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Chemically Programmed Antibody is a Long-lasting and Potent Inhibitor of Influenza Neuraminidase

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influenza; antibodies; neuraminidase inhibitor; click chemistry; conjugation

Globalization makes reoccurring influenza pandemics a probable health care concern for the foreseeable future.^[1] Although vaccination plays a primary role in the prevention of influenza epidemics and pandemics, vaccines must be designed and produced in advance of the influenza season and cannot be supplied on-demand in response to virus mutation.^[2] Thus, efficient anti-influenza drugs are as important as vaccination in epidemic management.^[3] Currently, the neuraminidase inhibitors zanamivir (1)^[4] and oseltamivir (2)^[5] are the mainstay drugs for treatment of infected patients (Figure 1). Each must be administered twice-daily due to their rapid excretion from the body. Therefore, long-lasting and potent anti-influenza drugs are highly attractive alternatives for treatment of influenza infection as well as for prophylaxis.^[6]

We have developed a strategy to prepare chemically programmed antibodies that have the long half-life and effector function of the antibody and therapeutic activity of the conjugated small-molecule drug, peptide, or oligonucleotide (Figure 2).^[7] The agent to be conjugated to the antibody is first functionalized with a β -lactam and then is selectively reacted with the low pKa lysine residues key to the catalytic activity of aldolase monoclonal antibody (mAb) 38C2 to form an amide bond. As part of a project aimed at developing novel anti-influenza agents and chemically programmed vaccine strategies, we have chemically programmed an antibody with a small molecule enzyme inhibitor that targets neuraminidase. Our goal was to create a novel potent neuraminidase inhibitor that maintains long-term systemic exposure with the potential for enhanced activity through antibody associated effector function and valency. To date, chemically programmed antibodies have not utilized enzyme inhibitors as specificity programming agents. Successful recruitment of enzyme inhibitors as programming agents would make a wide-range of enzyme inhibitors effective new therapeutic tools for targeting an immune response.

Influenza virions present two virus-encoded glycoproteins on their surface that are the targets of vaccines and small molecule drugs. These are hemagglutinin, which is responsible

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for binding sialic acid and fusion to the host cell, and neuraminidase, which is a glycosidase responsible for de novo virion release and virus spread within the host organism.^[8] Current vaccines rely on inducing potent antibody responses against particular hemagglutinin variants,^[9] whereas small molecule drugs aim to inhibit the enzymatic activity of neuraminidase. Co-targeting of the immune response to both of these proteins might have favourable prophylactic and therapeutic effects, however, antibody targeting of neuraminidase through vaccination has not proven to be highly effective perhaps due to the antigenic variation of the surface of this enzyme.^[10] In general, the active sites of enzymes tend to tolerate fewer mutations since catalytic activity must be retained, and they therefore present more stable targets as compared to the surface residues of an enzyme. Antibodies, however, are sterically constrained against reaching into the active sites of most enzymes, and to the best of our knowledge, antibodies that make direct contact with the catalytic residues of an enzyme are not known. Antibodies that block access to active sites are known,^[11] however, such epitopes are more tolerant to mutations than the conserved catalytic residues of the enzyme. In order to explore the potential of antibodies that directly engage the conserved catalytic mechanism of an therapeutically significant enzyme, we selected zanamivir as the chemical programming agent because it maintains antiviral activity against oseltamivir-resistant mutant viruses.^[12] Several groups have reported that dimeric, trimeric, tetrameric, and polymeric zanamivir derivatives linked through the C-7 position have long-acting and strong antiviral activities (Figure 3).^[13] Moreover, C-7 alkyl modified analogues of zanamivir reported by Honda et al. retained their inhibitory activities against neuraminidase.^[14] In the X-ray structure of a complex of zanamivir and neuraminidase, the 7-hydroxy group of zanamivir is directed toward solvent (Figure 4).^[13d,15] Thus, we selected the 7-hydroxy group as the point for attachment of a β -lactam group and designed chemical programming agents **3a** and **3b** (Figure 5). The linker length is nearly 40 Å. Even though the lysine of mAb 38C2, LysH93, is located at the bottom of a narrow 11 Å-deep pocket, this linker should present the zanamivir targeting module in a manner that is unhindered by the antibody and accessible to the enzymatic active site of neuraminidase.^[16]

The syntheses of **3a** and **3b** are shown in Scheme 1. Starting material **4** was prepared as described.^[13a] The 7-hydroxy group of compound **4** was activated with 4-nitrophenyl chloroformate and DMAP in pyridine, followed by the addition of amine **5a** or **5b** to give compound **6a** or **6b**, respectively. Deprotection of the Boc group with trifluoroacetic acid provided guanidine compounds **7a** and **7b**. Hydrolysis of methyl esters in **7a** and **7b** was performed with aqueous triethylamine. After reaction completion, the solution was neutralized with hydrochloric acid and lyophilized to give the corresponding carboxylic acids. The carboxylic acids then were used in the copper-catalyzed click reaction^[17] with β -lactam molecule **8** to afford the target compounds **3a** and **3b**.

