NMR), and JEOL ECS400 (400 MHz for ^{1}H NMR) spectrometers. Chemical shifts were referenced to the solvent used (δ = 7.26 ppm for $CDCl_3$, 3.31 ppm for CD_3OD). ESI-mass spectra were measured on a Waters ZQ4000 spectrometer (for LRMS).

EZ-Link® NHS-Iminobiotin was purchased from Thermo Scientific. Other reagents were purchased from Aldrich, Tokyo Chemical Industry Co., Ltd. (TCI), Kanto Chemical Co., Inc. and Wako Pure Chemical Industries, Ltd. and used without further purification.

Synthesis of artificial biotin analogues

Fig. S1 shows synthetic route of iminobiotin long tail (IMNtail), 6-(5-((3aS,4S,6aR)-2-iminohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)hexanoic acid. To a solution of 6-aminohexanoic acid **2** (3 mg, 0.023 mmol) in dioxane/H_2O (500 □l/500 □l) was added NaOH aq until the pH of the solution reached ~9. EZ-Link® NHS-Iminobiotin **1** (10 mg, 0.023 mmol) was added to the solution and the resulting mixture was stirred for 12 h at room temperature. The reaction mixture was washed with Et_2O and the aqueous phase was neutralized with aqueous HCl to afford a white precipitate. The precipitate was collected by filtration and washed with acetone.

The crude mixture was dissolved in dioxane/H_2O (500 □l/500 □l). 29% NH_3 aq was added to the solution and stirred for 3 h at room temperature, which evaporated to give a white solid. The crude product was washed with a CH_2Cl_2/MeOH solvent to give IMNtail **3** (4 mg, 49% yield, white solid).

^{1}H NMR (CD_3OD): δ =1.32–1.68 (m, 12H), 1.71–1.82 (m, 1H), 2.20 (t, J = 7.3 Hz, 2H), 2.29 (t, J = 7.3 Hz, 2H), 2.81 (d, J = 13 Hz, 1H), 3.0 (dd, J = 13, 4.6 Hz, 1H), 3.16 (t, J = 6.9 Hz , 2H), 4.52 (dd, J = 8.2, 4.6 Hz, 1H), 4.72 (dd, J = 8.2, 4.6 Hz, 1H); LRMS (ESI): m/z 357 $[M+H]^+$

The biotin long tail (BTNtail), 6-(5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)hexanoic acid (BTNtail) was prepared by following a reported procedure (*31*).

Expression and purification of V21 mutant

The nucleotide sequence of the V21 mutant, which contains amino-acid substitutions Y22S/Y83S/R84K/E101D/R103K/E116N/N23D/S27D, was subcloned into the pCold TF vector (Takara Bio). The HRV 3C protease cleavage site, the coding sequence of Trigger Factor (TF) and the 6×His tag were fused at the N-terminus of V21. *Escherichia coli* BL21Star(DE3) cells harboring the V21 plasmid were grown at 37 °C in Luria Broth containing 100 μg/ml ampicillin up to an OD of 0.5–0.6, induced with isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.4 mM and further grown at 15 °C for 24 h. Cells were harvested by centrifugation at 8000 × g for 20 min and resuspended in a lysis buffer containing 1× PBS, 500 mM NaCl, 20 mM imidazole, cOmplete EDTA-free protease inhibitor cocktail tablets (Roche) and Benzonase (Novagen). The sample was lysed by passing through an EmulsiFlex (AVESTIN) and centrifuged at 10,000 × g for 30 min. The

supernatant was loaded onto Ni agarose (Invitrogen) and washed with the lysis buffer. V21 was eluted from the column with elution buffer (1× PBS and 250 mM imidazole). HRV 3C protease was added to the sample at 1:50 molar ratio. After incubation at 4 °C for 2 d, the solution was dialyzed against buffer (1× PBS, 500 mM NaCl and 20 mM imidazole) and loaded onto a HisTrap HP column (Invitrogen) to remove His-tagged material. The flow-through was collected and reloaded onto the column. The flow-through was then recollected and dialyzed against buffer (25 mM Tris-HCl, 500 mM NaCl and 4 M GdnHCl). The dialysate was then passed through a HisTrap HP column. The flow-through was collected and dialyzed against buffer (10 mM Tris-HCl, pH 7.5 and 250 mM NaCl). Ligand (BTN, BTNtail, or IMNtail) was added to the V21 sample at a 20:1 molar ratio. The proteins were then concentrated to approximately 10 mg/ml, as estimated by absorbance at 280 nm.

