candidates, each displaying distinct sequences at the CDR3 region compared to the template sequence (before the site-directed mutagenesis), as illustrated in Figure 2b.

Other clones were found to possess identical oligonucleotide sequences to the template (designated as clone no. 14), while some clones displayed deletions and additions of nucleotide sequences.

Selected clones were analyzed for their binding affinity to the monoclonal anti-serotonin antibody using a fluorescence-labeled secondary antibody. As shown in Figure 2c, the selected clones (clone nos. 7, 33, and 40) displayed a significantly high fluorescence signal at the gate region upon binding to the monoclonal antibody. *E. coli* with a high fluorescence signal indicated that the autodisplayed Fv-antibody on the outer membrane specifically interacted with the fluorescence-labeled monoclonal antibody. The control strain, with only CDR1 and CDR2 regions (without the randomized CDR3 region), did not exhibit a high fluorescence signal within the specified gate region. The absence of a fluorescence signal in the control strain unequivocally indicated the vital role of the CDR3 regions of these *E. coli* strains in mediating their interaction with the monoclonal antibody.

Quantitative binding analysis was performed for the other three clones, and three of the four clones showed quantitatively increased fluorescence signals, as shown in Figure 2d. Native *E. coli* without autodisplayed Fv-antibodies and the control strain with only CDR1 and CDR2 regions showed a response at the baseline level over the whole concentration range of the monoclonal antibody. The binding constants (K_D) of the screened clones to the monoclonal antibody were estimated to be 21.6, 7.31, and 19.9 nM for clone nos. 7, 33, and 40, respectively. Information about the selected clones is summarized in Table 2.

The Fv-antibodies from the screened clones were expressed as soluble proteins. As shown in Figure 3a, the Fv-antibodies comprised four FRs and three CDRs, including the CDR3 regions (11 residues) of the screened Fv-antibodies. The Fv-antibodies were expressed as a fusion protein with a red fluorescent protein (tdTomato) with a molecular weight of 69.2 kDa. The binding affinities (K_D) of the expressed Fv-antibodies were estimated by using an SPR biosensor. As shown in Figure 3b, monoclonal anti-serotonin antibodies were immobilized on the gold surface of the SPR biosensor; subsequently, the expressed Fv-antibodies in the concentration range of 3.7 nM−0.3 μM were added to the immobilized anti-serotonin antibodies. As shown in Figure 3c, the association and the

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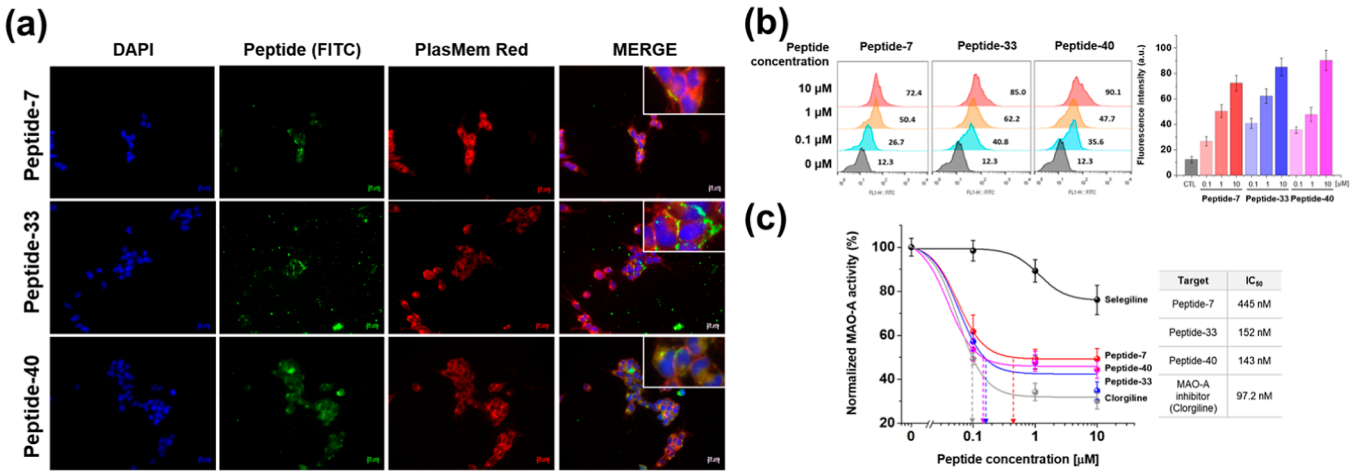

