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Sialic Acid-Mimic Peptides As Hemagglutinin Inhibitors for Anti-Influenza Therapy

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Influenza is an infectious disease caused by the influenza virus, and each year many people suffer from this disease. Hemagglutinin (HA) in the membrane of type A influenza viruses recognizes sialylglycoconjugate receptors on the host cell surface at an initial step in the infection process; consequently, HA inhibitors are considered potential candidates for antiviral drugs. We identified peptides that bind to receptor-binding sites through a multiple serial selection from phage-displayed random peptide libraries. Using the HA of the H1 and H3 strains as target proteins, we obtained peptides that bind to both HAs. The binding affinities of peptides for these HAs were improved by secondary and tertiary selections from the corresponding sublibraries. A docking simulation suggested that, similar to sialic acid, the peptides are recognized by the receptor-binding site in HA, which indicates that these peptides mimic the sialic acid structure. *N*-stearoyl peptides inhibited infections by the A/Puerto Rico/8/34 (H1N1) and A/Aichi/2/68 (H3N2) strains of influenza virus. Such HA-inhibitors are promising candidates for novel antiviral drugs.

Introduction

The influenza virus is a negative-sense single-stranded RNA virus of the family *Orthomyxoviridae* and causes epidemics and pandemics of influenza.[1,2] The virus has two major antigenic glycoproteins, namely hemagglutinin (HA[a]) and neuraminidase (NA). Three HA subtypes (H1, H2, and H3) and two NA subtypes (N1 and N2) have frequently been found in human influenza virus.[1] Since 1997, the highly pathogenic H5N1 strain has spread throughout the human population, with a mortality rate of 33%.[2] Thus, humans are currently being exposed to the hazard of a highly pathogenic influenza virus.

Influenza can usually be prevented by vaccination. Furthermore, antiviral drugs are effective in defending the body against infection. We can use several antiviral drugs for the treatment of influenza, particularly in those patients for whom it is not possible to administer an influenza vaccine, e.g., those having an egg allergy, or when a new type of virus, such as swine H1N1 virus in 2009, enters circulation.[3] NA inhibitors, such as oseltamivir[4] and zanamivir, which inhibit virus budding,[5] are available for clinical use. Further, M2 protein inhibitors, amantadine and rimantadine, have been used for over 50 years.[6] However, the appearance of drug-resistant strains has been reported,[6,7] emphasizing the fact that we are constantly required to develop new antiviral drugs.

These antiviral drugs inhibit different steps of the virus life cycle. To mount a defense against a new type of influenza, it is preferable that we have many types of antiviral drugs with a variety of pharmacological properties.[8] In influenza virus infection, the viral HA binds to sialylglycoconjugates on the host cell surface, and a subsequent membrane fusion is induced by a conformational change in HA.[9] Among the promising agents are entry blockers that inhibit the entry of viruses into cells during the infection process. To date, sialic acid-containing lipids[10] and polymers[11] have been developed as entry blockers. In particular, many types of sialic acid-containing polymers have been developed, including sialic acid-conjugated dendritic polymers,[12,13] sialyloligosaccharides containing poly L-glutamic acid backbones,[14,15] and sialyllactose-carrying polystyrene.[16] Whitesides and co-workers synthesized many sialic acid-containing polymers, and the detailed mechanism of hemagglutination has been investigated.[17,18]

As alternatives to sialic acids, we have developed "peptides" that inhibit the interaction between HA and sialylglycoconjugates by using a random library selection. We previously reported that sialylgalactose-binding peptides are capable of inhibiting virus infection but that their inhibitory activity is specific to the A/Puerto Rico/8/34 (H1N1) strain.[19] In the

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[a] Abbreviations: HA, hemagglutinin; NA, neuraminidase; Glc, glucose; Gal, galactose; Fuc, fucose; GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine; Neu5Ac, *N*-acetylneuraminic acid; Cer, ceramide; GM3, Neu5Acα2−3Galβ1−4Glcβ1−1′Cer; 6′GM3, Neu5Acα2−6Galβ1−4Glcβ1−1′Cer; SLX, sialyl Lewis^X; Neu5Ac-Lac, Neu5Acα2−3Galβ1−4Glc; WGA, wheat germ agglutinin; ELISA, enzyme-linked immunosorbent assay; SPR, surface plasmon resonance; ABC, avidin−biotin−peroxidase complex; K_d, dissociation constant; MDCK, Mardin−Darby canine kidney; IC_{50}, 50% inhibitory concentration; PDB, protein data bank; TBS, tris-buffered saline; BSA, bovine serum albumin; PBS, phosphate-buffered saline; RP-HPLC, reversed phase high-performance liquid chromatography; TU, transducing unit; pfu, plaque-forming unit; ΔA, absorbance change.

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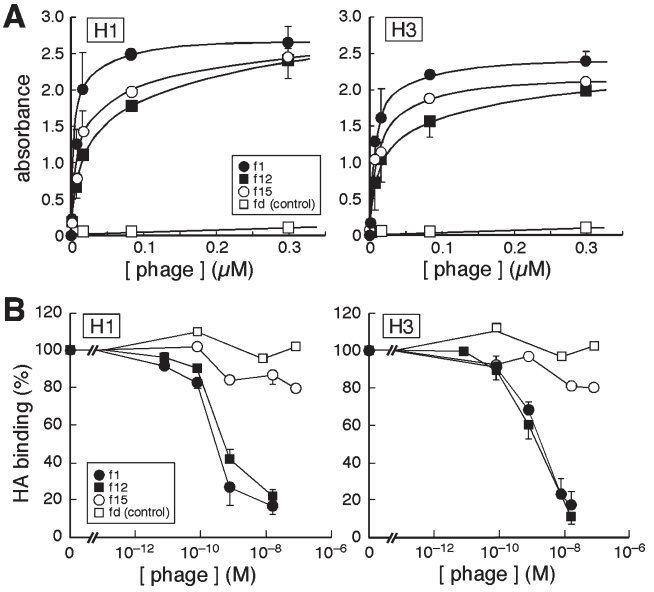