Expression and purification of S45-substituted mutants of V21

The nucleotide sequences of S45-substituted mutants (S45T, S45N, S45Q, S45H, S45L, S45I, S45A; V212 is the S45N mutant) of V21 were subcloned into the pET21a(+) vector (Novagen). For preparation of the muteins, the isolation and refolding protocols were performed as described in previous reports (*32-35*) with modifications. The T7 tag was introduced at the N-terminus of the muteins and a 6×His tag at the C-terminus. BL21Star(DE3) cells harboring the mutant plasmid were grown at 37 °C in 2×YT medium containing 100 μg/ml ampicillin to an OD of 0.8, and protein

expression induced by adding IPTG to a final concentration of 1 mM and cells grown at 37 °C for a further 5 h. Cells were harvested by centrifugation at 8000 × *g* for 20 min and resuspended in a lysis buffer containing 50 mM Tris-HCl, pH 8.0, and ruptured by sonication. The lysed cells were centrifuged at 16,500 × *g* for 20 min. The insoluble fraction was washed three times with buffer (50 mM Tris-HCl, pH 8.0 and 2% Triton X-100) and then washed twice with ultra-pure water. The inclusion bodies were dissolved in 6 M guanidine hydrochloride, pH 1.5, and dialyzed against the dissolution buffer at 4 °C overnight. Insoluble material was removed by ultracentrifugation at 16,500 × *g* and 4 °C for 30 min, and the supernatant was added to the refolding buffer (50 mM Tris-HCl, pH 8.0, 400 mM L-arginine hydrochloride, 200 mM NaCl and 1 mM EDTA) by the rapid refolding method and left for 2 d. The sample was loaded onto Complete His-tag Purification resin (Roche) and eluted with a refolding buffer containing 400 mM imidazole. The eluted sample was buffer-exchanged into gel-filtration buffer (1×PBS) by dialysis. Further purification was carried out by gel-filtration chromatography using a HiLoad 16/600 Superdex 75 column. IMNtail was added to the sample at a 8:1 molar ratio. Finally, the purified protein was buffer-exchanged and concentrated to 10 mg/ml in 20 mM Tris-HCl buffer (pH 7.5) containing 200 mM NaCl using Vivaspin 10-kDa cutoff (GE Healthcare)

Binding assays by SPR

The interactions between SA mutants and biocytin, BTN, or IMNtail were analyzed by SPR with a Biacore T200 instrument (GE Healthcare). We used sensor chip NTA and NTA reagent kit (GE Healthcare). C-terminal His-tagged LISA314, V21 and V212 were immobilized on sensor chip NTA through His tag. 1× HBS-P+ buffer (GE Healthcare) was used for running buffer. Each immobilized levels of ligand were between 2400 RU and 3500 RU. Each analyte were dissolved and diluted from 1 nM to 10 nM in running buffer. Steady-state affinity analysis was done by Biacore evaluation software.

Crystallization

Crystallization was performed by the sitting-drop vapor-diffusion method at 20 °C in Violamo 96-well plates (As One, Osaka, Japan). Sixty microliters of the reservoir solution was added to each well of the 96-well plates. Crystals of V21 complexed with BTN were obtained by mixing 0.5 μl of the protein solution and 0.5 μl of the reservoir solution (100 mM sodium acetate trihydrate, pH 4.6 and 2 M ammonium sulfate). Crystals of V21 complexed with BTNtail were grown by mixing 0.1 μl of the protein solution with 0.1 μl of the reservoir solution (0.1 M cadmium chloride hydrate, 0.1 M sodium acetate trihydrate, pH 4.6 and 30% (w/v) PEG400). Crystals of V21 complexed with IMNtail were prepared by mixing 0.5 μl of the protein solution and 0.5 μl of the reservoir solution (0.2 M sodium citrate tribasic dehydrate, 0.1 M HEPES sodium, pH 7.5 and 35% w/v (+/-)-2-methyl-2,

4-pentandiol). Crystals of V212 complexed with IMNtail were obtained by mixing 0.5 μl of the protein solution and 0.5 μl of the reservoir solution (60 mM sodium cacodylate trihydrate, pH 6.8 and 27% (w/v) PEG300).