Figure 6. MAO-A inhibitory activity in SH-SY5Y cells. (a) Fluorescence image of SH-SY5Y cells after treatment with the serotonin-like mimotopes (Peptide-7, Peptide-33, and Peptide-40). (b) Binding affinity of peptides for SH-SY5Y cells by flow cytometry at a concentration of 0.1−10 μM. (c) MAO-A inhibitory activity of peptides selegiline and clorgiline.

dissociation profiles of the SPR biosensor were obtained, and the binding affinity (K_D) of the expressed Fv-antibodies were estimated to be 20.0, 27.0, and 12.4 nM for Fv-7, Fv-33, and Fv-40, respectively. These results indicated that the expressed Fv-antibodies exhibited a binding affinity comparable to that of the *E. coli* cells, which suggested that the expressed Fv-antibodies could be used as serotonin-like mimotopes. The binding constants (K_D) of the three synthesized peptides are summarized in Table 2.

The CDR3 regions (11 residues) of the screened Fv-antibodies were synthesized into peptides (12 residues) by inserting linked amino acid residues. The binding affinities (K_D) of the synthesized peptides were estimated by using an SPR biosensor. As shown in Figure 3d, monoclonal anti-serotonin antibodies were immobilized on the gold surface of the SPR biosensor, followed by the addition of the synthesized peptides in the concentration range of 37 nM−3.0 μM to the immobilized antibodies. As shown in Figure 3e, the association and the dissociation profiles of the SPR biosensor were obtained, and the binding affinities (K_D) of the synthesized peptides were estimated to be 681, 693, and 733 nM for Peptide -7, Peptide-33, and Peptide-40, respectively.

These results demonstrate that the synthesized peptides exhibited significantly enhanced binding affinity compared with the selected clones (*E. coli* cells). This improvement can be attributed to the increased degree of freedom in the binding interaction with the monoclonal antibodies. These results indicated that the synthesized peptides could be used as serotonin-like mimotopes. The binding constants (K_D) of the three synthesized peptides are summarized in Table 2.

Inhibition of MAO-A by Synthetic Peptides. Being a neurotransmitter, serotonin is released into the synapse and is reuptaken into the presynapse. MAO-A has been reported to inhibit the reuptake of serotonin through the oxidation-based breakdown of serotonin utilizing the coenzyme (FAD). An inhibitor of MAO-A, such as clorgiline, has been utilized to maintain the serotonin concentration.[6] Since the serotonin-like mimotopes were screened using a monoclonal anti-serotonin antibody, it was anticipated that these mimotopes would be recognized by MAO-A as serotonin. Consequently, they would inhibit the oxidation activity of MAO-A by occupying the active site of MAO-A. Although MAO-A and MAO-B share high amino acid sequence homology, they exhibit different substrate specificities and inhibitor sensitivities.[3]

In the present study, we confirmed the selectivity of serotonin-like mimotopes for purified MAO-A and MAO-B. The inhibitory activity of the screened mimotopes was analyzed using an enzyme activity assay kit based on the purified MAO-A reaction, generating fluorescence through the production of hydrogen peroxide (λ_{ex} = 535 nm, λ_{ex} = 587 nm), as shown in Figure 4a. The expressed Fv-antibodies were utilized in the assay for the inhibition of MAO-A and MAO-B enzymes. After treatment with the three serotonin-like mimotopes (Fv-antibodies) and clorgiline, MAO-A activity was measured, revealing a concentration-dependent decrease in MAO-A activity corresponding to the Fv antibody concentration, as shown in Figure 4b. The inhibition activity (IC_{50}) of the expressed Fv-antibodies were estimated to be 73.7, 162, and 110 nM for Fv-7, Fv-33, and Fv-40, respectively, and 30.1 nM for the conventional inhibitor (IC_{50} of 42 nM for clorgiline[34]). The MAO-A activity was not altered when it was treated with selegiline, which is an MAO-B inhibitor. Another kind of monoamine oxidase called MAO-B, which has been previously identified to oxidize dopamine, was compared in terms of its inhibitory activity on serotonin-like mimotopes (Fv-antibodies).