Figure 1. Binding activities of the phage clones of f1, f12, and f15. (A) Dependence of phage concentration on the amount of phage clones bound to H1 (left) and H3 (right). (B) The inhibition of the HA−GM3 interaction by phage clones. HA and phages were incubated in GM3-coated wells, and the amount of HA bound to GM3 was detected using an anti-HA antibody. The amounts of H1 (left) and H3 (right) bound to GM3 were plotted against phage concentration.

present study, we identified HA-binding peptides that are able to inhibit the internalization of H1N1 and H3N2 influenza virus into host cells. An HA-binding 15-mer peptide was considered to mimic sialic acid and was repeatedly optimized by subsequent affinity selections from sublibraries. We accordingly believe that HA-binding peptides are promising candidates as novel antiviral drugs.

Results

First Selection of HA-Binding Peptides from a Random Library. To obtain HA-binding peptides, affinity selection with HA was performed using a phage library containing a random pentadecapeptide sequence (see Supporting Information, Table S1). For affinity selection, a phage peptide library was interacted with HA immobilized on a solid support. Phages bound to the HA were collected by eluting with sialyl Lewis^X (SLX) or synthetic 6′GM3 (Neu5Acα2−6Galβ1−4Glcβ1−1′Cer), which can bind with the receptor-binding site of HA (Supporting Information, Table S2). From the first selection, 16 types of phage clone were identified from the 39 clones isolated (Supporting Information, Table S3). The four experimental conditions of the first selection are summarized in Table S2 (Supporting Information). It is noted that the f1 clone was found with the highest frequency (15 copies from 39 isolated clones) and isolated under all conditions (Supporting Information, Table S3).

The bindings of isolated phage clones against HAs were evaluated by phage enzyme-linked immunosorbent assay (ELISA) (Figure S1, Supporting Information), and five clones (f1, f9, f12, f15, and f16) were found to have a high affinity, with dissociation constant (K_d) values of 7.8−30 nM against H1 and H3 (Figure 1A and Table 1). To confirm that these phage clones are recognized by the Neu5Ac-binding site, a competitive assay was performed. The HA and phage clones were incubated with GM3 immobilized in 96-well plates, and the amount of HA bound to GM3 was determined by ELISA. Figure 1B shows that f1 and f12 phage clones strongly inhibited the binding of HAs to GM3. Furthermore, the binding affinities of f1 phage clone against Neu5Ac-binding proteins, wheat germ agglutinin (WGA), and anti-GM3 antibody were also determined. The K_d values of the f1 clone for WGA and anti-GM3 antibody were 16 and 150 nM, respectively (Table 1). The affinities of the f1 phage clone for these targets were slightly lower than that for HAs; however, these results suggest that the selected peptides bind to a receptor-binding site of HA by mimicking Neu5Ac-containing sugar chains.

Table 1. Binding Affinity of Phage Clones Determined by Phage ELISA

		K_d (nM)			
code	peptide sequence	H1[a]	H3[a]	WGA	anti-GM3 antibody
f1	ARLSPTMVHPNGAQP	7.8	8.6	16	150
f9	EPYGFIAFSRAAHSP	30	20	30	140
f12	GRPPDSVFRSRGWLS	11	8.9	20	76
f15	GRVPVFGLSPLFKVE	7.8	16	19	58
f16	IDIAFSSLALADISR	19	13	30	180
fd[b]		nb[c]	nb	nb	nb

[a] H1, A/Puerto Rico/8/34; H3, A/Wuhan/359/95. [b] Wild-type phage prepared from fUSE5 vector. [c] Not bound.

Secondary Selection of HA-Binding Peptides from Mutational Sublibraries. To improve the binding affinity of the f1 peptide sequence (ARLSPTMVHPNGAQP) for HAs, two mutational sublibraries with different substitution frequencies were prepared and used for a second selection (Supporting Information, Figure S2 and Table S1). The substitution frequencies of the mutational f1 libraries were 5.0 residues (library no. 1) and 1.25 residues (library no. 2) per 15 amino acids. In the second selection, H1 and H3 were alternately used (Supporting Information, Table S4) to collect phage clones that bind to both H1 and H3. This is because peptides having an affinity for a broad spectrum of virus strains would be useful as inhibitors. The second selection resulted in the identification of 17 types of phage clone from 58 clones (Supporting Information, Table S5). Despite the libraries having 5.0 (library no. 1) and 1.25 (library no. 2) substitutions, the average number of substitutions of the peptides selected was 3.0 ± 1.4. Phage ELISA showed that the binding affinities of many clones were improved compared to the original f1 clone (Figure 2A). The s2 phage clone showed the highest binding affinities, which were 2.6- and 3.6-fold higher than that of the f1 clone for H1 and H3, respectively. The s1 clone showed the highest frequency, but the binding affinity was lower than that of s2. A further four clones (s11, s12, s15, and s20) were also identified, the binding affinities of which against H1 were 2-fold higher than that of the f1 clone.