X-ray data collection and structure determination

The dataset for V21 complexed with BTNtail were collected on the beamline BL17A at the Photon Factory (Tsukuba, Japan), and all the other datasets were collected on the BL44XU at SPring-8 (Harima, Japan) at −173 °C. A crystal of V21 complexed with BTNtail was cryoprotected by coating with a solution (66.5% Paratone-N, 28.5% Paraffin Oil and 5% Glycerol). Crystals of V21 complexed with BTN and IMNtail and V212 complexed with IMNtail were cryoprotected by the well solution containing 20% (v/v) glycerol. Data were indexed and scaled with the programs *DENZO* and *SCALEPACK* from the *HKL2000* program suite (HKL Research). The structures were solved by molecular replacement with the program *Phaser (36)* from the *CCP4i* package (*37*) using the crystal structure of LISA-314 (PDB ID: 3WYQ, unreleased data (*38*)) as the search model. The resultant structures were manually modified to fit into the experimental electron density maps, using the program *Coot (39)*, then refined with the program *Refmac5* (*40*) from the *CCP4i* package. The results of the structural analysis are summarized in Table I. Figures were prepared with *Pymol* (http://www.pymol.org/). The final structure coordinates and structure factor amplitudes were

deposited into the Protein Data Bank with IDs 3WZN for V21 complexed with BTN, 3WZO for V21 complexed with BTNtail, 3WZP for V21 complexed with IMNtail, and 3WZQ for V212 complexed with IMNtail.

Results and Discussion

Construction of V21 from the original LISA-314

To develop a pre-targeting method using the SA binding system, we must solve the problem that BTN present as a vitamin in the body strongly binds to LISA-314. Thus, a novel binding system is required, in which the original LISA-314 selectively binds to a non-natural BTN analogue without binding endogenous BTN species. We may therefore consider two strategies: the modification of LISA-314 and the development of a new, non-natural BTN analogue.

Reznik *et al.* (*28*) prepared a double mutant of N23A/S27D that acquired a higher binding affinity for IMN than BTN with K_d values of 1.0×10^{-6} M and 7.1×10^{-5} M, respectively (*28*). Considering the report, we introduced a double substitution N23D/S27D into LISA-314 (naming the resulting mutant "V21"). The reason why we adopted N23D instead of N23A is as follows. We presumed that N23D would induce electrostatic repulsion against the ureido oxygen of BTN and make the affinity lower for BTN than N23A, because the ureido oxygen is highly polarized in positive

(*41*). Besides, negatively charged aspartate residue would interact with the guanidino nitrogen of IMN, which is protonated at neutral pH (*42*). Although more electrostatic repulsion against the ureido oxygen of BTN was introduced in V21 (Fig. 1A), a lower K_d value of 1.0×10^{-7} M for BTN (cf. 7.1×10^{-5} M from Reznik *et al.*) and that of 3.5×10^{-7} M for biocytin was measured by surface plasmon resonance (SPR) (Table II). We are now crystallizing the N23A/S27D mutant to elucidate structural basis explaining the affinity difference from V21.

Crystal structure of V21 in complex with BTN

We then solved the crystal structure of V21 in complex with BTN at 1.3 Å resolution. The structure forms a tetramer, which is composed of a dimer of dimers. One BTN molecule binds to each protomer. No major conformational changes occurred in the overall structure when compared with LISA-314.

A comparison between the binding motif of BTN in V21 and that in LISA-314 showed that three residues 23, 27 and 128 interacted differently with BTN (Fig. 1). The distance between the carboxyl oxygen of Asp23 in V21 and the ureido oxygen of BTN is 4.47 Å. The carboxyl oxygen of Asp27 forms van der Waals contact but not hydrogen bond with the ureido oxygen because of the improper orientation despite the relatively short distance between them (i.e., 3.19 Å, Fig. S2), showing that two hydrogen bonds around the ureido oxygen in LISA-314 were lost in V21. As a

result, the position of the ureido oxygen of BTN in V21 moved away from Asp23 and Asp27 by 0.68 Å relative to that in LISA-314. Moreover, the interaction between the carboxyl oxygen of Asp128 and the N^3 nitrogen of BTN was altered from a direct hydrogen bond in the LISA-314-BTN complex to interaction *via* a water molecule in the V21-BTN complex.