Formation of chemically programmed mAb 38C2 is shown in Figure 6. mAb 38C2 was incubated with chemical programming agent **3a** or **3b** (4 equiv) in 10 mM PBS (pH 7.4) at room temperature for 2 h. Complete conjugation between mAb 38C2 and chemical programming molecule was confirmed by loss of catalytic activity in the retro-aldol reaction.^[18] Conjugates **9a** and **9b** did not catalyze the retro-aldol reaction of methodol, whereas the parent antibody did (Figure 7A). MALDI mass spectra of **9a** and **9b** in comparison to that of mAb 38C2 are shown in Figure 7B and 7C. The conjugates had masses indicative of conjugation of two agents per antibody (one agent linked to each active site lysine). ESI mass spectral studies of **9a** and **9b** also demonstrated that mAb 38C2 was conjugated to two molecules of **3a** and **3b**, respectively (see supporting information). The chemically programmed antibodies **9a** and **9b** bound to neuraminidase in an ELISA (Figure 7D and 7E), whereas the parent antibody mAb 38C2 did not.

Biological activities of **3a**, **3b**, **9a**, **9b**, and zanamivir are summarized in Table 1. Small molecules **3a** and **3b** exhibited weaker inhibitory activity against influenza A neuraminidase compared to zanamivir. The reduction in inhibitory activity is likely due to steric hindrance caused by the addition of the long linker at the C-7 position of zanamivir. This reduction in inhibitory activity of zanamivir derivatives modified at the 7-hydroxy group is consistent with other reports of zanamivir derivatives (for details, see supporting information). In contrast, chemically programmed antibodies **9a** and **9b** showed inhibitory activity similar to zanamivir. It is likely that antibody conjugation enhanced the binding with the enzyme by an avidity effect since each antibody displays two molecules of zanamivir and neuraminidase is a tetramer. We have previously noted large avidity-based increases in activity following antibody conjugation in other systems.^[7] We selected the most potent chemically programmed antibody **9a** for further evaluation. Chemically programmed antibody **9a** was found to be more potent than zanamivir in a plaque reduction assay against influenza A/California/07/2009(H1N1) (Table 1). We believe the increase in potency of **9a** over zanamivir in this virus-based assay is a product of the bivalency of **9a** and may be due to crosslinking of the virus.

A pharmacokinetics study was conducted in mice: Mice were given a single intravenous injection of 10 mg/kg of chemically programmed antibody **9a**. The serum concentration of **9a** was measured using a neuraminidase-specific ELISA. The previously reported elimination half-life of zanamivir after intravenous injection in mice is approximately 10 min.^[4c] The chemically programmed antibody **9a** had a dramatically prolonged half-life of 72 h, approximating the half-life of the antibody itself in this model. This improvement in half-life, when scaled in humans, might prevent influenza infection with once or twice monthly dosing.

In summary, this study indicates that by using an appropriately designed enzyme inhibitor, an effective chemically programmed antibody can be created from an established scaffold antibody effectively allowing an antibody to directly engage the conserved catalytic residues of an enzymes' active site. The zanamivir derivative reported here encourages the development of chemically programmed vaccination approaches^[19] for influenza prophylaxis and therapy and opens up antibody targeting to a wide range of therapeutically relevant enzymes through the adaptation of known enzyme inhibitors.

Experimental Section

Experimental details and characterization are available in the Supporting Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Zanamivir (**1**)

Oseltamivir (**2**)

Figure 1.
Structure of neuraminidase inhibitors used clinically.

Figure 2.

Generation of chemically programmed antibody. Site-specific conjugation of aldolase mAb 38C2 is performed by reacting the antibody with a chemical programming agent composed of b-lactam moiety and a small molecule, peptide, or oligonucleotide (indicated by green sphere).

Figure 3.
Dimeric, trimeric, tetrameric, and polymeric neuraminidase inhibitors.

Figure 4.
Binding mode of zanamivir to neuraminidase N9.

Figure 5. Design of chemical programming agents. The β -lactam moiety was linked to the 7-hydroxy group of zanamivir through two different linkers, each approximately 40 Å.

Figure 7.

Analysis of formation of **9a** and **9b**. A) Catalytic activity of **9a**, **9b**, and mAb 38C2 for retro-aldol reaction of methodol. B) Overlay of MALDI mass spectral of **9a** (Mw = 152895, red) and mAb 38C2 (Mw = 151061, blue). C) Overlay of MALDI mass spectral of **9b** (Mw = 152591, red) and mAb 38C2 (Mw = 151059, blue). D) ELISA of **9a** against neuraminidase. E) ELISA of **9b** against neuraminidase.

Figure 8.
Serum concentration of **9a** following a single intravenous dose of 10 mg/kg in mouse (n=3).

Scheme 1.

Synthesis of programming agents **3a** and **3b**. Reagents and conditions: a) 4-nitrophenyl chloroformate, **5a** or **5b**, DMAP, pyridine, rt; **6a** (63%) and **6b** (67%); b) $\text{CF}_3\text{CO}_2\text{H}$, anisole, CH_2Cl_2 , rt; **7a** (91%) and **7b** (40%); c) (i) Et_3N , H_2O , MeOH , rt; (ii) **8**, CuSO_4 , sodium L-ascorbate, tris[(1-hydroxypropyl-1H-1,2,3-triazol-4-yl)methyl]amine, H_2O , $t\text{BuOH}$, rt; **3a** (50%) and **3b** (10%).

Table 1Neuraminidase inhibition and plaque reduction activities of compounds **3a**, **3b**, **9a**, and **9b**.

	Neuraminidase inhibition assay IC ₅₀ (nM)	Plaque reduction assay IC ₅₀ (nM)
	Influenza A	CA 2009 H1N1 influenza
Zanamivir	16.6	13.0 ± 3.0
3a	221.1	NT
3b	1269.3	NT
9a	26.6	3.5 ± 0.9
9b	38.9	NT

NT = not tested