Binding of Synthetic 15-mer Peptides to HA. Binding of synthetic peptides to HA was determined by surface plasmon resonance (SPR) analysis. The 15-mer peptide amides of f1, s2, s11, and cp8 were chemically synthesized (Supporting Information, Table S6), and the binding of these peptides to HA immobilized on an SPR chip was measured. Figure 2B shows the typical sensorgrams of f1 and s2 peptides bound to H3 (A/Panama/2007/99) in HBS-EP buffer. The amounts of s2 peptide bound to H3 increased depending on the peptide concentration, and the s2 peptide showed the highest binding affinity among these peptides (Figure 2C,D). The order of

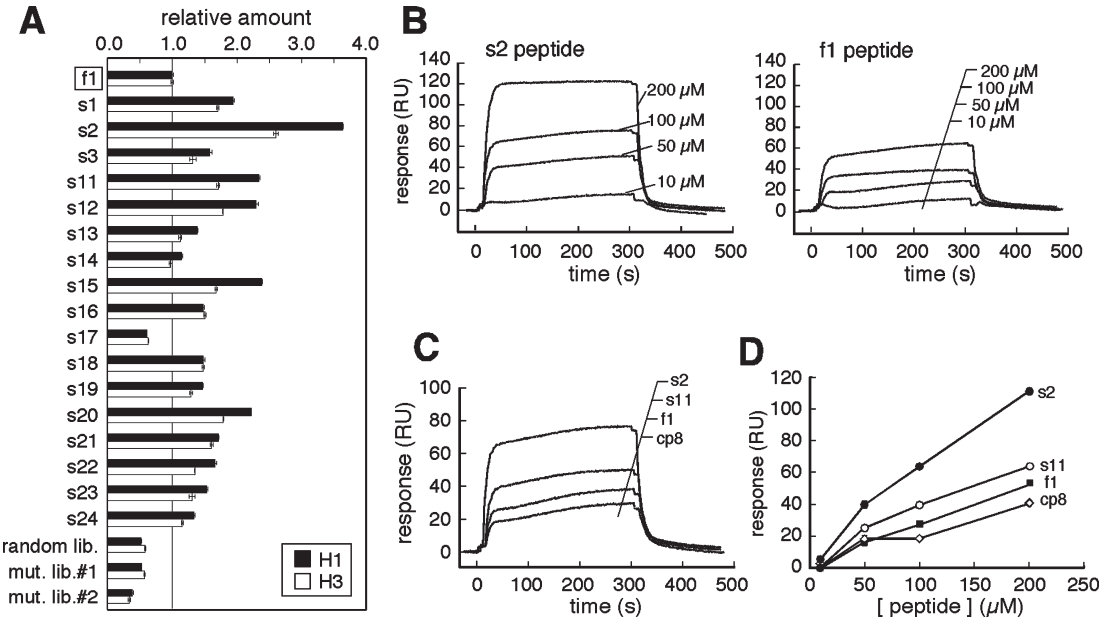

Figure 2. Binding activity of peptides isolated from the second selection. (A) Binding of phage clones to HAs determined by phage ELISA. The amounts of phage clones are shown as ratios to the amount of f1 clone. [phage] = 1.0 nM. random lib, random library; mut. lib, mutational library; H1, A/New Caledonia/20/99; H3, A/Panama/2007/99. The data are average values ± the standard deviation (n = 3). (B) SPR sensorgrams for the binding of s2 (left) and f1 (right) peptides to H3 (A/Panama/2007/99) at 10, 50, 100, and 200 μM. Association (0−300 s) and dissociation (300−500 s) phases are shown. (C) SPR sensorgrams for the binding of s2, s11, f1, and cp8 peptides to H3 at 100 μM. The cp8 peptide, AEGDDPAKAAFDSLQ, was used as a negative control. (D) The amounts of s2, s11, f1, and cp8 peptides bound to H3 at various peptide concentrations determined by SPR analysis. The equilibrium resonance units at 300 s in the association phase were plotted. The data are average values ± the standard deviation (n = 3).

peptide binding affinity was s2 > s11 > f1, which is consistent with the results of phage ELISA (Figure 2A).

The binding of s2 peptide was further investigated using the avidin−biotin−peroxidase complex (ABC) method.[20] The amounts of biotinyl s2 peptide bound to HA were determined using peroxidase-conjugated avidin. The K_d values of the s2 bound to H1 (A/New Caledonia/20/99) and H3 (A/Wyoming/3/2003) were determined by Scatchard analysis (Supporting Information, Figure S3). The K_d values of s2 for H1 and H3 were 31 and 22 μM, respectively.

Alanine Scanning Mutagenesis of s2 Peptide. To identify amino acid residues essential for the binding of the s2 peptide to HAs, the binding of 13 mutants substituted with alanine was investigated using the ABC method. On the basis of the K_d values of s2, 20 μM of biotinyl peptides (Supporting Information, Table S6) was used for this assay. Figure 3 indicates that five mutants (R2, L3, R5, M7, and K11) exhibited a significant decrease in binding. These five residues were therefore assumed to be essential for the binding of HAs, and RLxRxMxxxK is considered to be an HA-binding motif. Furthermore, it is considered that the substitutions of P5R and N11K in f1 contributed significantly to the increase in binding affinity.

Tertiary Selection of HA-Binding Peptides from a Motif-Conserved Library. To mature the s2 peptide sequence (ARLPRTMVHPKPAQP), a motif-conserved sublibrary was prepared and used for a tertiary selection (Supporting Information, Table S1). In this library, the five amino acids in the HA-binding motif, RLxRxMxxxK, were highly conserved (1.0 mutation per five amino acids) and the other 10 amino acids were moderately randomized (3.0 mutations per 10 amino acids) (Supporting Information, Figure S4). The phages tightly bound to HAs were eluted with 20 mM SLX after pre-elution with 2 mM SLX (Supporting Information,

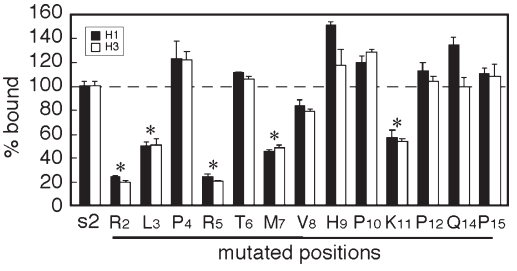

Figure 3. Effect of alanine substitutions on the binding of s2 peptides to HAs. The biotinyl peptides (s2 and s2 mutants, [biotinyl peptide] = 20 μM) were incubated with H1 (A/New Caledonia/20/99) and H3 (A/Wyoming/3/2003). The amounts of peptide (absorbance at 492 nm) bound to HAs were detected with peroxidase-conjugated avidin. An asterisk indicates statistical significance (p < 0.02). The data are average values ± standard deviation (n = 3).