Following treatment with the three serotonin-like mimotopes (Fv-antibodies) and selegiline, the measurement of MAO-B activity revealed no significant changes in response to the mimotope (Fv-antibodies) concentration, as shown in Figure 4c. MAO-B activity was inhibited by selegiline in a concentration-dependent manner. These results demonstrated that the three serotonin-like mimotopes (Fv-antibodies) could be recognized as serotonin by MAO-A. Additionally, these mimotopes (Fv-antibodies) exhibit selective inhibition of MAO-A activity while not affecting MAO-B.

The synthesized peptides were also utilized to assess the inhibition of MAO-A and MAO-B activity. Following treatment of MAO-A with the three serotonin-like mimotopes (peptides) and clorgiline, MAO-A activity was observed to decrease according to the mimotope (peptide) concentration, as shown in Figure 4d. The inhibition activity (IC_{50}) of the synthesized peptides was estimated to be 61.6, 242, and 114 nM for Peptide-7, Peptide-33, and Peptide-40, respectively, and 33.1 nM for the conventional inhibitor (IC_{50} value of 42 nM for clorgiline[34]).

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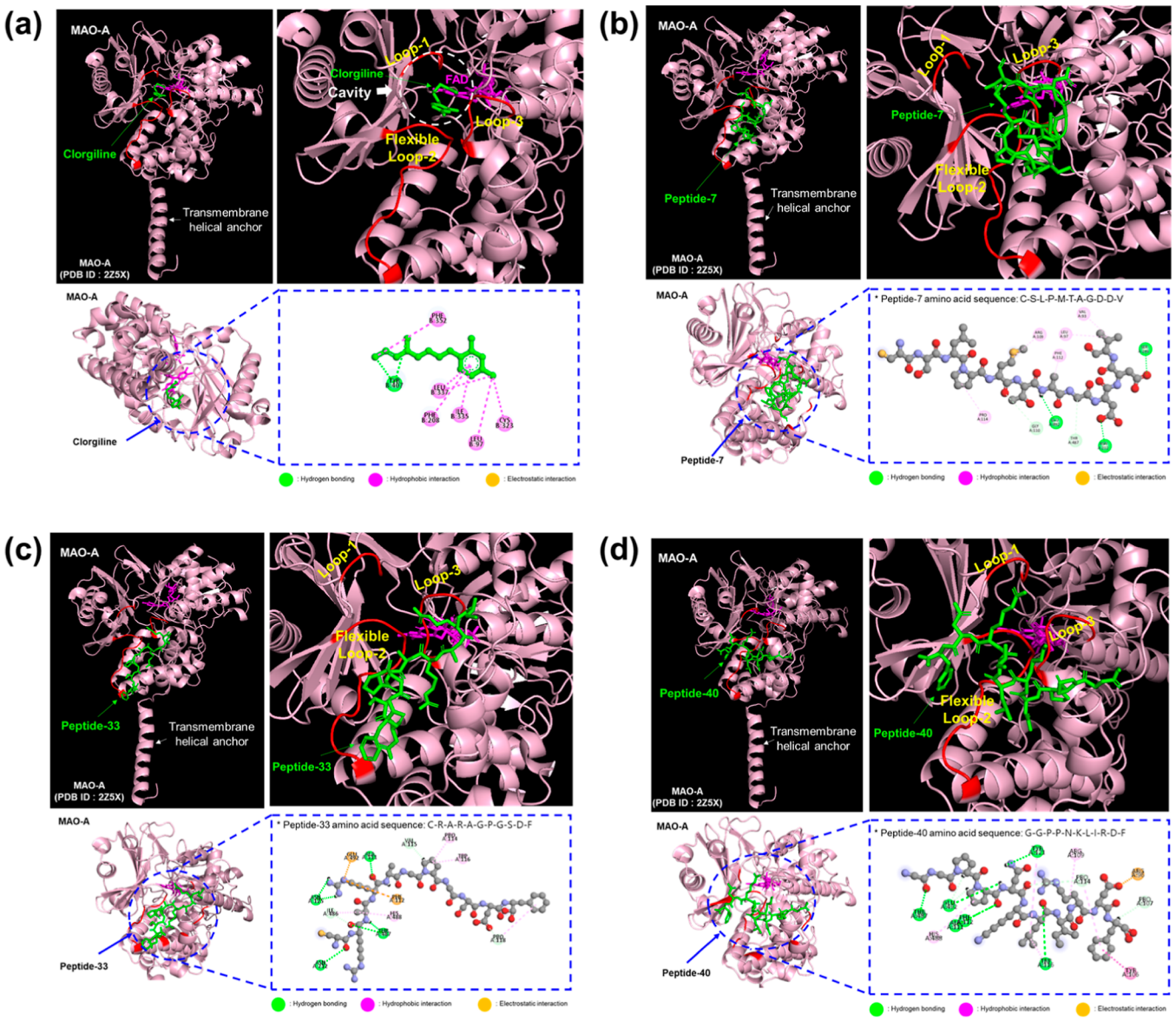

