

In this study, clinical specimens were collected from both NAI-untreated and NAI-treated patients, so later samples were collected after the exertion of selective pressure by drug treatment. However, frequency of detection of OR pandemic (H1N1) 2009 was low (1.4%). Because OR and OS pandemic (H1N1) 2009 isolates were genetically and antigenically indistinguishable, the current 2009–10 vaccine would be expected to be effective against recent OR pandemic (H1N1) 2009. No evidence of reassortment with seasonal influenza A (H1N1) virus was detected. Immunocompetent patients infected with OR pandemic (H1N1) 2009 showed typical uncomplicated influenza symptoms, similar to those caused by OS pandemic (H1N1) 2009 (19).

Early reports suggested that $\approx 70\%$ of the worldwide consumption of oseltamivir occurs in Japan (10), but long-term NAI surveillance in Japan from 1996 to 2007 (10) and previous NAI surveillance (16,20,21) showed a low frequency of resistant viruses, suggesting that the transmissibility of OR viruses selected by drug pressure was remarkably reduced. However, beginning in November 2007, an unexpectedly high frequency of OR seasonal influenza A (H1N1) viruses with the H275Y mutation was detected in Europe (11–15). Most were isolated from NAI-untreated patients and were more transmissible than OS influenza A (H1N1), resulting in rapid global dissemination (15). In contrast, even in the 2007–08 season, OR influenza A (H1N1) was detected only rarely (1.5%–2.6%) in Japan, despite the high level of oseltamivir use (13,22). However, OR influenza A (H1N1) virus was detected at a far higher frequency ($\approx 100\%$) the next year (13). Thus, the pattern of oseltamivir use did not correspond to the emergence and widespread distribution of OR influenza A (H1N1) viruses.

In contrast, this study and a recent report (8) found that OR pandemic (H1N1) 2009 has been detected predominantly in isolates from oseltamivir recipients. Unlike recent OR influenza A (H1N1) viruses, such OR pandemic (H1N1) 2009 viruses seemed to have restricted transmissibility among humans. These findings indicated that oseltamivir use was responsible for the emergence of OR pandemic (H1N1) 2009 viruses, but perhaps not for the widespread distribution of OR pandemic (H1N1) 2009.

Although the reason why recent OR seasonal influenza A (H1N1) isolates did not lose fitness remains unclear, a functional defect in NA proteins caused by H275Y may be counteracted by permissive secondary mutations. Two such mutations, R222Q and V234M, have been identified in seasonal influenza (H1N1) (23). Although whether the amino acids of the corresponding positions of pandemic (H1N1) 2009 play a similar role is unknown, the NA protein of this virus does have A and V residues at positions 222 and 234, respectively; a V at position 234 was identical to that in a nonpermissive amino acid sequence. Other

sporadic and some shared amino acid change(s) were observed in the NA protein of OR pandemic (H1N1) 2009 viruses, but these changes apparently did not restore viral fitness, because no efficiently transmissible OR pandemic (H1N1) virus was found. These observations suggest that the NA proteins of recent OR pandemic (H1N1) 2009 isolates likely did not possess such permissive secondary mutation(s) (online Appendix Table).

However, all recent animal studies of OR pandemic (H1N1) 2009 virus have shown that viral fitness and transmissibility did not differ from those of OS pandemic (H1N1) 2009 virus (24–26) and had a potential to supersede OS pandemic (H1N1) 2009 virus. Nevertheless, OR pandemic (H1N1) 2009 did not supersede OS pandemic (H1N1) 2009 in humans. This inconsistency may be explained by differences in infectious dose used in the animal models.

In an NAI assay of zanamivir susceptibility, statistical analysis identified 3 outliers. One OS pandemic (H1N1) 2009 possessed the D151D/N mutation, which has been reported to affect susceptibility to zanamivir in seasonal viruses (20,21). Two OR pandemic (H1N1) 2009 isolates had the substitutions I34V and I195V in NA; however, whether these affect zanamivir susceptibility is unclear. We also assayed peramivir susceptibility in representative OR and OS pandemic (H1N1) 2009 isolates. Data suggested that OR pandemic (H1N1) 2009 virus, which contained the H275Y substitution, possessed cross-resistance to peramivir, as reported by another group (27).

Both case reports and preliminary NAI surveillance (8,19,28–32) have indicated 2 groups are at high risk for the generation of resistant viruses. The first is patients with severely compromised or suppressed immune systems, who shed virus for prolonged periods and thus have an increased chance of developing resistant virus (33,34). WHO reported that 25% of 285 resistant cases (as of April 17, 2010) occurred in immunocompromised patients (35). The second group is persons who are receiving postexposure prophylaxis, who take a subtherapeutic dose of 75 mg 1 \times /day (treatment dosage is 75 mg 2 \times /day). This regimen may only partially inhibit viral replication, thus facilitating the emergence of OR pandemic (H1N1) 2009. WHO recommends chemoprophylaxis only for persons who have a higher risk for severe or complicated illness (19). These groups were observed in our study.

We found that most OR pandemic (H1N1) 2009 virus was detected in samples collected at least 4 days after oseltamivir treatment or prophylaxis (Table 1). However, the frequency of OR pandemic (H1N1) 2009 in each day of treatment with oseltamivir could not be calculated because of a lack of treatment history data. This timing is consistent with that for OR seasonal influenza A (H1N1, H3N2) and pandemic (H1N1) 2009 viruses, which typically emerge

3–6 days after oseltamivir treatment (36–38). Additionally, we also observed the rapid emergence of OR pandemic (H1N1) 2009 virus within 48 hours of oseltamivir exposure (39). Nonetheless, 6 cases occurred in untreated patients. Indeed, the OR pandemic (H1N1) 2009 in the Oita case may likely be a natural occurrence. We are aware of only 2 other reports, one from Vietnam and one from Hong Kong, of naturally occurring OR pandemic (H1N1) 2009 in untreated patients (7,40).

The greatest concern regarding OR pandemic (H1N1) 2009 is that drug-resistant viruses will acquire the ability to be transmitted efficiently among humans as has recent OR seasonal influenza A (H1N1). Two hospital outbreaks in the United Kingdom and the United States have been reported (31,32). In both, the immune systems of all patients were severely compromised or suppressed, indicating that these patients had an increased risk for not only the emergence of OR pandemic (H1N1) 2009 virus, but also OR seasonal influenza (H1N1) virus (31,32). In contrast, particular attention should be paid to the Vietnamese case because a naturally occurring OR pandemic (H1N1) 2009 virus caused a cluster of 7 cases in immunocompetent patients with no history of oseltamivir use (40).

Despite the high level of oseltamivir use in Japan, prevalence of OR pandemic (H1N1) 2009 remains low (1.4%). Thus, oseltamivir remains the first option for treating pandemic (H1N1) 2009, but zanamivir should be considered for immunocompromised patients. Additionally, as first priority for prophylaxis of both OR and OS pandemic (H1N1) 2009 infection should be vaccination, but not antiviral agents. Conversely, a preclinical animal model showed that OR pandemic (H1N1) 2009 had high potential to acquire transmissibility without losing viral fitness (24–26). Whether and how OR pandemic (H1N1) 2009 may acquire efficient transmissibility among humans are not known. Thus, vigilant monitoring of OR pandemic (H1N1) 2009 infection and alterations in its transmissibility and antigenic and genetic characteristics is essential.

Members of the Influenza Virus Surveillance Group of Japan: Hideki Nagano (Hokkaido Institute of Public Health), Masayuki Kikuchi (Sapporo City Institute of Public Health), Masaki Takahashi (Research Institute for Environmental Sciences and Public Health of Iwate Prefecture), Yuki Sato (Miyagi Prefectural Institute of Public Health and Environment), Masanori Katsumi (Sendai City Institute of Public Health), Hiroyuki Saito (Akita Research Center for Public Health and Environment), Katsumi Mizuta (Yamagata Prefectural Institute of Public Health), Syoko Hirose (Fukushima Prefectural Institute of Public Health), Setsuko Fukaya (Ibaraki Prefectural Institute of Public Health), Teruko Oogane (Tochigi Prefectural Institute of Public Health and Environmental Sciences), Hiroyuki Tsukagoshi (Gunma Prefectural Institute of Public Health and Environmental

Sciences), Yuka Uno (Saitama City Institute of Health Science and Research), Hiromi Maru (Chiba Prefectural Institute of Public Health), Hajime Yokoi (Chiba City Institute of Health and Environment), Mami Nagashima (Tokyo Metropolitan Institute of Public Health), Sumi Watanabe (Kanagawa Prefectural Institute of Public Health), Hideaki Shimizu (Kawasaki City Institute of Public Health), Sumiko Ueda (Sagamihara City Laboratory of Public Health), Miyako Kon (Niigata Prefectural Institute of Public Health and Environmental Sciences), Gen Kobayashi and Yoko Miyajima (Niigata City Institute of Public Health and Environment), Sanae Kuramoto (Ishikawa Prefectural Institute of Public Health and Environmental science), Masako Nakamura (Fukui Prefectural Institute of Public Health), Hiroyoshi Asakawa (Yamanashi Institute for Public Health), Seiko Sawatari (Gifu Prefectural Institute of Health and Environmental Sciences), Yasunori Tanaka (Gifu Municipal Institute of Public Health), Toshihiro Yamada (Shizuoka Institute of Environment and Hygiene), Shinobu Ide (Shizuoka City Institute of Environmental Sciences and Public Health), Yoshinori Kohno (Hamamatsu City Health Environment Research Center), Yoshihiro Yasui (Aichi Prefectural Institute of Public Health), Noriko Goto (Nagoya City Public Health Research Institute), Takuya Yano (Mie Prefecture Health and Environment Research Institute), Fumie Matsumoto (Shiga Prefectural Institute of Public Health), Tohru Ishizaki (Kyoto Prefectural Institute of Public Health and Environment), Satoshi Hiroi (Osaka Prefectural Institute of Public Health), Hideyuki Kubo (Osaka City Institute of Public Health and Environmental Sciences), Kiyoko Uchino (Sakai City Institute of Public Health), Tomohiro Oshibe (Hyogo Prefectural Institute of Public Health and Consumer Sciences), Ai Mori (Kobe Institute of Health), Yoshiteru Kitahori (Nara Prefectural Institute for Hygiene and Environment), Fumio Terasoma (Wakayama Prefectural Research Center of Environment and Public Health), Takayuki Hirooka and Hidenobu Egawa (Wakayama City Institute of Public Health), Yoshiaki Kimura (Tottori Prefectural Institute of Public Health and Environmental Science), Tamaki Omura (Shimane Prefectural Institute of Public Health and Environmental Science), Shinichi Takao (Center for Public Health and Environment, Hiroshima Prefectural Technology Research Institute), Katsuhiko Abe (Hiroshima City Institute of Public Health), Yumiko Kawakami (Tokushima Prefectural Centre for Public Health and Environmental Sciences), Satomi Aoki (Ehime Prefecture Institute of Public Health and Environmental Science), Daisuke Kawamoto (Fukuoka City Institute for Hygiene and the Environment), Takayuki Hirano (Saga Prefectural Institute of Public Health and Pharmaceutical Research), Manabu Hirano (Nagasaki Prefectural Institute for Environment Research and Public Health), Seiya Harada (Kumamoto Prefectural Institute of Public-Health and Environmental Science), Miki Kato (Oita Prefectural Institute of Health and Environment), Miho Miura (Miyazaki Prefectural Institute for Public Health and Environment), Kanji Ishitani (Kagoshima Prefectural Institute for Environmental Research and Public Health), Katsuya Taira (Okinawa

Prefectural Institute of Health and Environment), Mitsutaka Kuzuya (Okayama Prefectural Institute for Environmental Science and Public Health), Shoichi Toda (Yamaguchi Prefectural Institute of Public Health and Environment), Chiharu Kawakami (Yokohama City Institute of Health), Mayumi Konno (Kyoto City Institute of Health and Environmental Sciences), Hiroya Komoda (Kagawa Prefectural Research Institute for Environmental Sciences and Public Health), Tae Taniwaki (Kochi Public Health and Sanitation Institute), Toshitaka Minegishi (Saitama Institute of Public Health), Rika Tsutsui (Aomori Prefectural Institute of Public Health and Environment), Shizuko Kasuo (Nagano Environmental Conservation Research Institute), Yuichiro Okamura (Nagano City Health Center), Eiji Horimoto (Toyama Institute of Health), Nobuyuki Sera (Fukuoka Institute of Health and Environmental Sciences), Kotaro Murase (Kitakyushu City Institute of Environmental Sciences).

Acknowledgments

We thank Mariko Tokunaga for producing the figures.

This study was supported by Grants-in-Aid for Emerging and Reemerging Infectious Diseases (H21-Shinko-Ippan 005, H22-Shinko-Ippan 005) from the Ministry of Health, Labour and Welfare of Japan.

Dr Ujike is a virologist and the senior researcher of Center for Influenza Virus Research, the National Institute of Infectious Diseases, Tokyo, Japan. His Research interests are influenza virus and SARS corona virus.