Table S7). After two and four rounds of affinity selection, 58 types of phage clone were identified from 78 clones (Supporting Information, Table S8). Eleven phage clones that can bind to HAs with similar strength as well as the s2 clone were screened by phage ELISA (Supporting Information, Figure S5 and Table S8). The average substitution frequency of these peptides was 2.7 ± 1.2 residues; however, the binding motif was highly conserved. Although Pro4 and Pro12 were not responsible for the HA-binding (Figure 3), Pro4 was conserved, except in clone t8. A mutant series of the s2 peptide having a high binding affinity for HAs was thus identified.

Inhibition of Influenza Virus Infection by Pentadecapeptides. To investigate the inhibitory activity of the identified peptides, inhibition of the infection of Mardin−Darby canine kidney (MDCK) cells by influenza virus was determined by

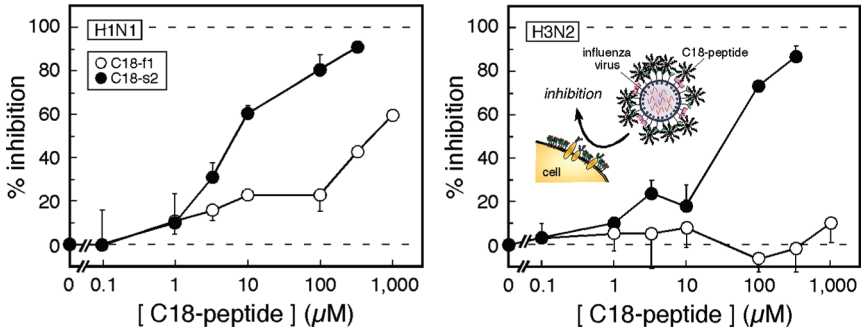

Figure 4. Inhibition of the influenza virus infection of MDCK cells by *N*-stearoyl peptides (C18-peptides). Prior to a plaque assay, C18-peptides (C18-f1 and C18-s2) were incubated with H1N1 (A/Puerto Rico/8/34) or H3N2 (A/Aichi/2/68) strain for 30 min. The mixture of C18-peptide and virus was incubated with MDCK cells for 30 min. After washing, the MDCK cells were grown for 2 days. The plaque number was counted and the percentage inhibition was plotted against the concentrations of C18-peptide. The data are average values ± standard deviation (n = 3).

Table 2. Inhibitory Activities of *N*-Stearoyl Peptides (C18-Ppeptides) against Influenza Virus Infection of MDCK Cells

clone code	structure[a]	H1N1[b] IC_{50} (μM)	H3N2[b] IC_{50} (μM)
C18-f1	$C_{17}H_{35}CO$-ARLSPTMVHPNGAQP-NH_2	530	> 1000
C18-s2	$C_{17}H_{35}CO$-ARLPRTMVHPKPAQP-NH_2	11	15
C18-t2	$C_{17}H_{35}CO$-ARLPRSMVHHKPAQP-NH_2	8.9	22
C18-t4	$C_{17}H_{35}CO$-ARLPRTLVHPKHAQA-NH_2	7.6	13.5
C18-t5	$C_{17}H_{35}CO$-ARLPRNMVHHKTAQP-NH_2	6.2	15
C18-t6	$C_{17}H_{35}CO$-ARLPRTMVHSKPAQP-NH_2	6.8	41
C18-t7	$C_{17}H_{35}CO$-TRLPRTMLHHKPAQA-NH_2	10	5.1
C18-t9	$C_{17}H_{35}CO$-ARLPRTMVHPKTAHQ-NH_2	8.0	13
C18-s2(1−8)	$C_{17}H_{35}CO$-ARLPRTMV-NH_2	3.0	4.2
C18-s2(5−11)	$C_{17}H_{35}CO$-RTMVHPK-NH_2	11	> 100
C18-s2(8−15)	$C_{17}H_{35}CO$-VHPKPAQP-NH_2	59	10
C18-s2(r1−8)	$C_{17}H_{35}CO$-VMTRPLRA-NH_2	> 100	> 100
C18-s2(1−5)	$C_{17}H_{35}CO$-ARLPR-NH_2	1.9	1.6

[a] Mutated residues are denoted by underline and bold. [b] H1N1, A/Puerto Rico/8/34; H3N2, A/Aichi/2/68.

plaque assay.[21] In accordance with Matsubara et al.,[19] *N*-stearoyl peptides (C18-peptide) were chemically prepared and used for this assay (Supporting Information, Table S6). The alkylation of a peptide induces its molecular assembly (formation of micelles and incorporation into liposomes), which is expected to enhance the inhibitory activity of the peptide through multivalency. Briefly, influenza virus A/Puerto Rico/8/34 (H1N1) or A/Aichi/2/68 (H3N2) (50−200 pfu) was mixed with C18-peptide (0.1−1000 μM) for 30 min at room temperature (preincubation), and the mixed solution was incubated for 30 min at 37 °C with MDCK cells. After washing to remove unbound virus and peptides, the MDCK cells were grown for 48 h. The numbers of plaques formed were counted, and the percentage inhibition was plotted against C18-peptide concentration (Figure 4). The C18-s2 peptide showed a higher inhibitory activity for both H1N1 and H3N2 strains than the C18-f1 peptide.

The IC_{50} values of C18-s2 for H1N1 and H3N2 were 11 and 15 μM, respectively, whereas those of C18-f1 were > 500 μM for both strains (Table 2). The inhibitory activities of six C18-peptides (t2, t4, t5, t6, t7, and t9) identified from the tertiary selection were also determined. The inhibitory activities of these six C18-peptides were similar to those of C18-s2 against both strains; the activities of these C18-peptides for H1N1 and H3N2 were 1.1−1.8 times and 0.4−2.9 times higher than that of C18-s2, respectively. The inhibitory activity of the s2 peptide (identified from the secondary selection) and the six peptides from the tertiary selection were also greater than that of the f1 peptide, consistent with the phage ELISA results (Figure 2A and Supporting Information, Figure S5). These results suggest that the inhibition of influenza virus infection is related to HA-binding activity.

