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Heptapeptide ligands against receptor-binding sites of influenza hemagglutinin toward anti-influenza therapy

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ABSTRACT

The initial attachment of influenza virus to cells is the binding of hemagglutinin (HA) to the sialyloligosaccharide receptor; therefore, the small molecules that inhibit the sugar–protein interaction are promising as HA inhibitors to prevent the infection. We herein demonstrate that sialic acid-mimic heptapeptides are identified through a selection from a primary library against influenza virus HA. In order to obtain lead peptides, an affinity selection from a phage-displayed random heptapeptide library was performed with the HAs of the H1 and H3 strains, and two kinds of the HA-binding peptides were identified. The binding of the peptides to HAs was inhibited in the presence of sialic acid, and plaque assays indicated that the corresponding *N*-stearoyl peptide strongly inhibited infections by the A/Aichi/2/68 (H3N2) strain of the virus. Alanine scanning of the peptides indicated that arginine and proline were responsible for binding. The affinities of several mutant peptides with single-amino-acid substitutions against H3 HA were determined, and corresponding docking studies were performed. A Spearman analysis revealed a correlation between the affinity of the peptides and the docking study. These results provide a practicable method to design of peptide-based HA inhibitors that are promising as anti-influenza drugs.

1. Introduction

A large number of people are infected by seasonal influenza every year, and an influenza pandemic occurs every few decades, for example, the A (H1N1) 2009 pandemic.[1] Influenza virus infects and proliferates in the upper respiratory tract epithelial cells. While healthy individuals typically recover within a week of the onset of influenza symptoms, high risk individuals, including very young children and the elderly, may develop more severe symptoms or die.[2] Although influenza can be prevented by vaccinations, their production requires more than 6 months.[3] A sufficient amount of time may not be available to produce vaccines against pandemic viruses before they spread to other countries. In the 2009 pandemic, the Director-General of the World Health Organization (WHO) increased the level of the influenza pandemic alert to phase 5, indicating widespread human infection, within one month of the first report of an influenza-like illness (April, 2009).[4] Antiviral drugs were previously found to be more effective than vaccines for protecting against the spread of influenza in a pandemic.[2]

The first anti-influenza drugs were the M2 ion channel inhibitors, amantadine and rimantadine. These have now been replaced by neuraminidase inhibitors (NAI) because all types of circulating viral strains have developed resistance to amantadine and rimantadine.[5,6] Several NAIs such as oseltamivir,[7] zanamivir,[8] peramivir,[9] and laninamivir,[10] are now administered for clinical treatment. NA is one of the main glycoproteins on the influenza virus surface, and this enzyme plays an important role in the budding step.[11] NA releases progeny viral particles from cells by digesting sialic acid on the cell surface, and NAIs inhibit the budding step. However, influenza viruses that are resistance to NAIs started to be isolated in 1996,[12,13] and the crystal structures of the NA mutants were also solved.[14] Several types of substitutions were identified in the catalytic site and/or framework of NA, and new antiviral drugs should be developed based on these findings.[15,16]

Random screening and mechanism-based drug designs against influenza have been performed since the 1960s, and structure-based drug design was initiated in 1983 based on the crystal structure of NA.[17] The antiviral drug zanamivir, 4-guanidino-Neu5Ac2en, was produced for the first time in 1993.[18] This compound is a transition-state analog of NA, and is recognized by the sialic acid-binding site of NA. The NA–zanamivir complex was estimated by the GRID program,[19] with structure-based design becoming an important part of the strategy of molecular design in addition to

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conventional synthetic chemistry techniques. NA inhibitors are chemically synthesized by several steps;[20,21] however, numerous candidates are needed in order to develop new antiviral drugs. If the burdens (cost and time) associated with the preparation of candidates are reduced, the development of these drugs will become easier.

The technology involved in preparing a diverse library of peptides has been improved in the past two decades;[22] therefore, peptide-based drugs are increasingly expected to be used for clinical applications.[23] We have focused on the inhibitory activity of carbohydrate-mimetic peptide ligands to design anti-influenza drugs,[24] with the target protein being the hemagglutinin (HA) of type-A influenza virus instead of NAIs.[25] HA in the viral membrane recognizes sialylglycoconjugate receptors on the host cell surface in the initial step of the infection process. We previously identified pentadecapeptides that mimic sialic acid and bind to the receptor-binding sites of multiple HAs through multiple serial selections.[26] Since these peptides are able to bind to multiple HAs, a broad spectrum of inhibitory activities was expected. The HA-binding peptides were linked with stearic acid[26] or the carbosilane dendrimer core scaffold,[27] and these modified peptides inhibited the infection of cells by the influenza virus. These results suggest that HA-binding peptides (sialic acid-mimic peptides) were the preferred candidates for anti-influenza drugs.