Figure 7. Molecular docking analysis of MAO-A (PDB ID: 2Z5X) and serotonin-like mimotopes (Peptide-7, Peptide-33, and Peptide-40). Molecular docking was performed to analyze the interaction between MAO-A and (a) MAO-A inhibitor (clorgiline), (b) Peptide-7, (c) Peptide-33, and (d) Peptide-40.

The MAO-A activity was not altered when selegiline was used, which is an MAO-B inhibitor. As described earlier, the inhibitory activity of the serotonin-like mimotopes (peptides) against MAO-B was compared. Similar to the observation for the serotonin-like mimotopes (Fv antibodies), no alterations in the activity of MAO-B were observed after the treatment with the three serotonin-like mimotopes (peptides) and clorgiline, as shown in Figure 4e. MAO-B activity was inhibited by selegiline in a concentration-dependent manner. These results demonstrated that the three serotonin-like mimotopes (peptides) could be recognized as serotonin by MAO-A, and they could selectively inhibit MAO-A activity but not the MAO-B activity.

The BBB permeation test of the three synthesized peptides (Peptide-7, Peptide-33, and Peptide-40) was performed using a commercial BBB permeability test kit from BioAssay Systems (Hayward, CA, USA), as shown in Figure 5a.[28−30] The BBB permeability rate (P_e) of the three peptides was calculated and compared with three reference standards, such as high (promazine), medium (clonidine), and low (diclofenac) permeability. From the measurement of fluorescence intensities of peptides before and after the artificial BBB membrane, the permeability rate (P_e) was calculated to be 3.33×10^{-6} cm/s for Peptide-7, 3.42×10^{-6} cm/s for Peptide-33, and 3.69×10^{-6} cm/s for Peptide-40. Also, the P_e value for the three reference standards was calculated as 5.12×10^{-6} cm/s for promazine (high permeability), 3.76×10^{-6} cm/s for clonidine (medium permeability), and 1.39×10^{-6} cm/s for diclofenac (low permeability). In comparison with the three reference standards, the permeability rate (P_e) of the three peptides was estimated to be between low and medium permeability, as shown in Figure 5b.

The screened serotonin-like mimotopes were treated with a cell line expressing a serotonin receptor (SH-SY5Y; ATCC code: CRL-2266). Three types of serotonin-like mimotopes (labeled with fluorescein) were used to treat the cell line and confirm their binding to serotonin receptors on the cell surface.

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As shown in Figure 6a, the fluorescence signals from mimotopes were observed, and to facilitate a comparison of images, a fluorescent dye [PlasMem Red from Dojindo (Kumamoto, Japan)] was concurrently utilized for plasma membrane staining. Upon merging the images, it was observed that the mimotopes (labeled with fluorescein) were bound to the cell membrane, and some of them exhibited partial penetration into the cell. Peptide-40 exhibited the highest fluorescence signal within the cytosol among the three mimotopes (peptides), which represented the highest concentration of the penetrated mimotope. The fluorescence signal from the total number of cells after treatment with mimotopes was analyzed using FACS. As shown in Figure 6b, the fluorescence signal from the cells was significantly higher than that of the control (without mimotope treatment), and the signal increased with increasing concentration (from 0.1 to 10 μM) for the three types of mimotopes. For Peptide-40, the fluorescence signal was observed to be the highest among the three types of mimotopes. As previously mentioned, mimotopes (synthesized peptides labeled with fluorescein) bind to the cell membrane and partially penetrate the cell. MAO-A activity was estimated using cell homogenates treated with serotonin-like mimotopes at a concentration of 0.1−10 μM. As shown in Figure 6c, the MAO-A activity was reduced to 50.7, 65.2, and 55.6% by 10 μM each of Peptide-7, Peptide-33, and Peptide-40, respectively. Treatment with the conventional MAO-A inhibitor clorgiline (positive control, at a concentration of 10 μM) caused a reduction in the MAO-A activity to 69.8%. These results indicated that the serotonin-like mimotopes (peptides) penetrated the cells and inhibited MAO-A activity at varying ratios. The inhibition ratio of Peptide-40 was estimated to be as high as that of conventional MAO-A inhibitors.