Minimization of s2 Peptide. To determine the minimum sequence of s2 necessary for its activity, fragments of s2 were designed and the inhibitory activities of the corresponding C18-peptides were examined. The s2 peptide was initially separated into the following three fragments: N-terminal sequence (amino acids 1−8), middle sequence (5−11), and C-terminal sequence (8−15). The three C18-s2 fragments, C18-s2(1−8), C18-s2(5−11), and C18-s2(8−15), inhibited influenza virus infection with IC_{50} values of 3.0, 11, and 59 μM for H1N1, respectively (Table 2). The activity of the C18-s2(1−8) fragment (3.0 μM for H1N1) was slightly enhanced compared to that of the full-length C18-s2 (11 μM for H1N1). The reverse sequence of s2(1−8), namely C18-s2(r1−8), had no activity against either strain (> 100 μM), suggesting that the amino acid sequence of s2(1−8) was responsible for its activity. Furthermore, C18-s2(1−5) was found to be the minimum-sized fragment having inhibitory activities against both strains (1.9 and 1.6 μM for H1N1 and H3N2, respectively) (Table 2). The s2(1−5) sequence, ARLPR, was conserved among the five peptides that were selected from the tertiary selection, and this fragment contains three residues of the HA-binding motif. Therefore, it is reasonable to assume that the s2(1−5) fragment is a minimum sequence.

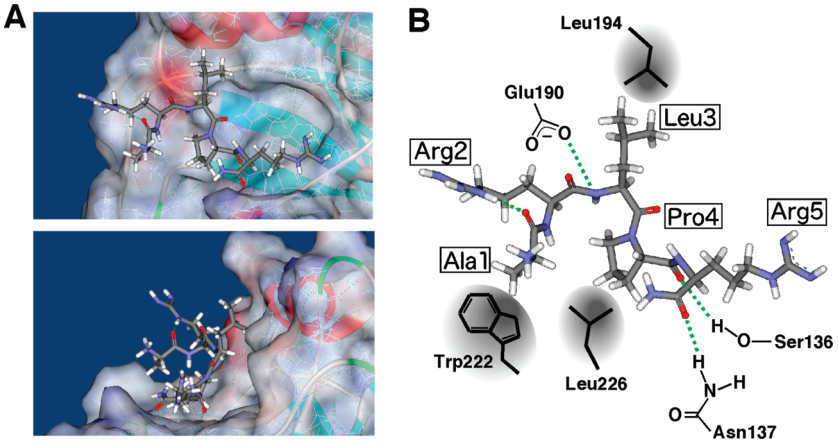

Figure 5. A proposed complex of s2(1−5) peptide with HA predicted by docking simulation. (A) A docking pose of the s2(1−5)−HA complex. The H3 protein structure of A/Aichi/2/68 (H3N2) was obtained from the PDB (entry 1HGG).[38] The s2(1−5) peptide is shown as stick models. Top view (upper) and side view (lower). (B) Schematic diagram of the binding site of HA. Three potential hydrogen bonds (green dotted lines) between H3 and s2(1−5) are proposed (Glu190−Leu3, Ser136−Pro4, and Asn137−Arg5), which are similar to those of the H3−Neu5Ac complex.[38] An intrapeptide hydrogen bond, Ala1−Arg2, is also proposed.

Possible Interaction between Peptide and Receptor-Binding Site of HA. On the basis of the plaque assay, we identified the five residues at the N terminus of s2 as probably having the most significant influence on the interaction with HA. To demonstrate the interaction between the s2(1−5) sequence and the receptor-binding site of HA, a docking simulation was performed using the LigandFit program in Discovery Studio 1.2.[22,23] The HA structure used was that of 1HGG (H3N2) obtained from the Protein Data Bank (PDB), and a naturally occurring receptor, Neu5Ac-Lac (Neu5Acα2−3Galβ1−4Glc), was used as control. Figure 5 shows a possible interaction of the HA−s2(1−5) complex, where s2(1−5) is recognized by the receptor-binding site of HA. Three side chains of HA, at Ser136, Asn137, and Glu190, were able to interact with the main-chain carbonyl groups (Pro4 and Arg5) and main-chain amino group (Leu3) of s2(1−5). It has been reported that the three side-chains of HA form hydrogen bonds with sialic acid (2-carboxylate and 9-OH).[24] Intramolecular hydrogen bonding between a guanidium group of Arg2 and a main-chain Ala1 is accordingly proposed. Furthermore, two hydrophobic Leu residues (Leu194 and Leu226) and an aromatic Trp residue (Trp222) were close to Leu3, Pro4, and Ala1, respectively, which suggests that hydrophobic interactions occur. The docking results supported the results of alanine scanning; three amino acids at Arg2, Leu3, and Arg5 are responsible for the HA binding and a hydrophobic Pro4 can be replaced by alanine (Figure 3). Under the same docking conditions, the Ligscore[22,23] of s2(1−5) (Ligscore = 5.7) was consistent with that of Neu5Ac-Lac (Ligscore = 6.0).