References

- Novel Swine-Origin Influenza A (H1N1) Virus Investigation Team, Dawood FS, Jain S, Finelli L, Shaw MW, Lindstrom S, et al. Emergence of a novel swine-origin influenza A (H1N1) virus in humans. *N Engl J Med*. 2009;360:2605–15. DOI: 10.1056/NEJMoa0903810
- Garten RJ, Davis CT, Russell CA, Shu B, Lindstrom S, Balish A, et al. Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. *Science*. 2009;325:197–201. DOI: 10.1126/science.1176225
- Smith GJ, Vijaykrishna D, Bahl J, Lycett SJ, Worobey M, Pybus OG, et al. Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. *Nature*. 2009;459:1122–5. DOI: 10.1038/nature08182
- Center for Diseases Control and Prevention. Drug susceptibility of swine-origin influenza A (H1N1) viruses, April 2009. *MMWR Morb Mortal Wkly Rep*. 2009;58:433–5.
- World Health Organization. Pandemic (H1N1) 2009: antiviral drug resistance. 2009 Dec 22 [cited 2010 Apr 22]. http://www.who.int/csr/disease/swineflu/frequently_asked_questions/antivirals/resistance/en/index.html.
- World Health Organization. Viruses resistant to oseltamivir (Tamiflu) identified. 2009 Jul 8 [cited 2009 Jul 13]. http://www.who.int/csr/disease/swineflu/notes/h1n1_antiviral_resistance_20090708/en/index.html
- Chen H, Cheung CL, Tai H, Zhao P, Chan JF, Cheng VC, et al. Oseltamivir-resistant influenza A pandemic (H1N1) 2009 virus, Hong Kong, China. *Emerg Infect Dis*. 2009;15:1970–2. DOI: 10.3201/eid1512.091057
- World Health Organization. Oseltamivir-resistant pandemic (H1N1) 2009 influenza virus, October. *Wkly Epidemiol Rec*. 2009;84:453–9.
- World Health Organization. Pandemic (H1N1) 2009—update 82. 2010 Jan 8 [cited 2010 Apr 13]. http://www.who.int/csr/disease/swineflu/laboratory08_01_2010/en/index.html.
- Tashiro M, McKimm-Breschkin JL, Saito T, Klimov A, Macken C, Zambon M, et al. Surveillance for neuraminidase-inhibitor-resistant influenza viruses in Japan, 1996–2007. *Antivir Ther*. 2009;14:751–61. DOI: 10.3851/IMP1194
- Meijer A, Lackenby A, Hungnes O, Lina B, van-der-Werf S, Schweiger B, et al. Oseltamivir-resistant influenza virus A (H1N1), Europe, 2007/08 season. *Emerg Infect Dis*. 2009;15:552–60. DOI: 10.3201/eid1504.081280
- Dharan NJ, Gubareva LV, Meyer JJ, Okomo-Adhiambo M, McClinton RC, Marshall SA, et al. Infections with oseltamivir-resistant influenza A(H1N1) virus in the United States. *JAMA*. 2009;301:1034–41. DOI: 10.1001/jama.2009.294
- Ujike M, Shimabukuro K, Mochizuki K, Obuchi M, Kageyama T, Shirakura M, et al. Oseltamivir-resistant A/H1N1 viruses during the 2007–2009 influenza seasons, Japan. *Emerg Infect Dis*. 2010;16:926–35.
- World Health Organization. WHO/ECDC frequently asked questions for oseltamivir resistance. 2008 Feb 15 [cited 2008 Feb 15]. http://www.who.int/csr/disease/influenza/oseltamivir_faqs/en/index.html
- World Health Organization. Influenza A(H1N1) virus resistance to oseltamivir—2008 influenza season, southern hemisphere. 2009 Mar 18 [cited 2009 Apr 15]. http://www.who.int/csr/disease/influenza/H1N1_webupdate20090318%20ed_ns.pdf
- Monto AS, McKimm-Breschkin JL, Macken C, Hampson AW, Hay A, Klimov A, et al. Detection of influenza viruses resistant to neuraminidase inhibitors in global surveillance during the first 3 years of their use. *Antimicrob Agents Chemother*. 2006;50:2395–402. DOI: 10.1128/AAC.01339-05
- World Health Organization. Manual on animal influenza diagnosis and surveillance, 2nd ed. 2002 [cited 2010 Apr 15]. http://www.who.int/vaccine_research/diseases/influenza/WHO_manual_on_animal-diagnosis_and_surveillance_2002_5.pdf
- Wetherall NT, Trivedi T, Zeller J, Hodges-Savola C, McKimm-Breschkin JL, Zambon M, et al. Evaluation of neuraminidase enzyme assays using different substrates to measure susceptibility of influenza virus clinical isolates to neuraminidase inhibitors: report of the neuraminidase inhibitor susceptibility network. *J Clin Microbiol*. 2003;41:742–50. DOI: 10.1128/JCM.41.2.742-750.2003
- World Health Organization. Antiviral use and the risk of drug resistance. 2009 Sep 25 [cited 2010 Apr 19]. http://www.who.int/csr/disease/swineflu/notes/h1n1_antiviral_use_20090925/en/index.html
- McKimm-Breschkin J, Trivedi T, Hampson A, Hay A, Klimov A, Tashiro M, et al. Neuraminidase sequence analysis and susceptibilities of influenza virus clinical isolates to zanamivir and oseltamivir. *Antimicrob Agents Chemother*. 2003;47:2264–72. DOI: 10.1128/AAC.47.7.2264-2272.2003
- Sheu TG, Deyde VM, Okomo-Adhiambo M, Garten RJ, Xu X, Bright RA, et al. Surveillance for neuraminidase inhibitor resistance among human influenza A and B viruses circulating worldwide from 2004 to 2008. *Antimicrob Agents Chemother*. 2008;52:3284–92. DOI: 10.1128/AAC.00555-08
- Tamura D, Mitamura K, Yamazaki M, Fujino M, Nirasawa M, Kimura K, et al. Oseltamivir-resistant influenza A viruses circulating in Japan. *J Clin Microbiol*. 2009;47:1424–7. DOI: 10.1128/JCM.02396-08
- Matsuzaki Y, Mizuta K, Aoki Y, Suto A, Abiko C, Sanjoh K, et al. A two-year survey of the oseltamivir-resistant influenza A(H1N1) virus in Yamagata, Japan and the clinical effectiveness of oseltamivir and zanamivir. *Virology*. 2010;7:53. DOI: 10.1186/1743-422X-7-53

23. Bloom JD, Gong LI, Baltimore D. Permissive secondary mutations enable the evolution of influenza oseltamivir resistance. *Science*. 2010;328:1272–5. DOI: 10.1126/science.1187816
24. Hamelin ME, Baz M, Abed Y, Couture C, Joubert P, Beaulieu E, et al. Oseltamivir-resistant pandemic A/H1N1 virus is as virulent as its wild-type counterpart in mice and ferrets. *PLoS Pathog*. 2010;6:e1001015. DOI: 10.1371/journal.ppat.1001015
25. Kiso M, Shinya K, Shimajima M, Takano R, Takahashi K, Katsura H, et al. Characterization of oseltamivir-resistant 2009 H1N1 pandemic influenza A viruses. *PLoS Pathog*. 2010;6:e1001079. DOI: 10.1371/journal.ppat.1001079
26. Seibert CW, Kaminski M, Philipp J, Rubbenstroth D, Albrecht RA, Schwalm F, et al. Oseltamivir-resistant variants of the 2009 pandemic H1N1 influenza A virus are not attenuated in the guinea pig and ferret transmission models. *J Virol*. 2010;84:11219–26. DOI: 10.1128/JVI.01424-10
27. Nguyen HT, Sheu TG, Mishin VP, Klimov AI, Gubareva LV. Assessment of pandemic and seasonal influenza A (H1N1) virus susceptibility to neuraminidase inhibitors in three enzyme activity inhibition assays. *Antimicrob Agents Chemother*. 2010;54:3671–7. DOI: 10.1128/AAC.00581-10
28. Baz M, Abed Y, Papenburg J, Bouhy X, Hamelin M, Boivin G. Emergence of oseltamivir-resistant pandemic H1N1 virus during prophylaxis. *N Engl J Med*. 2009;361:2296–7. DOI: 10.1056/NEJMc0910060
29. Centers for Disease Control and Prevention. Oseltamivir-resistant 2009 pandemic influenza A (H1N1) virus infection in two summer campers receiving prophylaxis—North Carolina, September 11. *MMWR Morb Mortal Wkly Rep*. 2009;58:969–72.
30. Centers for Disease Control and Prevention. Oseltamivir-resistant novel influenza A (H1N1) virus infection in two immunosuppressed patients—Seattle, Washington, 2009. *MMWR Morb Mortal Wkly Rep*. 2009;58:893–6.
31. Wolfe C, Greenwald I, Chen L. Pandemic (H1N1) 2009 and oseltamivir resistance in hematology/oncology patients. *Emerg Infect Dis*. 2010;16:1809–11.
32. Moore C, Galiano M, Lackenby A, Abdelrahman T, Barnes R, Evans MR, et al. Evidence of person-to-person transmission of oseltamivir-resistant pandemic influenza A(H1N1) 2009 virus in a hematology unit. *J Infect Dis*. 2011;203:18–24. DOI: 10.1093/infdis/jiq007
33. Ison MG, Gubareva LV, Atmar RL, Treanor J, Hayden FG. Recovery of drug-resistant influenza virus from immunocompromised patients: a case series. *J Infect Dis*. 2006;193:760–4. DOI: 10.1086/500465
34. Weinstock DM, Gubareva LV, Zuccotti G. Prolonged shedding of multidrug-resistant influenza A virus in an immunocompromised patient. *N Engl J Med*. 2003;348:867–8. DOI: 10.1056/NEJM200302273480923
35. World Health Organization. Pandemic (H1N1) 2009—update 96. 2010 Apr 16 [cited 2010 Apr 19]. http://www.who.int/csr/disease/swineflu/laboratory16_04_2010/en/index.html
36. Gubareva LV, Kaiser L, Matrosovich MN, Soo-Hoo Y, Hayden FG. Selection of influenza virus mutants in experimentally infected volunteers treated with oseltamivir. *J Infect Dis*. 2001;183:523–31. DOI: 10.1086/318537
37. Kiso M, Mitamura K, Sakai-Tagawa Y, Shiraishi K, Kawakami C, Kimura K, et al. Resistant influenza A viruses in children treated with oseltamivir: descriptive study. *Lancet*. 2004;364:759–65. DOI: 10.1016/S0140-6736(04)16934-1
38. Sy CL, Lee SS-J, Liu M-T, Tsai H-C, Chen Y-S. Rapid emergence of oseltamivir resistance. *Emerg Infect Dis*. 2010;16:723–5.
39. Inoue M, Barkham T, Leo YS, Chan KP, Chow A, Wong CW, et al. Emergence of oseltamivir-resistant pandemic (H1N1) 2009 virus within 48 hours. *Emerg Infect Dis*. 2010;16:1633–6.
40. Le QM, Wertheim HF, Tran ND, van Doorn HR, Nguyen TH, Horby P, et al. A community cluster of oseltamivir-resistant cases of 2009 H1N1 influenza. *N Engl J Med*. 2010;362:86–7. DOI: 10.1056/NEJMc0910448

Address for correspondence: Takato Odagiri, Center for Influenza Virus Research, National Institute of Infectious Diseases, 4-7-1, Gakuen, Musashi-murayama, Tokyo 208-0011, Japan; email: todagiri@nih.go.jp

Get the content you want
delivered to your inbox.

Table of contents
Podcasts
Ahead of Print
CME
Specialized topics



Mechanism of Drug Resistance of Hemagglutinin of Influenza Virus and Potent Scaffolds Inhibiting Its Function

Hiroshi Yanagita,^{†,#} Norio Yamamoto,^{‡,§,#,*} Hideyoshi Fuji,[†] Xinli Liu,[†] Masakazu Ogata,[†] Mizuho Yokota,[†] Hiroshi Takaku,^{||} Hideki Hasegawa,[⊥] Takato Odagiri,[‡] Masato Tashiro,[‡] and Tyuji Hoshino^{†,*}

[†]Graduate School of Pharmaceutical Sciences, Chiba University, Inohana 1-8-1, Chuo-ku, Chiba 260-8675, Japan

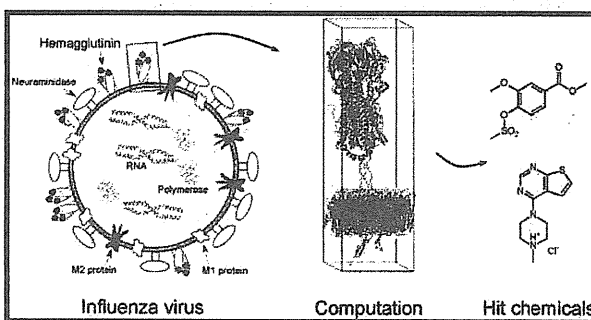
[‡]Influenza Virus Research Center and [⊥]Department of Pathology, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama, Tokyo 208-0011, Japan

[§]Department of General Medicine, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan

^{||}Department of Life and Environmental Science, Chiba Institute of Technology, 2-17-1 Tsudanuma, Narashino-shi, Chiba 275-0016, Japan

Supporting Information

ABSTRACT: Highly pathogenic influenza viruses have become a global threat to humans. It is important to select an effective therapeutic option suitable for the subtypes in an epidemic or pandemic. To increase the options, the development of novel antiviral agents acting on targets different from those of the currently approved drugs is required. In this study, we performed molecular dynamics simulations on a spike protein on the viral envelop, hemagglutinin, for the wild-type and three kinds of mutants using a model system consisting of a trimeric hemagglutinin complex, viral lipid membrane, solvation waters, and ions. A natural product, stachyflin, which shows a high level of antiviral activity specific to some subtypes of influenza viruses, was examined on binding to the wild-type hemagglutinin by docking simulation. The compound potency of stachyflin is, however, easily lost due to resistant mutations. From a comparison of simulation results between the wild-type and the resistant mutants, the reason for the drug resistance of hemagglutinin was clarified. Next, 8 compounds were selected from a chemical database by *in silico* screening, considering the findings from the simulations. Inhibitory activities to suppress the proliferation of influenza virus were measured by cell-based antiviral assays, and two chemical scaffolds were found to be potent for an inhibitor. More than 30 derivatives bearing either of these two chemical scaffolds were synthesized, and cell culture assays were carried out to evaluate the compound potency. Several derivatives displayed a high compound potency, and 50% effective concentrations of two synthesized compounds were below 1 μ M.