From the structural analysis results of V21, we conclude that N23D/S27D substitutions induce electrostatic repulsions against the ureido oxygen atom, breaking two main hydrogen bonds with BTN. Although the binding affinity of V21 for BTN was lower than that of the N23A/S27D mutant in Reznik *et al.* (*28*), no structural change was seen in the overall structure.

Design and synthesis of BTN analogues, BTNtail and IMNtail

We next designed non-natural BTN derivatives as new ligand molecules for SA. We predicted a need to add a long spacer between BTN and a drug molecule for future pre-targeting therapy. One study reported that the carboxyl terminus of BTN was modified for extension (*43*), and we designed a BTN molecule with a long tail (BTNtail) (Fig. S3A). A structural analysis of BTNtail-bound V21 at 1.5 Å resolution (Fig. S3B) demonstrated that the binding motif of BTNtail in V21 is very similar to that of BTN; although, the sulfur atom of the BTNtail was oxidized by unknown reason as observed in previous reports (*44, 45*). In summary, the addition of the long tail had no influence on the binding mode of BTNtail in V21 when compared with the binding mode of BTN. We also examined the

addition of a long tail to IMN (IMNtail) as a new ligand and this ligand was synthesized (Fig. 2A). SPR measurements showed that V21 had a K_d value of 1.5×10^{-7} M for IMNtail at neutral pH (Table II). Conversely, LISA-314 showed no binding affinity towards the IMNtail, which is interesting because SA-WT possessing an identical BTN binding motif to LISA-314 is known to have significant binding affinity toward IMN ($K_d = 1.3 \times 10^{-7}$ M at neutral pH) (*28*). The tail region of IMNtail might have dramatically lowered its binding ability to LISA-314.

Crystal structure of V21 in complex with the IMNtail

We then solved the crystal structure of V21 in complex with IMNtail at 1.2 Å resolution. One IMNtail molecule binds to each protomer (Fig. 2B). Superposition of the complex structures between V21-IMNtail and V21-BTNtail shows that a head group of IMNtail shifts toward Asp128 (Fig. 2C) compared to that of BTNtail. Besides, the N^2 nitrogen of IMNtail interacts with Asp27, with which the ureido oxygen of BTN has no interaction in V21 because of electrostatic repulsion. The shift of the head group to Asp128 causes a rearrangement of the hydrogen-bond network. The distances between the N^2 nitrogen of IMNtail and the carboxyl oxygen of Asp23 or the hydroxyl oxygen of Tyr43 are lengthened to 3.61 and 3.50 Å, respectively, indicating a weaker contribution to hydrogen bonding by Asp23 and Tyr43 than that observed in SA-WT (3.2 and 2.7 Å, respectively) (*42*). The S27D substitution, which gives a longer side chain, may push the N^2 nitrogen of IMNtail away from

Asp23 and Tyr43 and towards Asp128.

Interestingly, the most striking difference between the structures of V21-BTNtail and V21-IMNtail complexes was found at the hydrogen bond with Ser45. Although Ser45 directly forms a hydrogen bond to N^1 nitrogen of BTNtail in V21 (2.96 Å), as it does in SA-WT (2.90 Å), it indirectly forms a hydrogen bond to N^1 nitrogen of IMNtail *via* a water molecule (Ser45-N^1 distance: 4.39 Å). Because of this difference, we proposed that a substitution at Ser45 in V21 to a residue with a bulky side chain would eliminate the binding affinity for BTNtail through steric hindrance without affecting the binding of IMNtail., We made a single amino-acid substitution from serine to threonine, asparagine, glutamine, histidine, leucine, or isoleucine, with alanine as a negative control.

Evaluation of the amino-acid substitutions at Ser45 of V21

Out of the seven substitution mutants of V21, only S45L and S45I mutants could not be expressed in *E. coli*. The binding affinities of the V21 mutants for ligands were measured by SPR (Table □□). The S45A and S45T mutants had significantly decreased affinities for biocytin by 10–100-fold compared with V21, while maintaining binding affinities for IMNtail. As expected, the S45N, S45Q and S45H mutants, with more bulky residues, completely lost binding affinity for biocytin. Among these, S45N and S45H mutants retained binding affinity of about the order of 10^7 for the non-natural IMNtail, which is similar to the original V21. Since the isoelectric point of histidine

residue is near neutral pH and S45H mutant might show unstable binding property in wider pH range, the S45N mutant, termed V212, was examined in the subsequent development.