Discussion

An initial attachment of HA to sialylglycoconjugates on the host cell surface and a membrane fusion of HA matured by protease digestion are required for virus infection. The mechanism of neutralization depends on the HA site recognized by the HA-specific molecule; for example, antibodies against sequences around the receptor-binding site of HA inhibit virus cell attachment,[25] whereas antibodies against a hinge region[25] and a stem region[26] of HA inhibit membrane fusion. In the present study, to inhibit virus attachment to cells, we selected peptides that bind to the receptor-binding site of HA from a random peptide library using phage display technology. Replacement of a sugar by peptides is a useful strategy for drug discovery. For example, sugar-replica peptides that had been selected from a phage-displayed library inhibited the metastasis of tumor cells and exhibited an immunological response.[27] The peptide identified at the first selection from a phage library was further developed and matured by secondary and tertiary selections in an effort to increase HA-binding activity (Figure 2). On the basis of the results of the plaque assay, the binding affinity of peptides for HA was in good agreement with their inhibitory activity (Table 2). These results indicate that the HA-binding peptides are promising candidates for antiviral drugs as well as anti-HA antibodies.

In general random library selections, identification of target-specific peptides and antibodies is required. However, when dealing with many strains of virus, "selectivity" is an unfavorable characteristics for clinical use. In the present study, we attempted to identify peptides that bind to the receptor-binding sites of the H1 and H3 types of HA. Sialic acid is a general receptor against human influenza virus; therefore, the selected peptides play a role similar to that of a sialic acid and thus can be considered "sialic acid mimics." By using the H1 and H3 types of HA, we successfully identified the peptides that have inhibitory activities against the H1N1 and H3N2 strains of virus. The peptides showed higher affinity for H1N1 and H3N2 than anti-GM3 antibody and WGA (Table 1) and inhibited the interaction between HA and sialic acid-containing the glycolipid GM3 (Figure 1B). On the basis of these results, we consider that these peptides bind with the target receptor-binding site of HA. A docking simulation of the complex of s2(1−5) with HA (Figure 5) suggests that s2(1−5) is recognized by HA and involves three hydrogen bonds and hydrophobic interactions. To inhibit the attachment of virus to a cell, many types of sialic acid-containing lipids and polymers have been developed.[11] Although these polymers have been demonstrated to inhibit hemagglutination, reports on the inhibition of virus infection are limited.[8] Sialic acid-containing dendritic polymers inhibited H2N2 virus infection at a sialic acid concentration of 2.5 mM.[12] Guo et al. designed *C*-3-modified sialyl phosphatidylethanolamine derivatives to inhibit the hydrolysis by neuraminidase and demonstrated that the cytopathic effect of H3N2 infection was decreased ($TCID_{50}$ of 5.6 μM).[10]

We serially repeated affinity selection to identify superior HA-binding peptides. This is because when using a random library containing over seven residues ($20^7 = 1.3 \times 10^9$), it is not possible to cover the theoretical diversity of sequence space using phage-display technology.[28] However, the binding activities of the HA-binding peptides were saturated before the total theoretical diversity of the libraries used was reached. The binding affinities of the phage clones identified by tertiary selection exhibited no significant improvement compared to the phage clones of the s2 peptide (Supporting Information, Figure S5). Here, the diversity of the libraries used (primary library and secondary sublibrary) was 3.0×10^{13} (Supporting Information, Table S1), whereas the theoretical diversity of the pentadecapeptide library is 3.3×10^{19}. Of course, we cannot exclude the possibility of the peptide sequence falling into a local minimum of sequence space. However, the findings of the present study suggest that it is not always necessary to prepare a primary library (and sublibraries) with theoretical diversity.

Table 2 indicates that the shorter sequences, such as those containing five amino acids, s2(1−5), and eight amino acids, s2(1−8), at the N terminus of the s2 peptide also have inhibitory activity. This is reasonable because the four residues (Arg2, Leu3, Arg5, and Met7) at the N-terminus are responsible for HA-binding. The inhibitory activities of these shorter peptides (IC_{50} = 1.9−3.0 μM for H1N1) were slightly improved compared to the full-length s2 peptide (11 μM for H1N1) (Table 2). Significantly, the five amino acids at the N-terminus of the s2 peptide were highly conserved in the tertiary selection (Supporting Information, Table S8). Therefore, we determined the N-terminal five amino acids, s2(1−5), to be the minimum inhibitory sequence of the s2 peptide.

Conclusion

In the quest to develop antiviral drugs with a broad spectrum of inhibitory activity, the sialic acid-binding site in HA, which is highly conserved among many virus strains, is one of the potential targets for affinity selection. We repeatedly performed affinity selection and identified HA-binding peptides that mimicked sialic acid. Alkylation of the peptides was required for an improvement in their inhibitory activity against influenza virus infection. Eventually, we identified the pentapeptide s2(1−5) as having the highest inhibitory activity. Antiviral drugs based on such peptides could be used against any sugar-related virus infection. Since the mass production of peptides has already been established, peptide drugs can be made available for clinical use.[29] To counter the appearance of new influenza viruses and the emergence of resistant viral strains, anti-influenza drugs having different inhibitory mechanisms should be developed. HA-binding peptides are expected to be one type of novel anti-influenza drug having a mechanism of action that differs from that of neuraminidase inhibitors.

Methods

Materials. Neu5Acα2−3Galβ1−4Glcβ1−1′Cer (GM3) and sialyl Lewis^X tetrasaccharide (SLX), Neu5Acα2−3Galα1−4-(L-Fucα1−3)GlcNAc, were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). A synthetic 6′GM3 was prepared as previously described.[19]

A phage-displayed random pentadecapeptide library and *Escherichia coli* K91Kan were obtained as previously described.[30] The M13KE phage vector and *E. coli* ER2838 were purchased from New England BioLabs, Inc. (Beverly, MA). Wild-type phages (fd and M13) were prepared from fUSE5[31] and M13KE phage vectors,[32] respectively.