Influenza viruses cause acute respiratory infection in humans that occasionally progresses to a severe pulmonary condition. Even seasonal epidemics account for 300,000 or more deaths per a year all over the world. Recently, the emergence of highly pathogenic avian and swine influenza viruses has become a global threat to humans. Avian influenza H5N1 virus infections have been reported since 2003,¹ and a pandemic of transmissible H5N1 virus is a serious concern for public health.² The recent outbreak of swine influenza subtype H1N1 resulted in considerable mortalities for infants and the elderly. While several anti-influenza drugs are currently approved, their effectiveness for pandemic viruses may be limited due to drug resistance. Therefore, the development of additional antiviral agents against influenza virus infection is needed.

The currently available anti-influenza drugs target one of two viral proteins: M2 protein and neuraminidase. M2 protein is embedded in the lipid membrane of the viral envelope and functions as an ion channel to pump protons into the viral particles. Amantadine and Rimantadine block the function of M2 protein by combining at the center of the channel or the side domain of this enzyme.^{3,4} Neuraminidase is a kind of spike protein sticking out on the viral particle surface. Neuraminidase causes the hydrolysis of neuraminic acid of the glycan of the host cell. Zanamivir, Oseltamivir, Peramivir, and Laninamivir have been used as neuraminidase inhibitors.^{5,6} Emergence of drug-resistant viruses has been reported for the above approved

Received: September 1, 2011

Accepted: January 4, 2012

drugs. For example, resistance against Amantadine and Rimantadine was shown in the H3N2 and H1N1 viruses, and resistance against Oseltamivir was shown for the H1N1 virus.^{7–9} Since an RNA virus easily acquires amino acid mutations, the emergence rate of drug-resistant viruses is high. A drug-resistant virus is a serious issue in infectious diseases because a chemotherapeutic approach is restricted. In order to combat drug-resistant viruses, it is important to prepare many chemotherapeutic options and select an effective option suitable for subtypes in an epidemic. Accordingly, development of novel antiviral drugs that act on a target different from those of the currently approved drugs is needed.

A species of moss, *Stachybotrys* s. RF-7260, generates a unique natural product named stachyflin. Stachyflin was found to have strong antiviral activity against some subtypes of influenza viruses.^{10–12} The inhibitory mechanism of stachyflin is different from the inhibitory mechanism of the currently approved anti-influenza drugs. Stachyflin binds with hemagglutinin (HA) on the viral envelope and blocks conformation change of HA to prevent the lipid membrane of the viral envelope from merging with that of host cell. HA is one of the attractive targets of antiviral agents for the following reasons. First, HA is a key component in the viral infusio process that has no cellular counterparts and therefore has a potential advantage in selectivity and toxicity. Second, HA inhibitors will complement other currently approved drugs since they act on a different molecular target in the virus life cycle.

Stachyflin is highly effective for influenza virus of A/WSN/33 H1N1 subtype, and the 50% inhibitory concentration (IC₅₀) value for the WSN strain was reported to be 3 nM.¹² However, its inhibitory activity for other strains including other H1N1 virus strains is not so high, and its inhibitory activity is easily decreased by amino acid mutations of the virus. Since several amino acid mutations involved in drug resistance are not localized in one domain of HA, it is difficult to understand the mechanism of drug resistance caused by mutations straightforwardly. The chemical structure of stachyflin is complicated. Five rings merge to form a structure called the 3*H*-naphthopyrano-isoindol-3-one scaffold, and stachyflin also contains five chiral centers (Figure 1a). This complexity in its chemical structure is another reason for the difficulty in improving compound potency for stachyflin derivatives.

Yoshimoto and co-workers performed an experiment on resistance induction with stachyflin¹³ and demonstrated that K51R, K121E, S206L in the HA2 subunit and V176I in the HA1 subunit appeared in resistant viruses (Figure 1b). K51R and K121E mutations were suggested to be essential for drug resistance. They suggested from a docking simulation that stachyflin was bound to a position close to Lys51 or Phe110 of HA. This simulation, however, provided no clear explanation for the mechanism of drug resistance due to the above amino acid mutations. The mechanism of the drug resistance of HA should be clarified for producing promising inhibitory compounds.

In this study, we performed computational analysis, screening to find candidate compounds, a cell-based antiviral assay, and synthesis of analogue compounds in the following manner: (1) Molecular dynamics (MD) simulations were carried out for the wild-type and three kinds of variants containing amino acid mutations responsible for drug resistance in order to clarify the mechanism. (2) A point for designing a potential inhibitor was deduced from the concept of minimizing the influence of the drug-resistance-related conformational change of HA. (3) An *in*

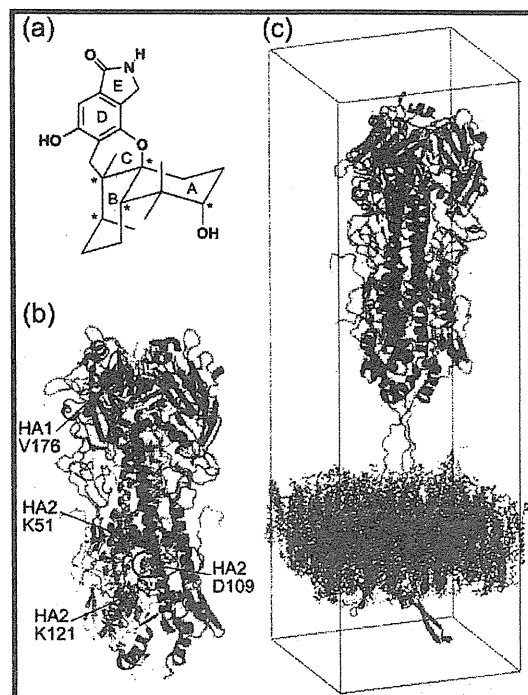


Figure 1. (a) Chemical structure of stachyflin. Stachyflin is composed of five complex rings and contains five chiral centers. The rings are labeled A–E, and chiral carbon atoms are marked by asterisks. (b) Structure of hemagglutinin in a trimer conformation. Spheres denote the residues introducing amino mutations in the respective mutants. The center residue targeted in the ligand docking simulation is indicated by a circle. (c) Calculation model of a complex of hemagglutinin trimer and lipid membrane. Hemagglutinin subunits HA1 and HA2 are colored blue and green, respectively. The membrane consists of 6 different kinds of lipid molecules, and its composition is presented in Supplementary Table S1. No water molecules or ions are shown for visual clarity.

silico screening was performed to find low-molecular-weight compounds showing inhibitory activity against HA, considering the above point and using the pharmacophore of stachyflin. (4) A cell-based assay was carried out to evaluate the inhibitory potencies of the compounds collected by the *in silico* screening. (5) Derivatives of the hit compounds found from the screening were synthesized, and their inhibitory activities were measured to elucidate the antiviral potency of the scaffold proposed in this work.

RESULTS AND DISCUSSION

Structural Difference in HA between the Wild-Type and Mutants. MD simulation was carried out for 30 ns to obtain the probable protein structure of HA in a trimer form for the wild-type and three mutants, using the model system containing a trimeric hemagglutinin and viral lipid membrane as shown in Figure 1c. The respective mutants contain the resistant mutations K51R and K121E in HA2 for mutant 1, V176I in HA1 and K51R, K121E in HA2 for mutant 2, and V176I in HA1 and K51R in HA2 for mutant 3. Root mean square deviation (rmsd) relative to the structure after heating is shown in Supplementary Figure S1. The rmsd value for the wild-type (Figure S1a) was scarcely changed during 30 ns. Each of the rmsd curves for the mutants (Figures S1b–d) shows a

gradual increase up to 15 ns and seems to be almost constant after 20 ns. Plots of rmsd in Figure S1 were obtained from the coordinates of main chain atoms of the whole HA. The N-terminal domain of the HA1 subunit is so flexible that rmsd values are considerably large. Hence, rmsd values were calculated again with respect to the main chain atoms of only the HA2 subunit with excluding the C-terminal region, aa 176–222. The rmsd curves only for HA2 in Supplementary Figure S2 also became constant after 20 ns for every model. Accordingly, protein conformations for the respective models were judged to be equilibrated.

Principal component analysis (PCA) in Supplementary Figure S3 indicates that the trajectory structures for the last 5 ns are in a single conformation for every model. The equilibration of the simulation is also confirmed from these PCA plots. In order to extract the plausible protein structure, the averaged structure was obtained using 500 trajectory structures from the last 5 ns of MD simulation. The rmsd between each trajectory structure and the average structure was calculated, and then one trajectory structure with the smallest rmsd value was determined to be the plausible protein structure. At a glance, there is no prominent difference among the 4 models in terms of shape of the trimer, conformation of the HA1 and HA2 subunits, or position of helices. Although no significant apparent change is seen in the backbone of HA, there appears a notable difference in the location of side chains. The differences in the side chain will be responsible for the change in binding affinity and inhibitory activity of inhibitors.

Binding of an Inhibitor to HA. By means of docking simulation, an inhibitor, stachyflin, was bound to the HAs, using the respective plausible protein structures obtained by MD calculations. In the wild-type HA, stachyflin was bound near Asp109 of the HA2 subunit (Figure 2). Hydrophobic interactions were observed between the B ring of stachyflin and Phe37 of HA1, between the C ring of stachyflin and Phe110 of HA2, and between the D ring and Leu113. Hydrogen bonds were formed between the O atom on the D ring of stachyflin and the amino group of Asn114 in HA2 and between the O atom on the E ring and the amino group of Asn117.

In mutant 2, stachyflin was bound to a location similar to that of the wild-type (Supplementary Figure S4c), while the docking simulation showed binding of stachyflin at the central space among three helices of the HA2 trimer in mutants 1 and 3 (Figures S4b and d). Judging from the binding affinity evaluated by ASP score function (Supplementary Table S2), the binding of stachyflin to the wild-type HA is the most stable. All of the mutants showed notable decrease in binding affinity compared to that of the wild-type.

An amino acid mutation of K51R in HA2 commonly appeared in the three mutants, suggesting that K51R was the primary mutation for drug resistance. Other mutations, K121E and V176L, will enhance the resistance. All of these amino mutations are, however, distant from the stachyflin binding site. Our MD simulation clearly indicated that inner helices of HA2 subunit were rotated (Figure 3a). One of the three inner helices, chain D in nomenclature in PDB 1RD8, was rotated by 10.8° in mutant 1 and by 15.0° in mutant 2 compared to the wild-type (Figure 3b). The amino group on the side chain of Lys51 makes a strong hydrogen bond with the hydroxy group of Thr107 of HA2 (Figure 3c). Arg is also a positively charged amino acid residue, but the length of the side chain is longer than that of Lys. When Lys is converted into Arg, the side chain

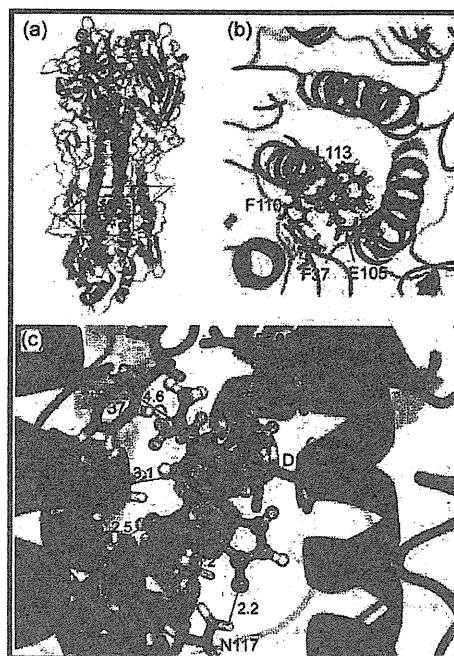


Figure 2. (a) Complex structure of stachyflin and hemagglutinin obtained by the ligand docking simulation. (b) Binding site of stachyflin viewed in a plane perpendicular to the helices of the HA2 subunit. (c) Binding mode of stachyflin, shown in a magnified view of the area indicated by a red frame in panel a. Stachyflin is bound to the space between two helices of HA2 subunit, making strong interaction with side chains of Asp109, Phe110, and Leu113. The interaction distances are in Å.

of the residue at codon 51 expands and pushes T107. The side chain of Thr107 serves as a lever to rotate the helix. The position of the side chain of Asp109 is largely deviated from that of the wild-type because of the closeness to Thr107. Lys121 has a strong interaction with the carboxy group of Asp18 of the HA1 subunit. When Lys is converted into Glu in the K121E mutation, the side chain of Glu121 and Asp18 of HA1 causes repulsion to increase the distance between them. This repulsion assists the helix rotation, and the deviation of Leu113 and Asn114 from the wild-type will be enhanced because of the closeness to Glu121. To monitor the helix rotation, the angle between the line connecting the $C\alpha$ and $C\beta$ atoms of Asp109 on the inner helix chain D and the line connecting the Asp109 $C\alpha$ atom on chain D and the Phe110 $C\alpha$ atom on another inner helix chain B was measured through the simulation as shown in Supplementary Figure S5. A significant angle change was observed after 10 ns for every model. The distances between the $N\zeta$ atom of Lys51 (or $C\zeta$ in K51R) of HA2 and the $O\gamma$ atom of Thr107 of HA2 and also between the $N\zeta$ atom of Lys121 (or $C\delta$ in K121E) of HA2 and $C\gamma$ of Asp18 of HA1 were monitored as shown in Supplementary Figure S6. Because HA is a trimer and there exist three HA1 and three HA2 subunits in the calculation models, three combinations of those interatomic distances were measured through the simulation. The distance plots in Figure S6 indicate that the interaction between the residues at codon 51 and codon 107 is quite stable in the wild-type HA. In contrast, some of these distances occasionally increased in the mutants. The distance between the residues at codon 121 of HA2 and at codon 18 of HA1 ceaselessly fluctuated in all