To investigate the interaction between the two mimotopes from the screened clones and MAO-A, a docking simulation was performed using AutoDock Vina software from Scripps Research.[35−37] The interactions were visualized and analyzed using PyMOL software, version 0.99rc6, from DeLano Scientific LLC (South San Francisco, CA, USA) and Discovery Studio Visualizer version 21.1.0.20298 from Dassault Systèmes BIOVIA (San Diego, CA, USA). The structure of MAO-A (PDB ID:2BXR and 2Z5X) was obtained from Protein Data Bank (PDB, www.rcsb.org), and the interaction with the conventional MAO-A inhibitor, clorgiline, was performed, as shown in Figure 7a. The docking results showed that the inhibitor was bound to the entrance gate region of MAO-A, where a substrate cavity (V_{210}−E_{216}) is surrounded by three additional loops (loop 1: V_{93}−V_{95}, loop 2: Y_{108}−P_{118}, and loop 3: F_{208}−N_{212}). The inhibitor effectively blocked the interaction between MAO-A and its cofactor (FAD) responsible for serotonin oxidation. Among the three loops, flexible loop-2 is essential for controlling substrate access to the active site of MAO-A.[5] The amino acids of MAO-A that hydrophobically interacted with clorgiline were L_{97}, F_{208}, C_{323}, I_{335}, L_{337}, and F_{352}, while the amino acid Y_{407} formed hydrogen bonds with clorgiline. The docking results revealed that serotonin-like mimotope-1 (Peptide-7) interacted with MAO-A, specifically binding to the middle section of the entrance gate region, as shown in Figure 7b. At this specific location, Peptide-7 interacts with three loops surrounding the cavity (loop-1, flexible loop-2, and loop-3), as previously described. These loops are known to play a pivotal role in regulating substrate access to the active site of MAO-A, thereby influencing the approach of the substrate (serotonin) to the MAO-A cofactor (FAD) during serotonin oxidation.

The amino acids of MAO-A, including V_{93}, L_{97}, R_{109}, and F_{112}, were found to engage in hydrophobic interactions with the amino acids P_{11}, V_{11}, G_8, M_5, and P_4 of Peptide-7. Additionally, the amino acids G_{110}, S_{209}, N_{212}, T_{487}, and E_{492} of MAO-A formed hydrogen bonds with M_5, D_{10}, D_9, G_8, and M_7 of the mimotope. The docking results revealed that serotonin-like mimotope-2 (Peptide-33) interacted with MAO-A and was specifically bound to the lower side of the single cavity site of MAO-A, as shown in Figure 7c. At this specific location, Peptide-33 interacts with two loops present in the substrate cavity, specifically the flexible loop-2 and loop-3. This interaction significantly impacts the approach of the substrate (serotonin) to the cofactor of MAO-A (FAD) during serotonin oxidation. The amino acids, including P_{114}, W_{116}, and H_{488} of MAO-A, exhibited hydrophobic interactions with the amino acids P_7 and A_3 of the serotonin-like mimotope-2 (Peptide-33). Additionally, the MAO-A amino acids A_{111}, V_{115}, P_{118}, N_{125}, N_{212}, I_{486}, and T_{487} formed hydrogen bonds with the amino acids R_4, P_7, F_{11}, R_4, C_1, and A_3 of the mimotope. Moreover, the amino acids F_{112} and E_{492} of MAO-A exhibited electrostatic interactions with the R_4 amino acid of the mimotope. A docking simulation of serotonin-like mimotope-3 (Peptide-40) with MAO-A was performed, and the results revealed that Peptide-40 was bound to the lower side of the entrance gate region of MAO-A, as shown in Figure 7d. At this specific location, Peptide-40 interacts with the substrate cavity surrounding loop-3 and influences the approach of the substrate (serotonin) to the cofactor of MAO-A (FAD) for serotonin oxidation. The amino acids of MAO-A, including Y_{106}, R_{109}, and H_{488}, hydrophobically interact with the amino acids F_{11} and P_4 of the serotonin-like mimotope-3 (Peptide-40). Additionally, the MAO-A amino acids, including P_{107}, A_{111}, F_{112}, P_{114}, W_{116}, Y_{121}, and T_{487} formed hydrogen bonds with the amino acids F_{11}, P_4, P_4, N_5, L_7, N_5, and G_1 of Peptide-40. Moreover, the R_{96} of MAO-A electrostatically interacts with the D_{10} of the mimotope. The interactions of MAO-A with clorgiline and the three mimotopes are summarized in Table 3.