The findings of a previously conducted docking simulation suggested that several residues are well enough to be recognized by receptor-binding sites instead of sialic acid.[26] In the present study, a lead heptapeptide ligand was identified from a phage-displayed random peptide library, and the binding of the peptides for HA was determined using the avidin–biotin-peroxidase complex method. A competitive binding assay suggested that the peptides bind to the receptor-binding site and mimic sialylglycoconjugates, and the corresponding *N*-stearoyl derivative exhibited strong inhibitory activity against the H3N2 virus. The binding affinities of mutant peptides with single amino acid substitutions were determined for H3 HA, and the rank of binding was correlated with that predicted by the docking simulation using the three-dimensional structure of the target protein. The properties of the receptor-mimic peptide shown in the present study are considered to be useful for designing the strategies of anti-viral drugs.

2. Results and discussion

2.1. Affinity selection of HA-binding peptides from a randomized heptapeptide library

We previously identified HA-binding pentadecapeptides and demonstrated that five- and eight-amino acid peptides at the N-terminals of these pentadecapeptides exhibited high inhibitory activity against influenza infection.[26] Therefore, in order to obtain HA-binding peptides in the present study, a heptapeptide (7-mer) library was chosen as a primary library and phage display selection was performed against influenza virus HAs.[26] Prior to the affinity selection, the H1 and H3 HAs used were extracted from influenza viruses A/New Caledonia/20/99 (H1N1) and A/Panama/2007/99 (H3N2).[28] Briefly, a phage library was interacted with HA immobilized on a 96-well plate and the phages that bound to HA were collected by eluting with the ganglioside GM3, Neu5Acα2–3Galβ1–4Glcβ1–1′ceramide. GM3 was used to obtain phages that bound with the receptor-binding site of HA because the sialylgalactose moiety of GM3 is one of the receptors of HA. In addition, H1 and H3 HAs were alternately used in the selection to obtain peptides that bind to multiple HAs. The phages collected were amplified by an infection with *Escherichia coli*, and this procedure was repeated six times. The relative yield of collected phages

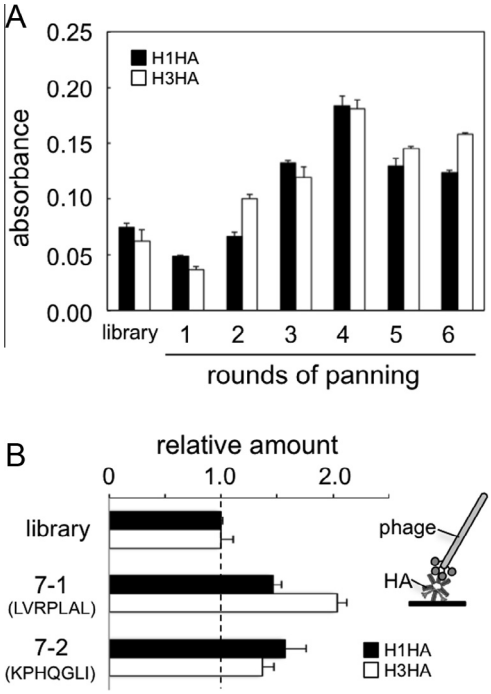

Figure 1. Binding of phages to HAs determined by phage ELISA. (A) Dependence of rounds on the amount of phages that bound to H1 and H3 HAs. The enrichment of HA-binding phages was observed ([phage] = 1.0 nM). (B) The amounts of phage clones are shown as ratios to the amount of the primary library ([phage] = 5 nM). H1 and H3 HAs were obtained from A/New Caledonia/20/99 (H1N1) and A/Panama/2007/99 (H3N2), respectively. Data are average values ± the standard deviation (n = 3).

increased from 0.0029% to 0.18% from the first to the fourth round of affinity selection (see Supporting information, Table S1 and Fig. S1A, online).

The enrichment of HA-binding phages was confirmed by an enzyme-linked immunosorbent assay (ELISA). The phage ELISA results indicated that the amount of fourth round phages that bound to both H1 and H3 HAs was the highest among the six rounds (Fig. 1A). Two types of individual phage clones, LVRPLAL (7-1) and KPHQGLI (7-2), were identified from the 16 clones isolated in the fourth round phages. These peptides have a cationic amino acid (Arg or Lys), Pro, and Leu-containing hydrophobic C-terminal (RPxxL and KPxxxL). This composition was similar to that of the s2(1–5) peptide (Ala-Arg-Leu-Pro-Arg) identified previously.[26] Although these peptides are considered to mimic sialic acid-containing oligosaccharides, cationic amino acids having the opposite charge of sialic acid were contained.

The phage clones 7-1 and 7-2 were detected at frequencies of 15/16 (15 copies from 16 isolated clones) and 1/16, respectively. The binding of isolated phage clones to HAs was evaluated by phage ELISA. The amounts of the phage clones bound to HAs increased in a phage concentration-dependent manner (Fig. S1B), with phage clone 7-1 showing higher affinities for H1 and H3 HAs than 7-2 (Fig. 1B). Two kinds of HA-binding peptides were successfully obtained, and further investigations were conducted using the 7-1 peptide sequence. The amounts of phage clone 7-1 at 5 nM were 1.5- and 2.0-fold higher than those of the library for H1 and H3, respectively.

2.2. Binding of synthetic 7-mer peptides to HA

The binding of the 7-1 peptide to HA was detected using the avidin–biotin-peroxidase complex (ABC) system.[29] The biotinyl