C

dx.doi.org/10.1021/cb200332k | ACS Chem. Biol. XXXX, XXX, XXX–XXX

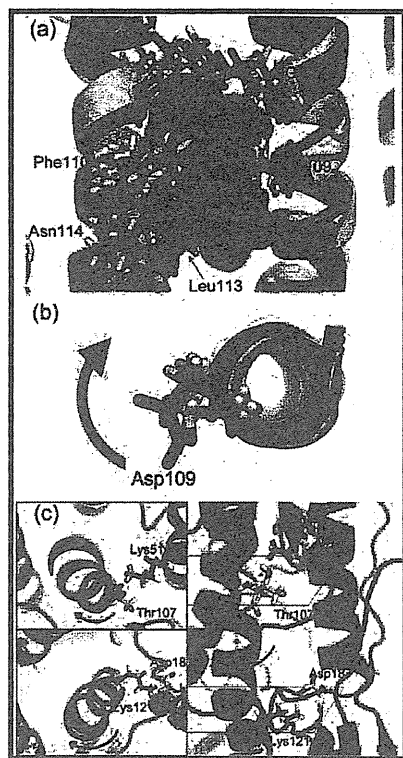


Figure 3. (a) Superposition of the hemagglutinin structures of mutants on that of the wild-type. The wild type is colored green and mutants 1, 2, and 3 are colored cyan, magenta, yellow, respectively. The binding area of stachyflin to the wild-type HA is shown in a mesh representation. There is no significant change in positions of helices. However, the amino acid mutations cause rotation of helices. The rotation angle is estimated from the position of $C\beta$ atom of Asp109, as shown in panel b. Thr107 in HA2 subunit and Asp18 in HA1 subunit are deeply involved in the helix rotation induced by K51R and K121E mutations, respectively, as shown in panel c. Two illustrations on the left side are depicted around the planes perpendicular to the helices and containing Thr107 or Lys121, respectively. The planes are indicated by blue and red frames in the right side illustration in panel c.

models. A strong interaction was, however, established at least for one combination of Lys121 and Asp18 in the wild-type.

The helix rotation pointed out above is the reason for the decrease in inhibitory activity of stachyflin to the resistant mutants. In the mutants, the side chains of Asp109 and Leu113 are displaced and occupy the space that stachyflin was bound to in the wild-type HA. The side chains of Phe110 and Asn114 are also displaced and move away from the stachyflin binding site as shown in Supplementary Figure S7. Therefore, stachyflin would not be bound to HA stably any more. The helix rotation in mutant 3 is small. This is naturally understood because the influence of V176I mutation in the HA1 subunit is slight. This finding suggests that some degree of flexibility is favorable to HA inhibitors for releasing the strain due to the helix rotation. Therefore, compounds bearing complicated heteroring structure are disadvantageous. Instead, a single bond connection of separated ring domains can be a good chemical frame to maintain structural flexibility.

Conformational change accompanying helix rotation has been reported for other kinds of transmembrane proteins. For example, an X-ray crystallographic analysis of the human $\beta 2$

adrenergic G-protein-coupled receptor¹⁴ suggested that a rotational motion was observed for one of the transmembrane helices owing to the binding of a ligand for the receptor. An electron paramagnetic resonance measurement¹⁵ and a computational analysis¹⁶ indicated that light adsorption induced a conformational change of retinal chromophore in sensory rhodopsin and this conformational change caused the rotation of transmembrane helix TM1 to lead signal transduction of the sensory protein.

Search for Active Compounds. The natural product stachyflin possesses a unique pentacyclo structure in which each ring is labeled as A, B, C, D, and E, respectively. Rings AB are composed of a naphthol skeleton, and rings DE are composed of an indol frame. Ring C bears a pyran structure and connects rings AB and rings DE. Rings ABC are fused in *cis*-form, and the oxygen of pyran is bonded to the carbon at the junction of rings AB. Furthermore, stachyflin contains 5 chiral centers, in which all of the chiral carbons are located on rings AB. Therefore, the naphthol moiety and its connection to pyran make the chemical structure of stachyflin highly complicated. A hydroxy group and carbonyl oxygen are bound to the indol ring, which characterizes the electrostatic property of stachyflin. The hydroxy group of naphthol is another factor to characterize the polar feature of this natural product.

Eight chemical compounds were selected by an *in silico* screening using the pharmacophore of stachyflin (Table 1). All of the selected compounds were produced by organic synthesis and are available by purchase. The molecular weights of these compounds range from 304 to 341. Compound 1 bears two ester bonds and a benzofuran moiety corresponding to rings C and D of stachyflin in superimposition. Compound 2 bears a thieno-pyrimidine, which corresponds to rings D and E of stachyflin and a phenyl-cyclopentane corresponding to rings A and B. Two benzene rings are connected *via* a dichloromethyl-carbonyl group in compound 3. Methylbenzoic acid methyl-ester in compound 4 corresponds to rings D and E, and dimethyl-diazolane is connected by a sulfonyloxy group. Thieno-pyrimidine in compound 5 corresponds to rings D and E, and another thiophene corresponds to ring A. The molecular weight of compound 6 is the largest among the selected compounds, and methylester-benzene corresponds to rings D and E and pyridyl-triazole corresponds to rings A and B. Compound 7 bears a seven-membered ring containing two nitrogen atoms. Hydroxybenzene in compound 8 corresponds to ring B of stachyflin.

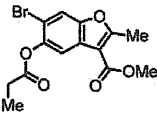

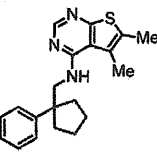

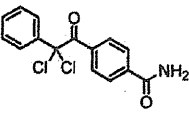

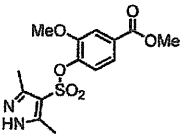
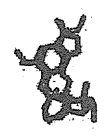
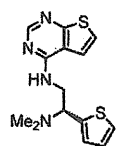

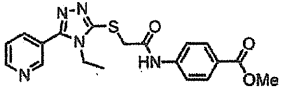

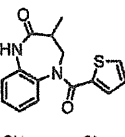

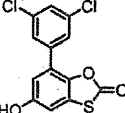

Assay for Antiviral Activity. The 8 selected compounds were tested in an influenza virus cell culture assay (Table 1). Compounds 4 and 5 were found to have significant antiviral activity ($EC_{50} < 5 \mu M$). Although compound 2 was the most highly potent with an EC_{50} of $3 \mu M$, compound 2 exhibited significant cytotoxicity at a concentration of $6 \mu M$, measured by an MTT assay with MDCK cells. Since compounds 4 and 5 showed no noticeable cytotoxicity, these two compounds were chosen as the structural core in the next step for organic synthesis.

The binding modes of compounds 4 and 5 to the wild-type HA were predicted by performing docking simulation. The most probable docking structures are shown in Figure 4a and b, which were determined from the score ranking for 50 docking poses. The two compounds are bound to almost the same position as stachyflin is. That is, these compounds are located not in the central space of HA2 trimer but at a position in the middle of two helices of the HA2 subunit. The calculated

D

dx.doi.org/10.1021/cb200332k | ACS Chem. Biol. XXXX, XXX, XXX–XXX

Table 1. Structures and Inhibitory Activities of the Chemical Compounds Obtained through an *in Silico* Screening

Compound	Structure	Superimposition ^{a)}	EC ₅₀ (μM)	CC ₅₀ (μM)
1			9.0	> 60
2			3.0	> 5.9
3			> 32	> 60
4			4.4	> 60
5			4.9	> 60
6			17.9	> 60
7			31.4	> 60
8			14.9	> 60

^aStachyflin is depicted in green stick representation, while compounds are blue.

binding affinities of these two compounds in ASP score are lower than that of stachyflin (TSupplementary table S2) but higher than that in the cases of stachyflin bound to the three kinds of mutants.

Synthesis of Analogue Compounds. Compound 4 bears a structural core of vanillic acid. Based on the vanillic acid methylester core, 22 derivatives from compound 4 were synthesized as shown in Table 2, where the functional group at the fourth position of the benzoic acid was modulated. A highly potent compound (9) was found in the analogues in which methylsulfonyl was connected to the fourth position. The incorporation of benzyl or methylphenyl (10, 12) showed no inhibitory activity, while moderate activity was observed in case of phenyl only (11). This means that a small chemical group is favorable for the substitute connecting *via* the sulfonyloxy group. Conversion of the sulfonyl group into an ester bond (13–15) resulted in complete loss of compound potency, regardless of the size of the substitutes connected to

the ester. Substitution of the sulfonyl group by an alkyl chain (16–20) resulted in loss of inhibitory activity. Only compound 18 in which the sulfonyl group was substituted by benzyl showed slight inhibitory activity. Then the effect of addition of functional groups to the benzyl (21–28) was surveyed. Compound 22 containing an oxybenzyl group at the *para*-position of the benzyl exhibited high compound potency. The inhibitory activity was maintained with the addition of methoxy or trifluoromethyl at the *para*-position, while the incorporation of other kinds of functional groups (23–25) or the addition of trifluoromethyl at the *ortho*- or *meta*-position (27, 28) resulted in loss of inhibitory activity. Conversion of the sulfonyloxy group into an amino group (29, 30) was tested. None of the derivatives showed noticeable increase in compound potency.

Compound 5 bears a heteroring core of thieno-pyrimidine, and a dimethyl-aminy-thienyl group is connected to the heteroring *via* amine. Keeping the heteroring core, 9 derivatives from compound 5 were synthesized as shown in Table 3. The

E

dx.doi.org/10.1021/cb200332k | ACS Chem. Biol. XXXX, XXX, XXX–XXX

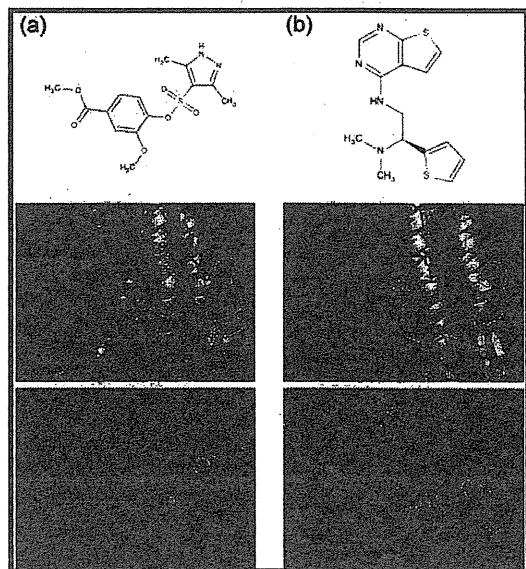


Figure 4. Hit chemicals found through *in silico* screening: (a) compound 4 and (b) compound 5. Top: chemical structure. Middle: binding site of the compound predicted by the docking simulation. Bottom: binding position of the compound viewed from the direction of subunit HA1.

introduction of benzyl *via* amine group (31) resulted in an increase in inhibitory activity. In contrast, conversion into dimethylamine (32, 33) resulted in loss of compound potency. Interestingly, while conversion into methyl-piperazine (34) resulted in a decrease in inhibitory activity, its hydrochloride salt (35) exhibited a high compound potency. Phenyl-piperazine (36) also exhibited a considerably high inhibitory activity. The conversion of thiophene of compound 5 into benzene (37) exhibited a high compound potency, while its chiral analogue (38) showed no inhibitory activity. This chiral-selective compound potency was confirmed by substitution of a Boc protection group (39) for the dimethylamine of compound 37.

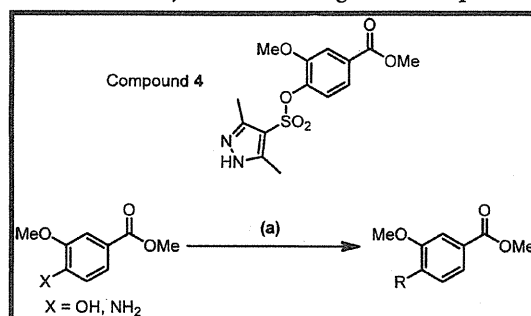
Actions of Active Compounds for Blocking HA. Fusion of the membrane is an essential process in the entry of influenza virus into the host cell. HA mediates this process through two functions in the early stage of the viral life cycle.¹⁷ One is anchoring at sialylated glycoprotein receptors on the cell membrane surface. The other is the low-pH induced conformational change that initiates the exposure of a fusion peptide, a hydrophobic N-terminal segment buried in the HA trimer interface, to be inserted into the endosomal membrane. Stachyflin is assumed to block this fusogenic process of HA.^{12,13} Indeed, in the predicted binding structure shown in Figure 2, stachyflin is combined with HA with the formation of two strong hydrogen bonds and three strong aromatic ring-involved hydrophobic interactions.