The HAs of influenza virus, A/Puerto Rico/8/34 (H1N1), A/New Caledonia/20/99 (H1N1), A/Wuhan/359/95 (H3N2), A/Panama/2007/99 (H3N2), and A/Wyoming/3/2003 (H3N2), were kindly provided by Dr. Yujiro Suzuki (the Kitasato Institute, Japan).[33]

Affinity Selection of HA-Binding Peptides. The different experimental conditions are summarized in Supporting Information, Tables S2, S4, and S7. Typically, HA-coated wells (Supporting Information) were filled with 100 μL of phage solution containing 1×10^{10}−4×10^{11} transducing units (TU) or 1.5×10^8 plaque-forming units (pfu) in tris-buffered saline (TBS). After 2 h, the wells were washed five times with TBS. If required, the wells were filled with eluent solution at a low concentration (2 μM to 2 mM), incubated for 1 h, and washed off (pre-elution). The phages bound tightly to HA were collected by elution with eluent solution at high concentration (2−20 mM). Phages in the eluate were counted by titering in order to estimate the number of phages collected (output phage). The output phages were amplified, purified with polyethyleneglycol/NaCl, and subjected to the next round. This process was repeated 4−6 times, resulting in the enrichment of the HA-binding phages. The individual phage clones were amplified and their phage genomes (single-stranded DNA) were used as a template for sequencing in order to deduce the amino acid sequence, as described previously.[34]

Phage ELISA. HA-coated wells were blocked with 1% bovine serum albumin (BSA)/TBS in advance and washed three times with TBS. The wells were filled with 50 μL of phage in TBS and incubated for 2 h. The wells were washed five times with TBST (TBS containing 5% Tween20) and then blocked with 150 μL of 5% BSA/TBS. After washing, the phages bound to HA were incubated with a 1:1000 (v/v) dilution of antifd bacteriophage antibody (Sigma-Aldrich) for 1 h, then the primary antibody was labeled with a 1:1000 (v/v) dilution of peroxidase-conjugated antirabbit IgG antibody (Sigma-Aldrich) for 1 h. The color was developed using *o*-phenylenediamine, and the change in absorbance (ΔA) at 492 nm was determined. Each experiment was performed in triplicate. Wild-type (fd and M13) phage and primary libraries (see Supporting Information, Table S1) were used as controls.

The concentration of phage solution was estimated from the absorbance at 260 nm, e.g., when $A_{260} = 1$, [phage] = 1.1×10^{-8} M.[31] The ΔA value was plotted against the phage concentration (Figure 1A). To obtain the K_d value, ΔA was plotted against [phage], and simple saturation curves indicating Langmuir adsorption were obtained (data not shown). Reciprocal plots between [phage]/ΔA and ΔA gave straight lines according to the following equation:

$$[\text{phage}]/\Delta A = [\text{phage}]/\Delta A_{\max} + K_d/\Delta A_{\max}$$

where $\Delta A_{\max}$ is the maximum amount of phage that can bind, and K_d is the dissociation constant. $\Delta A_{\max}$ and K_d were calculated from the slope and intercept, respectively, of the linear relationship indicated by the equation.

The amounts of phages bound to WGA (or anti-GM3 antibody)-coated wells were also determined by phage ELISA, and the K_d value was calculated from these amounts.

Inhibition of GM3−HA Interaction by Phage Clone. The amount of HA bound to GM3-coated plates was determined by ELISA.[35] A methanol solution (10 μL) of GM3 (0.85 mg/mL) and a $CHCl_3$/hexane/methanol solution (50 μL) of 0.08% polyisobutylmethacrylate (1:5.25:25, v/v/v) were added to 96-well microplates. The organic solvents were evaporated using a blowdryer, and the wells were blocked with 50 μL of 1% BSA/TBS at 4 °C overnight. Then 50 μL of HA solution (4.4 μg/mL, 60 nM) and 50 μL of phage solution were mixed in advance.

The wells were washed with TBS, and the mixed solution was added to the wells (100 μL/well). After incubation for 3 h, the wells were washed and blocked again with 1% BSA/TBS. After washing with TBS, the HA bound to GM3 was incubated with a 1:1000 (v/v) dilution of anti-influenza virus type A (hemagglutinin) antibody (HyTest, Ltd.) in 1% BSA/TBS for 1 h, and then the primary antibody was labeled with a 1:1000 (v/v) dilution of peroxidase-conjugated antimouse IgG antibody (Chemicon) for 1 h. Each experiment was performed in triplicate. Wild-type fd phage was used as a control. The amounts of HA bound to GM3-coated wells were detected using a secondary antibody, and the ratio of these amounts to the amounts without inhibitor (phage clone) was plotted (Figure 1B).

Synthetic Peptides. All peptides were synthesized using standard 9-fluorenylmethoxycarbonyl (Fmoc) chemistry with C-terminal amidation.[19] For modification of the biotinyl and stearoyl groups, Fmoc-Lys(biotin)-OH and stearic acid ($C_{17}H_{35}COOH$) were linked to the C-terminal and N-terminal of the peptide, respectively. All synthetic peptide amides were purified by reversed phase high-performance liquid chromatography (RP-HPLC) and lyophilized. The purity (> 95%) and expected structure were verified by RP-HPLC and matrix-assisted laser desorption ionization/time-of-flight mass spectrometry, respectively. A list of the synthetic peptides used in the present study is shown in Supporting Information, Table S6.

The ABC Method for Peptide Binding Assay and Alanine Scanning. HA-coated wells were washed with phosphate-buffered saline (PBS), filled with 100 μL of HA solution (70 μg/mL), and incubated at 4 °C overnight. After blocking with 1% BSA/PBS, 100 μL of biotinyl peptide (5, 10, 20, 50, 75, 100, 200, and 400 μM) was added to the wells, and the plates were incubated for 2 h. After five washes with PBS, 100 μL of a 1:5000 (v/v) dilution of peroxidase-conjugated streptavidin in 1% BSA/PBS was added to each well, followed by an incubation of 1 h. After five washes with PBS, the color was developed using *o*-phenylenediamine, and ΔA at 492 nm was measured. Each experiment was performed in triplicate.