Table 3. Analysis of the Interactions between MAO-A and Mimotopes

	binding site of MAO-A		
	hydrophobic interaction	hydrogen bond	electrostatic interaction
clorgiline	L_{97}, F_{208}, C_{323}, I_{335}, L_{337}, F_{352}	Y_{407}	
Peptide-7	V_{93}, L_{97}, R_{109}, F_{112}, P_{114}	G_{110}, S_{209}, N_{212}, T_{487}, E_{492}	
Peptide-33	P_{114}, W_{116}, H_{488}	A_{111}, V_{115}, P_{118}, N_{125}, N_{212}, I_{486}, T_{487}	F_{112}, E_{492}
Peptide-40	Y_{106}, R_{109}, H_{488}	P_{107}, A_{111}, F_{112}, P_{114}, W_{116}, Y_{121}, T_{487}	R_{96}

CONCLUSIONS

Serotonin-like mimotopes were screened from the Fv-antibody library to be used as inhibitors against MAO-A. Target clones with binding affinity for the monoclonal anti-serotonin antibody were screened from the Fv-antibody library. Finally, three clones were screened by measuring the binding affinity of serotonin using FACS. The screened Fv-antibodies were expressed as soluble fusion proteins with a green fluorescent protein. Additionally, the CDR3 regions (11 residues) of the screened

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Fv-antibodies were synthesized into peptides (12 residues) by inserting linking amino acid residues. Three serotonin-like mimotopes each of Fv-antibodies and peptides were synthesized, and their binding constants were estimated using the SPR biosensor. The binding affinity (K_D) of the expressed Fv-antibodies was estimated to be 20.0, 27.0, and 12.4 nM for Fv-7, Fv-33, and Fv-40, respectively. The binding affinities (K_D) of the synthesized peptides were estimated to be 681, 693, and 733 nM for Peptide-7, Ppetide-33, and Peptide-40. The inhibitory activity of serotonin-like mimotopes against MAO-A and MAO-B was compared with that of conventional inhibitors, such as clorgiline for MAO-A and selegiline for MAO-B. Three serotonin-like mimotopes were recognized as serotonin by MAO-A, and these mimotopes could selectively inhibit MAO-A activity compared to MAO-B inhibitors. Finally, the screened serotonin-like mimotopes (peptides) were added to a cell line (SH-SY5Y, ATCC code: CRL-2266) with a serotonin receptor to confirm binding to the serotonin receptors on the cell surface. Analysis of the cell lysate after treatment with the three serotonin-like mimotopes (peptides, at a concentration of 10 μM) indicated a reduction in the MAO-A activity to 50.7, 65.2, and 55.6% by Peptide-7, Peptide-33, and Peptide-40, respectively. These results indicated that the serotonin-like mimotopes effectively penetrated the cells, leading to the inhibition of MAO-A to varying degrees. Notably, Peptide-40 exhibited a substantial inhibition ratio comparable to that of conventional MAO-A inhibitors.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsptsci.3c00204.

Sequences of screened oligonucleotide and amino acid of CDR3 regions for serotonin-like mimotopes and purity of the three synthesized peptides (Peptide-7, -33 and -40) (PDF)

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Notes

The authors declare no competing financial interest.

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