Several other compounds blocking the fusogenic activity of HA have been identified.^{18–22} An active compound¹⁸ bears a naphthoquinone skeleton, which is commonly seen in stachyflin as rings AB. Another compound¹⁹ contains a quinolizin moiety connected to a benzamide skeleton, and a one-step virus growth experiment indicated that this compound mainly inhibited virus proliferation at the early stage of the replication cycle. Therefore, a quinoline skeleton is one of the effective chemical cores for binding to HA. A compound

containing piperidine connected to trifluoro-methylbenzoyl also showed high inhibitory activity for influenza virus replication.²⁰ Ligand docking calculation suggested that this compound was bound to a site near Phe110 of the HA2 subunit, which is almost identical to the binding site of stachyflin obtained in this work as shown in Figure 2. A compound analogue to podocarpic acid was identified as an inhibitor of type A viruses²¹ and showed a high inhibitory activity especially for Kawasaki strain but was not so sensitive to WSN strain. In contrast, some recently identified blockers are targeted at another domain of HA.^{23,24} Some peptides mimicking sialic acid were shown to inhibit the entry of viruses into host cells, attached to the receptor-binding site of HA.²³ Synthesized macromolecules containing three sialyllactoses linked with trisphenol or trisaniline were reported to inhibit viral replication, combined with the receptor-binding site of HA.²⁴ Recently, an HA inhibitor bearing a benzenesulfonamide core was identified through structural modifications of a salicylamide derivative.²⁵ It is interesting to note that the chemical structure of this agent resembles that of compound 9 in Table 2 to some extent.

In most of the active compounds, susceptibility varies among strains of influenza viruses and the inhibitory activity is drastically decreased due to the acquirement of resistant mutation. Our MD simulation demonstrated that the rotation of helices is the reason for the reduction of compound potency. Due to the increase in performance of computers and the development of calculation methodology, several computational analyses have recently been carried out.^{26–32} Quantum mechanical calculations using the fragment molecular orbital method were used to investigate the role of key amino acid residues in recognizing sialoglycoproteins on the host cell surface²⁶ or in combining with neutralizing monoclonal antibodies.²⁷ Huge MD simulations were performed to examine the interaction between HA and sialoglycans²⁸ and clarify the reason for mutations at the receptor-recognizing site.²⁹ MD simulations were also employed to evaluate the binding free energy³⁰ and to interpret the difference in receptor specificity.³¹ A computational approach was further demonstrated to be quite helpful for designing protein peptides that strongly inhibit the function of HA.³²

Pharmaceutical Properties of Hit Chemicals. Since stachyflin exhibits a highly potent anti-influenza activity for the wild-type WSN strain and is a challenging synthetic target due to its unique alkaloid structure, two research groups have so far attempted total synthesis of stachyflin. The first total synthesis of racemic (\pm)-stachyflin was reported by Taishi *et al.*,¹¹ in which the characteristic structure of 5 heterorings was built one by one in 29 steps. The first enantioselective total synthesis of (+)-stachyflin was recently achieved by Watanabe *et al.*,³³ utilizing an acid-induced domino epoxide-opening, rearrangement, cyclization reaction.³⁴ These synthetic studies suggest the possibility for producing stachyflin analogues and encourage the development of stachyflin-based antiviral agents. The complexity in synthesis is, however, disadvantageous from the viewpoint of productivity in manufacturing. Carey and co-workers analyzed the reactions used for the preparation of drug candidate molecules,³⁵ surveying 128 compounds produced in the departments of process chemistry of three major pharmaceutical companies. According to their analysis, the average number of chemical steps for synthesizing one candidate is 8.1. Compounds containing a chiral center account for about half of the 128 molecules. Therefore, the structural

Table 2. Structures and Inhibitory Activities of the Synthesized Analogues to Compound 4, Which Bears a Vanillic Acid Core^a

Compound	X	R	Temp. (°C)	Time (h)	Yield (%)	EC ₅₀ (μM)
9		OSO ₂ Me	rt	3	71	0.9
10		OSO ₂ CH ₂ Ph	rt	3	75	>10
11	OH	OSO ₂ Ph	rt	6	81	7.0
12		OSO ₂ <i>p</i> -MeC ₆ H ₄	rt	6	82	>10
13		OCOMe	rt	3	35	>10
14	OH	OCOCH ₂ Cl	rt	3	66	>10
15		OCOPh	reflux	3	80	>10
16			reflux	24	93	>10
17	OH	OCH ₂ <i>t</i> -Bu	reflux	72	21	>10
18		OCH ₂ Ph	rt	8	81	8.3
19		OCH ₂ CH ₂ Ph	reflux	24	70	>10
20		OCH ₂ (CH ₂) ₂ Ph	reflux	24	91	>10
21		OCH ₂ <i>p</i> -MeOC ₆ H ₄	rt	12	72	8.1
22		OCH ₂ <i>p</i> -BnOC ₆ H ₄	rt	12	83	2.5
23		OCH ₂ <i>p</i> -NO ₂ C ₆ H ₄	rt	12	67	>10
24		OCH ₂ <i>p</i> -FC ₆ H ₄	rt	12	88	>10
25	OH	OCH ₂ 2,3,4,5,6-F ₅ C ₆	rt	12	82	>10
26		OCH ₂ <i>p</i> -CF ₃ C ₆ H ₄	rt	12	79	8.2
27		OCH ₂ <i>o</i> -CF ₃ C ₆ H ₄	rt	12	76	>10
28		OCH ₂ <i>m</i> -CF ₃ C ₆ H ₄	rt	12	68	>10
29	NH ₂	NHCH ₂ Ph	rt	12	54	8.5
30		N(CH ₂ Ph) ₂	rt	12	27	>10

^aCondition: (a) K₂CO₃, R-Cl, CH₃CN, temp, time, 21–93%.

core of stachyflin would not be suitable for scale-up synthesis in terms of the number of synthetic steps and the number of chiral centers. In this study, we provided two scaffolds exhibiting antiviral activity. Most of the compounds shown in Tables 2 and 3 were synthesized within 3 steps, except for compounds 37–39. Compounds 37–39 include chiral centers, and the synthesis of these compounds was achieved at most within 8 steps. Accordingly, the proposed scaffolds are feasible for diverse modulation of functional groups and then ones of the chemical bases for the development of HA inhibitors.

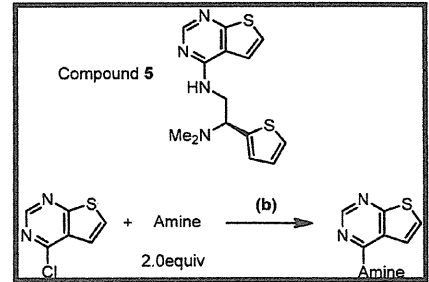
A requirement of antiviral drugs in clinical use is inhibitory activity for a broad range of viral strains. We tested the potency of several compounds synthesized in this study using A/Vietnam/1194/2004 (H5N1) strain,³⁶ which causes severe pathological conditions for humans and is one of the viruses attracting keen concern for the threat of a pandemic. A cell-based antiviral assay indicated that compounds 31 and 39 were effective for this H5N1 type virus with IC₅₀ values of 0.2 and 5.2 μM, respectively. This suggests that the scaffolds found in this study will maintain compound potency over different types of influenza viruses.

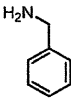


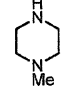
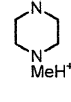
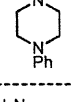
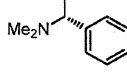
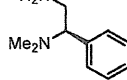
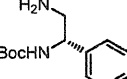
Plan of the Design of Potent Agents. In the *in silico* screening carried out in this study, 4 features, *i.e.*, hydrophobic region, hydrogen-bond donor, hydrogen-bond acceptor, and aromatic ring, were monitored for pharmacophore. Hit chemical compound 4 is compatible with stachyflin in the

three features of aromatic ring, hydrophobic region, and hydrogen-bond acceptor. Hit chemical compound 5 is compatible in all four features. All of the 8 selected compounds are compatible with stachyflin at least in two features, aromatic ring and hydrogen-bond acceptor. That is, these two features are commonly observed in pharmacophores of all selected compounds. This suggests that aromatic ring and hydrogen-bond acceptor are indispensable for inhibitors analogous to stachyflin. From the viewpoint of compatibility in the pharmacophore, compound 5 is the most advantageous for an HA inhibitor. In the binding modes of compounds 4 and 5 to the wild-type HA shown in Figure 3, compound 4 generated three hydrogen bonds with HA. In contrast, one hydrogen bond was observed between compound 5 and HA. The binding position of compound 5 is considerably close to that of stachyflin. These findings in binding mode suggest that the shape complementarity between compound 5 and the binding site in HA is high, while electrostatic complementarity is more significant in the binding of compound 4.

Methylester of compound 4 corresponds to carbonyl oxygen on ring E of stachyflin, and methoxy corresponds to the hydroxy group bound to ring D. It should be noted that rings DE of stachyflin compose a large flat region and that the vanillic acid core of compound 4 mimics the flat region and the distribution of polar atoms. Diazolane of compound 4 is compatible with ring A of stachyflin, and amine on the

Table 3. Structures and Inhibitory Activities of the Synthesized Analogues to Compound 5, Which Bears a Thieno-pyrimidine Core^a



Compound	Amine	Yield (%)	EC ₅₀ (μM)
31		45	3.6
32		62	>10
33		43	>10
34		54	>10
35		28	0.6
36		82	4.6
37		48	2.0
38		32	>10
39		7	4.8

^aCondition: (b) amine, NaOH, THF, reflux, 6 h.

diazolane corresponds to the hydroxy group bound to ring A. Compound 9 in Table 2, in which diazolane is replaced by methoxy, shows a considerably high inhibitory activity. Therefore, a polar group at the position of the diazolane is not necessarily required to maintain compound potency.

Sulfur and nitrogen atoms of thieno-pyrimidine moiety of compound 5 mimic the charge distribution of rings DE. It is notable that thieno-pyrimidine also composes a large flat

region. Another thiophene and dimethylamine are compatible with rings A and B of stachyflin, respectively. The high inhibitory activity of compound 35 in Table 3 suggests that thiophene is not necessarily required to maintain compound potency. This finding provides a sound explanation for the high inhibitory activity of compound 9. Accordingly, the region of ring A of stachyflin is not so important for inhibitory activity. Instead, the flat region at rings DE and the charge distribution on the flat region are essential for compound potency.

Compound 9 and 35 were docked to the wild-type HA and the three mutants in the same manner as stachyflin was. The predicted binding structures in Supplementary Figure S8 indicate that the binding site of compound 9 changed among the models. In contrast, compound 35 was bound to almost the same location among all models. The thieno-pyrimidine moiety is positioned near D109. This position is, however, different from that of stachyflin. The sizes of compounds 9 and 35 are small compared to stachyflin and compounds 4 and 5. Hence this smallness may be a reason for the incompatibility of the binding mode. To produce potent compounds appropriately fitted to the domain between inner helices of HA, compounds 9 and 35 should be converted with retention of the vanillic acid core and/or the thieno-pyrimidine moiety and with attachment of some chemical group containing a chiral center at the opposite side to cling to the helix round surface.

METHODS

Molecular Dynamics Simulation. Information on the structural difference between the wild-type HA and the mutants is essential for clarifying the reason why some amino acid mutations in HA diminish the inhibitory activity of stachyflin. The initial structure of the wild-type HA of WSN strain was constructed by homology modeling using Modeller ver. 9.4.³⁷ The multiple-alignment technique was employed, where the X-ray crystal structures of A/Puerto Rico/8/1934 H1N1 with PDB codes 1RVZ and 1RU7³⁸ and A/Brevig Mission/1/1918 H1N1 with code 1RD8³⁹ were selected for references in modeling. Homology modeling was also performed to build the initial structures for variants; K51R and K121E mutations were introduced in the HA2 subunit in mutant 1, V176I in HA1 and K51R, K121E in HA2 in mutant 2, and V176I in HA1 and K51R in HA2 in mutant 3. A lipid bilayer of 100 Å × 100 Å was generated by using an in-house software GLYMM implemented in VMD ver. 1.8⁴⁰ for the purpose of embedding the C-terminal side of HA2 subunit into the lipid membrane mimicking the viral envelope. The composition of lipid molecules in the membrane was set to be as compatible as possible with the composition of lipid molecules in the influenza viral envelope, as shown in Supplementary Table 1. Judging from the prediction results with UniProtKB,⁴¹ amino residues 186–206 were assumed to be the transmembrane region embedded in the viral envelope. It is natural to consider that an α helix structure is formed in the transmembrane region. However, there is no experimental ground for the formation of an α helix in this region. Accordingly, this region was set to have no secondary structure, that is, to be in a strand form in the initial structure of the present simulations. In our preliminary calculation without embedding the C-terminal side of HA2 subunit into the lipid membrane, each helix in HA2 gradually changed its conformation to separate itself from other helices. HA trimer was converted from a closed shape to an open one with the progress of simulation. The motion of the C-terminal region of HA2 was large in the preliminary calculation, which seemed to be a cause of structural instability and became a trigger for the drastic conformational change. That is, the helices of the HA trimer cannot maintain the closed form unless the C-terminal side of HA2 is embedded in the lipid membrane. Hence, calculation models in this work included the membrane mimicking the viral envelope and the transmembrane region of HA. TIP3P water molecules and ions to neutralize the calculation cell were generated to solvate the complex of HA and lipid membrane, making a

periodic boundary box of $100 \text{ \AA} \times 100 \text{ \AA} \times 250 \text{ \AA}$ in which the top and bottom parts of HA were set apart from the boundary by more than 10 \AA (Figure 1c). Consequently, the total number of atoms was about 259,300 in each model.

MD simulations were carried out for every model using NAMD ver. 2.6.⁴² Initially, energy minimization was executed for 1,000,000 steps with the conjugate gradient method. Next, the temperature of the model system was elevated up to 310 K. Then 30 ns equilibrating simulation was performed in the NTP ensemble condition to obtain the equilibrated structures of the wild-type HA and the three kinds of mutants. Nonbonded interaction terms were computed with a cutoff distance of 12 \AA , where a switching distance of 10 \AA was applied to make the nonbonded interaction zero at the cutoff distance smoothly. A periodic boundary condition was applied to all directions of the calculation cell, and the particle mesh Ewald method was employed to compute the long-distance nonbonded interaction. CHARMM27 force field⁴³ was adopted for all atoms.