Binding affinity was calculated from a Scatchard analysis of equilibrium binding using the equation:

$$\Delta A/[\text{peptide}] = \Delta A_{\max}/K_d - \Delta A/K_d$$

where [peptide], $\Delta A_{\max}$, and K_d are the concentration of peptide, the maximum absorbance, and dissociation constant, respectively. The ΔA values were plotted in the form ΔA/[peptide] versus ΔA, and K_d and $\Delta A_{\max}$ were calculated from the slope and intercept, respectively, of the linear relationship. The peptide concentration range (20−200 μM) in which the peptide amounts were analyzed as a single interaction by Scatchard plot was used to determine K_d values.

Construction of Mutational Libraries. The f1 phage mutational libraries were constructed as describe previously with minor modifications.[31,36] A phage vector containing a double-stranded replicative form of the f1 clone was amplified with K91Kan and purified using a QIAfilter Plasmid Maxi kit (QIAGEN). The phage vector was digested with *Eag*I and *Sfi*I prior to use. Two types of insert duplexes with different substitution frequencies were prepared as described in Supporting Information, Figure S2. The insert duplex was ligated into the predigested phage vector, and the product was electroporated into *E. coli* K91Kan. The cells were grown in NZY medium to prepare the phages, and the phages were isolated and checked by DNA sequencing. The diversities of libraries no. 1 and no. 2 were 1.2×10^5 TU (5.0 mutations per 15 amino acids) and 5.0×10^4 TU (1.25 mutations), respectively (Supporting Information, Table S1).

Construction of a Motif-Conserved Library. A motif-conserved library of the s2 phage was constructed as previously described with minor modifications.[32] A phage vector M13KE was digested with *Eag*I and *Acc65*I prior to use. The insert duplex (Supporting Information, Figure S4) was ligated into the predigested phage vector, and the product was electroporated into *E. coli* ER2738. The cells were grown in Luria−Bertani medium to prepare the phages, which were subsequently isolated and checked by DNA sequencing. The diversity of the library was 7.4×10^7 pfu (1.0 mutations in the RLxRxMxxxK motif and 3.0 mutations in the other 10 amino acids) (Supporting Information, Table S1).

SPR Analysis. The affinity of synthetic peptides for HA was determined by SPR using a Biacore X biosensor system (Biacore International). The H3 HA (500 μg/mL in acetate buffer, pH 4.7) was immobilized on a CM5 sensor chip (code BR-1000−14) using the EDC/NHS activation procedure according to the manufacturer's instruction manual, and the response was approximately 11000 RU. HBS-EP buffer (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, 0.005% Tween 20, pH 7.4) was used as a running buffer. After the immobilization of HA, HBS-EP buffer was injected over the chip at a flow rate of 5 μL/min for 60 min. A solution of peptide in HBS-EP buffer (10, 50, 100, and 200 μM) was injected at 25 °C with a flow rate of 1 μL/min for 5 min (association phase), and then the buffer alone was injected for 3 min (dissociation phase).

Preparation and Inactivation of Viruses. Influenza A/Puerto Rico/8/34 (H1N1) and A/Aichi/2/68 (H3N2) viruses were grown for 48 h at 34 °C in allantoic sacs of 11-day-old embryonated eggs.[37] To be safe to use in experiments, viruses were inactivated by ultraviolet (UV) radiation. To this end, viruses were exposed on ice at a distance of 10 cm to the UV radiation from a germicidal lamp for 5 min. The inactivation was confirmed by plaque assays using MDCK cells.

Plaque Assay. To determine the inhibitory activity of peptides, the infection of MDCK cells by the influenza virus was evaluated using a plaque assay.[19] Briefly, the peptide solution (2 mM) was serially diluted with PBS (0.3 mL each in a 1.5 mL tube) and mixed with 0.3 mL of influenza A/Puerto Rico/8/34 (H1N1) or A/Aichi/2/68 (H3N2) virus solution containing 50−200 pfu. After 30 min at room temperature, the mixture was incubated with MDCK monolayer for 30 min at 37 °C under 5% CO_2 (0.2 mL/well, $n = 3$). After washing, the MDCK cells were incubated for 2 days and the number of plaques was counted using crystal violet. The percentage of inhibition was calculated using the equation $100(1 - (N/N_0))$, where N and N_0 are the number of plaques in the presence and in the absence of inhibitor, respectively. The IC_{50} value (50% inhibitory concentration) of the peptide was obtained as described previously.[19]

Docking Study. Docking simulation was performed using the LigandFit program in Discovery Studio (DS) 1.2 (Accelrys, Inc., CA).[22] Prior to docking, the protein and peptide structures were determined using DS 1.1. The crystal structure of a target HA (H3N2) (PDB entry 1HGG) was retrieved from PDB.[38] All bound water and ligand molecules were removed from the proteins and then hydrogen atoms were added. To generate an initial peptide structure, a s2(1−5) peptide was constructed using the Biopolymer program. Minimization and molecular dynamics simulations of the peptide were performed using the CHARMm force field. In the LigandFit program using DS 1.2, the target HA and s2(1−5) were defined as protein and ligand, respectively, and then the sugar-binding site was defined as the binding site. Peptide ligand conformations were generated using Monte Carlo simulations (15000 trials). The docking poses were evaluated with Dockscore and Ludi3, and K_d values were obtained using the following equation; Ludi score = 100 log $(1/K_d)$.

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Supporting Information Available: Preparation of HA-coated wells, list of phage libraries, experimental conditions and results of the first selection with HAs, phage clones identified from the first selection with HAs, experimental conditions and results of the second selection with HAs, phage clones identified from the second selection with HAs, synthetic peptides used in the present study, experimental conditions and results of the third selection with HAs, phage clones identified from the third selection with HAs, the binding of phage clones to HAs isolated from the first affinity selection, construction of phage libraries using the fUSE5 vector system, HA-binding of s2 peptide determined using the ABC method, construction of a phage library using the M13KE vector system, and binding of phage clones isolated from the third selection determined by ELISA. This material is available free of charge via the Internet at http://pubs.acs.org.

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