Crystal structure of V212 in complex with IMNtail

To investigate the effect of the S45N substitution, we solved the crystal structure of V212 in complex with IMNtail at 1.7 Å resolution. V212 also forms a tetramer and one IMNtail molecule binds to each protomer. A comparison of the binding pocket of the V212-IMNtail complex to that of the V21-IMNtail complex showed that the binding loop composed of residues 45–52 in V212 was formed differently to the same loop in V21 (Fig. 3A). The binding loop in V212 does not interact with the IMNtail molecule. When a ligand binds, the binding loop usually changes from a flexible open conformation to a close conformation like a lid over the binding pocket (*46-48*). Even in the complex with IMNtail, the binding loop in V212 showed an open conformation as well as in the ligand-free SA-WT (Fig. 3B). Although the electron densities are not well ordered, the side chain of Asn45 in V212 appears to interact with Gly48, Asn49 and Ser52 to maintain the opened loop conformation (Fig. 3A, Fig. S4). This is similar to the characteristic interaction between Ser45 and Ser52 found in the ligand-free SA-WT to stabilize the opened loop (*46-48*). We infer that the S45N substitution sterically hinders the head group of IMNtail, so the ligands and Asn45 are unable to make interactions. Thus, the Asn45 flips away from IMNtail to force the binding loop to adopt the open conformation. We

initially expected that this steric hindrance effect by introducing the S45N substitution occurred only for BTNtail based on the distance between Ser45 and the N^1 nitrogen of BTNtail or IMNtail in V21, but it seems to occur also for IMNtail to some extent.

However, interestingly, the K_d values of IMNtail are comparable between V21 and V212 (Table II) despite the structural change of the binding loop from the closed form found in V21 to the open form observed for V212, resulting in loss of interactions between the binding loop and IMNtail (Fig. 3C). The interaction of Ser45 with the N^1 nitrogen of IMNtail *via* a water molecule observed in V21 was lost in V212. Instead, Asp27 forms a hydrogen bond with the N^1 nitrogen of IMNtail through a water molecule in V212. In addition, the hydrogen bond distances between the carboxyl oxygen of Asp23 and the N^2 nitrogen of IMNtail was 3.61 Å for V21 and 2.75 Å for V212, indicating that the weak hydrogen bond in V21 was strengthened in V212. We postulate that these unique interactions in V212 may compensate for the loss of the interaction between the binding loop and IMNtail and maintain affinity for IMNtail comparable to V21. In contrast to the IMNtail, the BTN and BTNtail molecules cannot form a hydrogen bond with Asp23 or Asp27 because of electrostatic repulsion as described above, and they would suffer severe steric hindrance by the S45N substitution. We conclude these differences decide the outcome; V212 abolishing affinity only for BTN species.

In the present study, we have created a novel SA binding system, V212 and IMNtail, by

modifying the low immunogenic mutant LISA-314. While abolishing the binding affinity for a BTN species, biocytin, V212 can bind the non-natural BTN analogue, IMNtail, with a K_d value of 5.2×10^{-7} M. This promises that V212 can capture the IMNtail in the human body without any binding competition from endogenous BTN species. Although it is necessary to increase the affinity towards IMNtail and evaluate the immunogenicity of V212 by animal testing before clinical application, our V212 and IMNtail system is a promising technology as a novel drug delivery tool.

Supplementary Data

Supplementary data are available at *JB* Online

Funding

This work was carried out under the Japanese research project Molecular Dynamics for Antibody Drug Development (MDADD), which is supported by the Cabinet Office, Government of Japan and the Japan Society for the Promotion of Science (JSPS) through the Funding Program for World-Leading Innovative R&D on Science and Technology (FIRST Program) initiated by the Council for Science and Technology Policy (CSTP). This work was partly supported by the JST-ERATO, Lipid Active Structure Project, the Grant-in-Aid for Scientific Research on Innovative Areas, the X-ray Free-Electron Laser Priority Strategy Program (MEXT), and the JSPS KAKENHI Grant Numbers 25282230, 24770096, and 22550152

Acknowledgements

The authors thank the beamline staff of BL44XU at SPring-8 (Proposal No. 2011B1595) and BL17A at Photon Factory for technical assistance during data collection, and Ms. Kyoko Watanabe and Ms. Maiko Nampo for help in crystallization.

Conflict of interest

None declared

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