Docking Simulation. The binding modes of stachyflin to the wild-type HA and its mutants were predicted by docking simulation using GOLD ver. 4.^{44,45} The equilibrated structure obtained from the MD simulation was utilized as a plausible protein structure of HA in the wild-type and three mutants. Binding score was also calculated to evaluate the difference in binding affinity due to the mutations. A preliminary docking calculation was executed to search for the docking area within 30 \AA from Phe110 of the HA2 subunit. Since an adequate docking space was found inside the area and stachyflin was positioned near Asp109 in the binding mode ranking first, recalculation of stachyflin docking was performed with the search area set within 10 \AA from Asp109 of HA2 subunit. Fifty binding poses were generated and the binding affinities of those binding poses were estimated by GOLD score function. On the basis of the ranking in the estimated GOLD score, the most probable binding pose was selected. The binding affinity for the selected binding pose was re-estimated using ASP score function. This two-step approach, *i.e.*, determination of the binding pose with GOLD score and subsequent estimation of binding affinity with ASP score, was reported to successfully provide reliable prediction in docking of low-molecular-weight ligands to an enzyme or receptor.^{46,47}

In Silico Screening. A search for compounds bearing chemical features similar to those of stachyflin was made by an *in silico* pharmacophore screening. A chemical database was provided by Namiki Co. Ltd., in which about 3 million synthesized compounds are listed and all of the compounds are available by purchase. First, conformations of every compound were generated by using OMEGA module of OpenEye software.⁴⁸ Totally, more than 200 million chemical conformations were generated. Second, the pharmacophore of stachyflin was extracted for setting queries, in which hydrogen-bond donor and acceptor, aromatic ring, and hydrophobic region appeared to be key features. Third, chemical screening was carried out from the viewpoint of structural similarity to stachyflin using ROCS module of OpenEye.⁴⁹ A total of 5094 compounds were extracted from the Namiki database under the condition of the Tanimoto coefficient being more than 0.75. Fourth, more condensed selection of chemicals from the 5094 compounds was performed using EON module⁴⁹ from the viewpoint of similarity in charge distribution. The compounds without structural flexibility were excluded. Consequently, 8 chemical compounds were selected as candidates for purchase.

Synthesis of Analogue Compounds. Two series of derivatives were synthesized in this work. One is an analogue containing a vanillic acid skeleton, and the other is one bearing thieno-pyrimidine. Compound 9, 3-methoxy-4-[(methyl-sulfonyl)oxy]-benzoic acid methoxy-ester, is a typical derivative of the former series. A mixture of vanillic acid methyl (1.0 g, 5.49 mmol), KCO_3 (1.13 g, 8.24 mmol), and acetonitrile (30 mL) was cooled in an ice bath under Ar atmosphere. Methane-sulfonyl chloride (0.51 mL, 6.59 mmol) was slowly added to the mixture, and then the solution was mechanically stirred for 3 h at RT. The reaction mixture was filtered with Celite, and the solvent was removed *in vacuo*. The resulting product was extracted with a solution of EtOAc (30 mL) and 1 M aqueous HCl (30 mL). The aqueous layer was treated with EtOAc (20 mL) two times, and

the combined organic layer was washed with brine, dried over MgSO_4 , and concentrated *in vacuo*. The product was purified by thin-layer chromatography with hexane/EtOAc in a ratio of 3:1. The solid obtained was resuspended with a solution of hexane and EtOAc, and recrystallization produced the final compound as a white solid (1.01 g, yield 71%).

A typical derivative of the latter series is compound 35, 4-(4-methyl-1-piperazinyl)-thieno[2,3-*d*]pyrimidine hydrochloride salt. 4-Chloro-thieno[2,3-*d*]pyrimidine (200 mg, 1.17 mmol) was solvated with THF (15 mL). After addition of 1-methyl-piperazine (0.26 mL, 2.34 mmol) and NaOH (0.94 g, 2.34 mmol), the mixture was heated under reflux for 6 h. The solvent was removed *in vacuo*, and the reaction mixture was treated with a solution of EtOAc (20 mL) and distilled H_2O (15 mL). The product was extracted with EtOAc two times, and the organic layer was washed with brine and dried over Na_2SO_4 . The solvent was evaporated *in vacuo*, and the resulting product was purified by two-dimensional thin-layer chromatography with hexane/EtOAc in ratios of 3:1 and 1:1. A brown solid of (phenyl piperazinyl)-thienopyrimidine was obtained in a yield of 54% (148 mg). The solid obtained was resuspended in a solution of toluene (20 mL) and 1 M aqueous HCl (0.6 mL) and heated under reflux for 1 h. The reaction solution was cooled to RT, and filtration gave the final compound as a brownish solid (127 mg, yield 45%).

Antiviral Assay. Compound potency was tested by an influenza virus cell culture assay with measurement of the quantity of viral RNA using real-time polymerase chain reaction (RT-PCR). Test compounds were mixed with minimum essential medium (MEM) containing bovine serum albumin (BSA). To prepare a virus-containing compound-mixed medium, 100 units of 50% tissue culture infective dose (TCID₅₀) of influenza virus A/Puerto Rico/8/34 strain (PR8) was suspended in 100 μL of the MEM-BSA containing test compounds and 10 $\mu\text{g}/\text{mL}$ of acetylated trypsin. Madin-Darby canine kidney (MDCK) cells were loaded in a 96-well plate. The cells were washed with phosphate-buffered saline (PBS), followed by 0.5–1 h incubation with compound-mixed medium. The virus-containing compound-mixed medium was also incubated for 0.5–1 h. After incubation, MDCK cells in the compound-mixed medium without viruses were transferred to the compound-mixed medium with viruses. Then the cells were incubated for 1 h at $37 \text{ }^\circ\text{C}$ in 100 μL of the virus-containing compound-mixed medium (100 TCID₅₀ influenza virus, 10 $\mu\text{g}/\text{mL}$ acetylated trypsin, and test compound at several concentrations). MDCK cells were washed with compound-mixed medium without viruses and incubated in the compound-mixed, acetylated trypsin-containing medium without influenza virus for 24 h. Culture supernatants of MDCK cells were collected after the incubation, and RNA was extracted from the supernatants. The amount of viral RNA was measured by the RT-PCR method. The measurement was compared to that of the control that was performed in a similar manner without any test compound. The compound concentration to suppress viral proliferation to 50% (EC_{50}) was estimated from the comparison.

RNA Extraction and RT-PCR. To monitor the efficiency of RNA purification, uninfected VeroE6 cells were mixed in the ISOGEN reagent as a source of 18S rRNA for normalization. Supernatants from MDCK cell culture medium were mixed with ISOGEN reagent and RNA was purified according to the manufacturer's protocol. For quantification of PR8 HA RNA, real-time RT-PCR was performed using the primers and the probe with the sequences of PR8-HA-F: 5'-GGCAAATGGAAATCTAATAGCACC-3', PR8-HA-R: 5'-TGATGCTTTTGAGGTGATGA-3', and PR8-HA-probe: 5'-FAM-TCGCACTGAGTAGAGGCTTTGGGTCC-TAMRA-3'. For monitoring efficiency of RNA purification, 18S rRNA was quantified using the primers and the probe with the sequences of 18S-F: 5'-GTAACCCGTTGAACCCATT-3', 18S-R: 5'-CCATCCAATCGG-TAGTAGCG-3', and 18S-probe: 5'-FAM-TGCGTTGAT-TAGTCCCTGCCCTTTGTA-TAMRA-3'. The intensity of fluorescence emitted from the probe was detected by the ABI-7700 sequence detector system (Applied Biosystems).

■ ASSOCIATED CONTENT

📄 Supporting Information

Composition of lipid membrane models, predicted binding affinities, RMSDs during MD simulation, predicted binding structures of stachyflin, superimposition of HA structures, chemical properties of synthesized compounds, and their NMR spectra. This material is available free of charge *via* the Internet at <http://pubs.acs.org>.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: (N.Y.) n-yama-5@nih.go.jp; (T.H.) hoshino@chiba-u.jp.

Author Contributions

#These authors contributed equally to this work.

■ ACKNOWLEDGMENTS

We thank L.Q. Mai (National Institute of Hygiene and Epidemiology, Hanoi) for providing the A/Vietnam/1194/04 virus strain. This work was partly supported by a Health and Labor Science Research Grant for Research from the Ministry of Health and Labor of Japan. This work was also supported by a Grant-in-Aid for Scientific Research (C) from Japan Society for the Promotion of Science (JSPS). This study was also supported in part by a grant-in-aid (S0991013) from the Ministry of Education, Culture, Sport, Science, and Technology, Japan (MEXT) for the Foundation of Strategic Research Projects in Private Universities. Theoretical calculations were performed at the Research Center for Computational Science, Okazaki, Japan and at the Information Technology Center of the University of Tokyo and also by the high-performance computer system at Institute for Media Information Technology in Chiba University.

■ REFERENCES

- Gambotto, A.; Barratt-Boyes, S. M.; de Jong, M. D.; Neumann, G.; and Kawaoka, Y. (2008) Human infection with highly pathogenic H5N1 influenza virus. *Lancet* 371, 1464–1475.
- Beigel, J. H., Farrar, J., Han, A. M., Hayden, F. G., Hyer, R., de Jong, M. D., Lochindarat, S., Nguyen, T. K. T., Nguyen, T. H., Tran, T. H., Nicoll, A., Touch, S., Yuen, K. Y., and Writing Committee of the World Health Organization Consultation on Human Influenza A/H5. (2005) Avian influenza A (H5N1) infection in humans. *N. Engl. J. Med.* 353, 1374–1385.
- Schnell, J. R., and Chou, J. J. (2008) Structure and mechanism of the M2 proton channel of influenza A virus. *Nature* 451, 591–595.
- Stouffer, A. L., Acharya, R., Salom, D., Levine, A. S., Di Costanzo, L., Soto, C. S., Tereshko, V., Nanda, V., Stayrook, S., and DeGrado, W. F. (2008) Structural basis for the function and inhibition of an influenza virus proton channel. *Nature* 451, 596–599.
- McNicholl, I. R., and McNicholl, J. J. (2001) Neuraminidase inhibitors: zanamivir and oseltamivir. *Ann. Pharmacother.* 35, 57–70.
- Moscona, A. (2005) Neuraminidase inhibitors for influenza. *N. Engl. J. Med.* 353, 1363–1373.
- Bright, R. A., Medina, M.-j., Xu, X., Perez-Orozco, G., Wallis, T. R., Davis, X. M., Povinelli, L., Cox, N. J., and Klimov, A. I. (2005) Incidence of adamantane resistance among influenza A (H3N2) viruses isolated worldwide from 1994 to 2005: a cause for concern. *Lancet* 366, 1175–1181.
- Deyde, V. M., Xu, X., Bright, R. A., Shaw, M., Smith, C. B., Zhang, Y., Shu, Y., Gubareva, L. V., Cox, N. J., and Klimov, A. I. (2007) Surveillance of resistance to adamantanes among influenza A(H3N2) and A(H1N1) viruses isolated worldwide. *J. Infect. Dis.* 196, 249–257.
- Kitahori, Y., Imanishi, Y., and Inoue, Y. (2008) High incidence of amantadine-resistant influenza H1N1 viruses isolated during the

2007–2008 season in Nara Prefecture, Japan. *Jpn. J. Infect. Dis.* 61, 253–254.

(10) Kamiguchi, T.; Fujiwara, T.; Tani, H.; Kawamura, Y.; Horibe, I. Shionogi & Co, Ltd. Sesquiterpene derivatives having antiviral activity, Patent WO/1997/011947, 1997.

(11) Taishi, T., Takechi, S., and Mori, S. (1998) First total synthesis of (±)-stachyflin. *Tetrahedron Lett.* 39, 4347–4350.

(12) Yoshimoto, J., Kakui, M., Iwasaki, H., Fujiwara, T., Sugimoto, H., and Hattori, N. (1999) Identification of a novel HA conformational change inhibitor of human influenza virus. *Arch. Virol.* 144, 865–878.

(13) Yoshimoto, J., Kakui, M., Iwasaki, H., Sugimoto, H., Fujiwara, T., and Hattori, N. (2000) Identification of amino acids of influenza virus HA responsible for resistance to a fusion inhibitor, Stachyflin. *Microbiol. Immunol.* 44, 677–685.

(14) Rosenbaum, D. M., Cherezov, V., Hanson, M. A., Rasmussen, S. G. F., Thian, F. S., Kobilka, T. S., Choi, H. J., Yao, H. J., Weis, W. I., Stevens, R. C., and Kobilka, B. K. (2007) GPCR engineering yields high-resolution structural insights into β 2-adrenergic receptor function. *Science* 318, 1266–1273.

(15) Wegener, A. A., Klare, J. P., Engelhard, M., and Steinhoff, H. J. (2001) Structural insights into the early steps of receptor-transducer signal transfer in archaeal phototaxis. *EMBO J.* 20, 5312–5319.

(16) Sato, Y., Hata, M., Neya, S., and Hoshino, T. (2005) Computational analysis of the transient movement of helices in sensory rhodopsin II. *Protein Sci.* 14, 183–192.

(17) Cross, K. J., Burleigh, L. M., and Steinhauer, D. A. (2001) Mechanisms of cell entry by influenza virus. *Exp. Rev. Mol. Med.* 3, 1–15.

(18) Bodian, D. L., Yamasaki, R. B., Buswell, R. L., Stearns, J. F., White, J. M., and Kuntz, I. D. (1993) Inhibition of the fusion-inducing conformational change of influenza hemagglutinin by benzoquinones and hydroquinones. *Biochemistry* 32, 2967–2978.

(19) Luo, G., Colonna, R., and Krystal, M. (1996) Characterization of hemagglutinin-specific inhibitor of influenza A virus. *Virology* 226, 66–76.

(20) Plotch, S. J., O'Hara, B., Morin, J., Palant, O., LaRocque, J., Bloom, J. D., Lang, S. A. Jr., DiGrandi, M. J., Bradley, M., Nilakantan, R., and Gluzman, Y. (1999) Inhibition of influenza A virus replication by compounds interfering with the fusogenic function of the viral hemagglutinin. *J. Virol.* 73, 140–151.

(21) Staschke, K. A., Hatch, S. D., Tang, J. C., Hornback, W. J., Munroe, J. E., Colacino, J. M., and Muesing, M. A. (1998) Inhibition of influenza virus hemagglutinin-mediated membrane fusion by a compound related to podocarpic acid. *Virology* 248, 264–274.

(22) Hoffman, L. R., Kuntz, I. D., and White, J. M. (1997) Structure-based identification of an inducer of the low-pH conformational change in the influenza virus hemagglutinin: Irreversible inhibition of infectivity. *J. Virol.* 71, 8808–8820.

(23) Matsubara, T., Onishi, A., Saito, T., Shimada, A., Inoue, H., Taki, T., Nagata, K., Okahata, Y., and Sato, T. (2010) Sialic acid-mimic peptides as hemagglutinin inhibitors for anti-influenza therapy. *J. Med. Chem.* 53, 4441–4449.

(24) Feng, F., Miura, N., Isoda, N., Sakoda, Y., Okamatsu, M., Kida, H., and Nishimura, S. (2010) Novel trivalent anti-influenza reagent. *Bioorg. Med. Chem. Lett.* 20, 3772–3776.

(25) Tang, G., Lin, X., Qiu, Z., Li, W., Zhu, L., Wang, L., Li, S., Li, H., Lin, W., Yang, M., Guo, T., Chen, L., Lee, D., Wu, J. Z., and Yang, W. (2011) Design and synthesis of benzenesulfonamide derivatives as potent anti-influenza hemagglutinin inhibitors. *ACS Med. Chem. Lett.* 2, 603–607.

(26) Sawada, T., Fedorov, D. G., and Kitaura, K. (2010) Role of the key mutation in the selective binding of avian and human influenza hemagglutinin to sialosides revealed by quantum-mechanical calculations. *J. Am. Chem. Soc.* 132, 16862–16872.

(27) Takematsu, K., Fukuzawa, K., Omagari, K., Nakajima, S., Nakajima, K., Mochizuki, Y., Nakano, T., Watanabe, H., and Tanaka, S. (2009) Possibility of mutation prediction of influenza hemagglutinin

by combination of hemadsorption experiment and quantum chemical calculation for antibody binding. *J. Phys. Chem. B* 113, 4991–4994.

- (28) Newhouse, E. I., Xu, D., Markwick, P. R., Amaro, R. E., Pao, H. C., Wu, K. J., Alam, M., McCammon, J. A., and Li, W. W. (2009) Mechanism of glycan receptor recognition and specificity switch for avian, swine, and human adapted influenza virus hemagglutinins: a molecular dynamics perspective. *J. Am. Chem. Soc.* 131, 17430–17442.
- (29) Kasson, P. M., Ensign, D. L., and Pande, V. S. (2009) Combining molecular dynamics with bayesian analysis to predict and evaluate ligand-binding mutations in influenza hemagglutinin. *J. Am. Chem. Soc.* 131, 11338–11340.
- (30) Das, P., Li, J., Royyuru, A. K., and Zhou, R. (2009) Free energy simulations reveal a double mutant avian H5N1 virus hemagglutinin with altered receptor binding specificity. *J. Comput. Chem.* 30, 1654–1663.
- (31) Xu, D., Newhouse, E. I., Amaro, R. E., Pao, H. C., Cheng, L. S., Markwick, P. R., McCammon, J. A., Li, W. W., and Arzberger, P. W. (2009) Distinct glycan topology for avian and human sialopentacaccharide receptor analogues upon binding different hemagglutinins: a molecular dynamics perspective. *J. Mol. Biol.* 387, 465–491.
- (32) Fleishman, S. J., Whitehead, T. A., Ekiert, D. C., Dreyfus, C., Corn, J. E., Strauch, E. M., Wilson, I. A., and Baker, D. (2011) Computational design of proteins targeting the conserved stem region of influenza hemagglutinin. *Science* 332, 816–821.
- (33) Watanabe, K., Sakurai, J., Abe, H., and Katoh, T. (2010) Total synthesis of (+)-stachyflin: a potential anti-influenza A virus agent. *Chem. Commun.* 46, 4055–4057.
- (34) Nakatani, M., Nakamura, M., Suzuki, A., Inoue, M., and Katoh, T. (2002) A new strategy toward the total synthesis of stachyflin, a potent anti-influenza A virus agent: concise route to the tetracyclic core structure. *Org. Lett.* 4, 4483–4486.
- (35) Carey, J. S., Laffan, D., Thomson, C., and Williams, M. T. (2006) Analysis of the reactions use for the preparation of drug candidate molecules. *Org. Biomol. Chem.* 4, 2337–2347.
- (36) Mizukami, T., Imai, J., Hamaguchi, I., Kawamura, M., Momose, H., Naito, S., Maeyama, J., Masumi, A., Kuramitsu, M., Takizawa, K., Nomura, N., Watanabe, S., and Yamaguchi, K. (2008) Application of DNA microarray technology to influenza A/Vietnam/1194/2004 (H5N1) vaccine safety evaluation. *Vaccine* 26, 2270–2283.
- (37) Sali, A., and Blundell, T. L. (1993) Comparative protein modelling by satisfaction of spatial restraints. *J. Mol. Biol.* 234, 779–815.
- (38) Gamblin, S. J., Haire, L. F., Russell, R. J., Stevens, D. J., Xiao, B., Ha, Y., Vasisht, N., Steinhauer, D. A., Daniels, R. S., Elliot, A., Wiley, D. C., and Skehel, J. J. (2004) The structure and receptor binding properties of the 1918 influenza hemagglutinin. *Science* 303, 1838–1842.
- (39) Stevens, J., Corper, A. L., Basler, C. F., Taubenberger, J. K., Palese, P., and Wilson, I. A. (2004) Structure of the uncleaved human H1 hemagglutinin from the extinct 1918 influenza virus. *Science* 303, 1866–1870.
- (40) Humphrey, W., Dalke, A., and Schulten, K. (1996) VMD: visual molecular dynamics. *J. Mol. Graph.* 14, 33–38.
- (41) The UniProt Consortium. (2011) Ongoing and future developments at the Universal Protein Resource. *Nucleic Acids Res.* 39, D214–D219.
- (42) Phillips, J. C., Braun, R., Wang, W., Gumbart, J., Tajkhorshid, E., Villa, E., Chipot, C., Skeel, R. D., Kalé, L., and Schulten, K. (2005) Scalable molecular dynamics with NAMD. *J. Comput. Chem.* 26, 1781–1802.
- (43) Feller, S. E., Gawrisch, K., and MacKerell, A. D. (2002) Polyunsaturated fatty acids in lipid bilayers: intrinsic and environmental contributions to their unique physical properties. *J. Am. Chem. Soc.* 124, 318–326.
- (44) Jones, G., Willett, P., Glen, R. C., Leach, A. R., and Taylor, R. (1997) Development and validation of a genetic algorithm for flexible docking. *J. Mol. Biol.* 267, 727–748.

(45) Jones, G., Willett, P., and Glen, R. C. (1995) Molecular recognition of receptor sites using a genetic algorithm with a description of desolvation. *J. Mol. Biol.* 245, 43–53.

(46) Verdonk, M. L., Cole, J. C., Hartshorn, M. J., Murray, C. W., and Taylor, R. D. (2003) Improved protein-ligand docking using GOLD. *Proteins: Struct., Funct., Genet.* 52, 609–623.

(47) Mooij, W. T. M., and Verdonk, M. L. (2005) General and targeted statistical potentials for protein-ligand interactions. *Proteins: Struct., Funct., Genet.* 61, 272–281.

(48) Bostrom, J., Greenwood, J. R., and Gottfries, J. (2003) Assessing the performance of OMEGA with respect to retrieving bioactive conformations. *J. Mol. Graphics Modell.* 21, 449–462.

(49) OpenEye Scientific Software, Inc., Santa Fe, NM, USA, www.eyesopen.com.

Original Article

Newly Established Monoclonal Antibodies for Immunological Detection of H5N1 Influenza Virus

Kazuo Ohnishi¹, Yoshimasa Takahashi¹, Naoko Kono², Noriko Nakajima³, Fuminori Mizukoshi¹, Shuhei Misawa⁴, Takuya Yamamoto¹, Yu-ya Mitsuki¹, Shu-ichi Fu¹, Nakami Hirayama¹, Masamichi Ohshima¹, Manabu Ato¹, Tsutomu Kageyama², Takato Odagiri², Masato Tashiro², Kazuo Kobayashi¹, Shigeyuki Itamura², and Yasuko Tsunetsugu-Yokota^{1*}

¹*Department of Immunology, ²Influenza Virus Research Center, and ³Department of Pathology, National Institute of Infectious Diseases, Tokyo 162-8640; and ⁴Tsuruga Institute of Biotechnology, Toyobo, Co., Ltd., Fukui 914-8550, Japan*

(Received October 4, 2011. Accepted October 28, 2011)

SUMMARY: The H5N1 subtype of the highly pathogenic (HP) avian influenza virus has been recognized for its ability to cause serious pandemics among humans. In the present study, new monoclonal antibodies (mAbs) against viral proteins were established for the immunological detection of H5N1 influenza virus for research and diagnostic purposes. B-cell hybridomas were generated from mice that had been hyperimmunized with purified A/Vietnam/1194/2004 (NIBRG-14) virion that had been inactivated by UV-irradiation or formaldehyde. After screening over 4,000 hybridomas, eight H5N1-specific clones were selected. Six were specific for hemagglutinin (HA) and had in vitro neutralization activity. Of these, four were able to broadly detect all tested clades of the H5N1 strains. Five HA-specific mAbs detected denatured HA epitope(s) in Western blot analysis, and two detected HP influenza virus by immunofluorescence and immunohistochemistry. A highly sensitive antigen-capture sandwich ELISA system was established by combining mAbs with different specificities. In conclusion, these mAbs may be useful for rapid and specific diagnosis of H5N1 influenza. Therapeutically, they may have a role in antibody-based treatment of the disease.

INTRODUCTION

The highly pathogenic (HP) H5N1 avian influenza virus caused the first outbreak in humans in Hong Kong in 1997. This outbreak resulted in the infection of 18 people and resulted in six deaths (1,2). Thereafter, it was determined that H5N1 avian influenza virus was continuously circulated among geese in Southeastern China. Eventually, it spread to other Southeast Asian countries, where it severely damaged poultry farms (3,4). Subsequent H5N1 outbreaks in humans occurred in China and Vietnam in 2003 and in Indonesia in 2005. The most recent endemic has occurred in Egypt. According to a World Health Organization report, the H5N1 avian influenza virus had infected 565 people and resulted in 331 deaths by August 19, 2011 (5). Therefore, although sporadic, this fatal human infection is persistent and has the potential to cause serious future pandemics.

In humans, infection with HP H5N1 avian influenza virus causes high fever, coughing, shortness of breath, and radiological findings of pneumonia (6–8). In severe cases, rapidly progressive bilateral pneumonia develops, causing respiratory failure and may be responsible for the high mortality associated with this virus. de Jong et

al. analyzed human cases of H5N1 infection and found that a high viral load and the resulting intense inflammatory response caused severe symptoms; furthermore, viral RNA was frequently detected in the rectum, blood, and nasopharynx (9). Thus, it is essential to detect HP influenza virus infection early and rapidly in order to provide early interventions that protect patients from devastating respiratory failure that arises from a high viral load. Additionally, early viral detection would facilitate rapid identification of infected patients and prevent unregulated contact with other people.

The present diagnostic standard for HP H5N1 influenza is the presence of the neutralization antibody. However, it takes more than 1 week for H5N1-specific antibodies to develop, and a well-equipped biosafety level 3 (BSL3) laboratory is required for the virus neutralization assay. A simpler method is the hemagglutination-inhibition assay using horse erythrocyte. This method has been widely performed on paired acute and convalescent sera from patients with HP H5N1 influenza virus infections. Although this method has acceptable sensitivity, its specificity has been questioned (7).

Isolating the virus from patient samples is the gold standard for diagnosing an infection; however, this is not always possible. For example, the method of sample preparation and preservation strongly influence the ability to isolate the virus. Moreover, a BSL3 laboratory is essential. At present, the most sensitive and rapid method for initial diagnosis of H5N1 virus infections is by conventional or real-time reverse-transcriptase polymerase chain reaction (RT-PCR). However, this proce-

*Corresponding author: Mailing address: Department of Immunology, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan. Tel: +81-3-5285-1111, Fax: +81-5285-1150, E-mail: yyokota@nih.go.jp

dures requires expertise in molecular virology and expensive equipment and reagents. Moreover, because of its high sequence specificity, this approach could fail to identify mutant influenza viruses that continually evolve due to a high mutation rate (8).

For screening suspected H5N1 influenza virus in the field, the ideal approach would be to employ an immunology-based technique that detects viral antigens. Such a method is simple and rapid. However, its sensitivity and specificity depend highly on the antibodies used. Thus, an immunological assay that uses appropriate specific antibodies against H5N1 in combination with specific antibodies against other subtypes of influenza virus or viruses that cause febrile diseases would be useful for screening in areas with endemic influenza-like illness. While there are several rapid influenza virus diagnostic systems available for seasonal influenza (10), few exist for H5N1 influenza. Therefore, we have developed a simple and rapid diagnostic system with high sensitivity and specificity for H5N1 influenza virus.

Influenza virus belongs to the family *Orthomyxoviridae*; its genome consists of a negative-sense, single-stranded RNA with eight segments, each encoding structural and non-structural proteins (11). Influenza A viruses are classified into several subtypes based on the hemagglutinin (HA) and neuraminidase (NA) serotypes. In total, there are 16 HA and 9 NA serotypes. The H5N1 viruses are divided into clades 1 and 2 based on their HA genotypes. Clade 2 has been further subdivided into five sub-clades (12). Clade 1 viruses were predominant in Vietnam, Thailand, and Cambodia in the early phase of the 2004–2005 outbreak, whereas clade 2.1 viruses were endemic in Indonesia at that time (8). These two viruses are the major prototypes for the preparation of pre-pandemic H5N1 vaccines. We used inactivated purified clade 1 virion [A/Vietnam/1194/2004 (NIBRG-14)] as an immunizing antigen to establish mouse monoclonal antibodies (mAbs) specific for H5N1 influenza virus. Characterization of these mAbs revealed that they could detect H5N1 viruses when used in an immunofluorescence staining assay (IFA), Western blotting analysis, immunohistochemistry, and antigen-capture sandwich ELISA. In addition, the mAbs had significant *in vitro* neutralization activity against H5N1 viruses, and some broadly detected both clade 1 and 2 viruses.

MATERIALS AND METHODS

Viruses and cell culture: The NIBRG-14 (H5N1) virus, which possesses modified HA and NA genes derived from the A/Vietnam/1194/2004 strain on the backbone of six internal genes of A/Puerto Rico/8/34 (PR8), was provided by the National Institute for Biological Standards and Controls (NIBSC; Potters Bar, UK). A/Indonesia/05/2005 (Indo5/PR-8-RG2), A/Turkey/1/2005 (NIBRG-23), A/Anhui/01/2005 (Anhui01/PR8-RG5) were also obtained from NIBSC. All non-H5N1 strains were obtained from a stockpile of seed vaccines of the Influenza Virus Research Center of the National Institute of Infectious Diseases. The live virus was manipulated in a BSL2 laboratory. To produce and purify the virion, the NIBRG-14 and PR8 viruses were propagated in the allantoic cavity of 10-day-old

embryonated hens' eggs and purified through a 10–50% discontinuous sucrose gradient by ultracentrifugation (13). The viruses were then resuspended in phosphate-buffered saline (PBS) and inactivated by ultraviolet (UV) irradiation or by treatment with 0.05% formalin at 4°C for 2 weeks. These preparations were served as the inactivated H5N1 virus fraction. These conditions have been previously shown to completely inactivate H5N1 viruses.

Production of mAbs: Nine-week-old female BALB/c mice (Japan SLC, Shizuoka, Japan) were immunized subcutaneously with 20 µg of UV- or formaldehyde-inactivated NIBRG-14 (H5N1) virus using Freund's Complete Adjuvant (Sigma, St. Louis, Mo., USA). Two weeks later, the mice were boosted with a subcutaneous injection of 5 µg of the inactivated virus emulsified with Freund's Incomplete Adjuvant (Sigma). Three days after the boost, sera from the mice were tested by ELISA to determine the antibody titer against the NIBRG-14 virus. The three mice with the highest antibody titers were given an additional boost 14 days after the first boost by intravenous injection of 5 µg of the inactivated virus. Three days later, the spleens of these three mice were excised, and the spleen cells were fused with Sp2/O-Ag14 myeloma cells using the polyethylene glycol method of Kozbor and Roder (14). The fused cells were cultured on twenty 96-well plates and selected with hypoxanthine-aminopterin-thymidine (HAT) medium. The first screening was conducted by ELISA using formalin-inactivated purified NIBRG-14 (H5N1) and PR-8 (H1N1) virions, which were lysed with 1% Triton X100. The lysates (1 mg/ml) were diluted 2,000-fold with ELISA-coating buffer (50 mM sodium bicarbonate, pH 9.6), and the ELISA plates (Dyna-tek, Chantilly, Va., USA) were coated at 4°C overnight. After blocking with 1% ovalbumin in PBS-Tween (10 mM phosphate buffer, 140 mM NaCl, 0.05% Tween 20, pH 7.5) for 1 h, the culture supernatants of the HAT-selected hybridomas were added and incubated for 1 h. After washing with PBS-Tween, the bound antibodies were detected using alkaline phosphatase-conjugated anti-mouse IgG (1:2,000; Zymed, South San Francisco, Calif., USA) and *p*-nitrophenyl phosphate, which served as a substrate. In this first screening, hybridomas that reacted to the H5N1 virus (NIBRG-14) but not to the H1N1 virus (PR-8) were selected.

Baculoviral expression of recombinant HA and NA: Recombinant HA (rHA) and NA (rNA) proteins were produced as previously described (13). Briefly, the HA- and NA-coding genes of NIBRG-14 were amplified by PCR to attach a 6x-His tag to the C terminus of HA and to the N terminus of NA. The amplified DNAs were then cloned into pBacPAK8 (Clontech, Mountain View, Calif., USA) and transfected into Sf-21 (*Spodoptera frugiperda*) insect cells. Recombinant baculoviruses containing the rHA and rNA genes were isolated and used to infect Sf-21 cells. The recombinant proteins tagged with 6x-His were purified with TALON columns (Clontech) according to the manufacturer's protocol.

Neutralization assay: For the neutralization assay, 100 TCID₅₀ of H5N1 virus, a standard tissue culture infectious dose for such assays, was incubated for 30 min at 37°C in the presence or absence of the purified mAbs, which had been serially diluted twofold. The viruses

were then added to MDCK cell cultures that had been grown to confluence in a 96-well microtiter plate. The virus strains used were A/Vietnam/1194/2004 (NIBRG-14) (H5N1) (clade 1), A/Indonesia/05/2005 (Ind05/PR8-RG2) (H5N1) (clade 2.1), A/Turkey/1/2005 (NIBRG-23) (H5N1) (clade 2.2), and A/Anhui/01/2005 (Anhui01/PR8-RG5) (H5N1) (clade 2.3). After 3–5 days, the cells were fixed with 10% formaldehyde and stained with crystal violet to visualize the cytopathic effects induced by the virus (15). Neutralization antibody titers were expressed as the minimum concentration of purified immunoglobulin that inhibited a cytopathic effect.

Western blot analysis: UV-inactivated purified H5N1 virus (0.5 $\mu\text{g}/\text{lane}$) was loaded on SDS-PAGE gels under reducing conditions. The proteins were then transferred to a PVDF membrane (Genetics, Tokyo, Japan). After blocking with BlockAce reagent (Snow Brand Milk Products Co., Tokyo, Japan), the membranes were detected with the mAbs or diluted sera (1:1,000) that had been obtained from mice immunized with UV-irradiated H5N1 virus. After washing, the membrane was reacted with the peroxidase-conjugated F(ab')₂ fragment of anti-mouse IgG (H + L) (1:20,000; Jackson ImmunoResearch, West Grove, Pa., USA), and the bands were visualized on X-ray film (Kodak, Rochester, N.Y., USA) with chemiluminescent reagents (Amersham Biosciences, Piscataway, N.J., USA).

Purification and biotinylation of mAbs: Hybridomas were grown in Hybridoma-SFM medium (Invitrogen, Carlsbad, Calif., USA) supplemented with recombinant IL-6, penicillin (100 U/mL), and streptomycin (100 $\mu\text{g}/\text{mL}$) (16). The culture supernatants were harvested, and 1/100 volume of 1 M Tris-HCl (pH 7.4) and 1/500 volume of 10% Na₂SO₄ were applied directly on a Protein G-Sepharose 6B column (Amersham Biosciences). The column was washed with PBS and eluted with glycine/HCl (pH 2.8). After measuring the OD₂₈₀ of the fractions, the protein-containing fractions were pooled, and an equal volume of saturated (NH₄)₂SO₄ was added. The precipitated proteins were dissolved in PBS, dialyzed against PBS, and stored at -20°C. The purified antibodies were biotinylated with sulfo-NHS-LC-biotin (Pierce, Rockford, Ill., USA) according to the manufacturer's protocol.

Antigen-capture ELISA: The purified antigen-capturing mAb was immobilized on a microplate (Immulon 2; Dynatech) by incubating 4 $\mu\text{g}/\text{mL}$ of the mAb in 50 mM sodium bicarbonate buffer (pH 8.6) at 4°C overnight. The microplate was blocked with 1% BSA, washed with PBS-Tween, and reacted with serial dilutions of UV-inactivated purified H5N1 virus for 1 h at room temperature. After washing with PBS-Tween, biotinylated probing mAb (0.1 $\mu\text{g}/\text{mL}$) was added to the wells for 1 h at room temperature. After washing, horseradish peroxidase (HRP)-labeled streptavidin (Zymed) was added to the wells for 1 h at room temperature. After washing, 0.4 mg/mL *o*-phenylenediamine (OPD Sigma P-8412) in OPD Buffer (0.05 M citrate-phosphate buffer pH 5.0, 0.04% H₂O₂) or TMB(+) substrate (DAKO, Kyoto, Japan) was added. The reaction was stopped by adding 2N H₂SO₄, and the OD₄₉₀ or OD₄₅₀ was measured using a multi-well plate reader (Flow Laboratories Inc., Inglewood, Calif., USA).

Immunohistochemistry: Lung tissues were harvested from mice infected with A/Vietnam/1194/2004 (NIBRG-14) or A/HongKong/483/97 (HK483). In addition, autopsied lung tissues of patients infected with influenza virus (H1N1 or 2009 H1N1pdm) were used. Formaldehyde- or formalin-fixed paraffin-embedded lung tissue sections were deparaffinized with xylene and graded ethanol and then autoclaved in 0.1 M citrate-buffer (pH 6.0) at 121°C for 10 min to retrieve the antigens. Endogenous peroxidase was inactivated with 0.3% hydrogen peroxide for 30 min at room temperature. After blocking with M.O.M. blocking reagent (Vector laboratories, Burlingame, Calif., USA) or 5% goat serum, the sections were incubated with each of the mouse mAbs or rabbit polyclonal antibody against type A influenza nucleoprotein at 4°C overnight. After washing off the excess antibodies, the sections were incubated with HRP-labeled anti-mouse IgG followed by tyramide signal amplification system (Biotin-free catalyzed amplification system, CSAII; DAKO) or biotinylated anti-rabbit IgG followed by streptavidin/HRP (LSAB kit; DAKO). The labeled peroxidase activity was detected using diaminobenzidine (DAB; Dojin, Kumamoto, Japan) in 0.015% hydrogen peroxide/0.05 M Tris-HCl (pH 7.6). The sections were counterstained with hematoxylin.

RESULTS

Generation of H5N1-specific mAbs: To establish hybridomas that secrete mAbs specific for the H5N1 virus, BALB/c mice were immunized with the whole virion fraction of purified A/Vietnam/1194/2004 (NIBRG-14) virus. The virus had been inactivated by conventional formaldehyde-fixation or by UV-irradiation to avoid possible changes in antigenicity caused by aldehyde fixation. A standard immunization protocol was used, where mice were boosted twice at 2-week intervals with antigen emulsified first in Freund's Complete Adjuvant and then in Freund's Incomplete Adjuvant. Three days after the final boost, a cell suspension was prepared from the spleens of three immunized mice and fused with SP-2/O myeloma using a polyethylene-glycol method. The fused cells were then selected with HAT (14). Hybridoma screening yielded eight hybridoma clones that reacted to NIBRG-14 lysate but not PR-8 lysate in ELISA (Table 1). Of these clones, seven were from mice immunized with UV-inactivated virion, and one was from mice immunized with formaldehyde-inactivated virion. Six clones (Niid_H5A, Niid_H5B, Niid_H5C, Niid_H5D, Niid_H5E, and Niid_H5F) reacted to rHA protein from a H5N1 virus (recHA_H5N1), while one clone (Niid_N1A) reacted to rNA protein from a H5N1 virus (recNA_H5N1). The remaining clone (Niid_150KA) did not react to either recHA_H5N1 or recNA_H5N1 by ELISA but did react to a 150-kDa molecule on Western blot analysis (described below). Interestingly, seven of the eight clones were from the mice immunized with UV-inactivated virus. The eight hybridomas were successfully cloned by a repeated limiting-dilution method and adapted to a serum-free hybridoma culture medium. The purified antibodies from each clone were biotinylated and used for further experiments.

Table 1. Summary of the eight H5N1-specific mAbs generated in this study

Clone name	Old name	Ig-subclass	ELISA				Western blot	IFA	Histology	Neutralization ($\mu\text{g}/\text{mL}$)	Hemagglutination inhibition
			H5N1 NIBRG-14	H1N1 PR-8	recHA-H5N1	recNA-H5N1					
Niid_H5A ¹⁾	YH-1A1	IgG2a	+++	-	+	-	57 kDa	++		1.5 (Clade-dep)	-
Niid_H5B ¹⁾	YH-2F11	IgG2a	+++	-	+++	-	57 kDa			25	+
Niid_H5C ¹⁾	OM-A	IgG2a	+++	-	++	-	57 kDa		+(mo/hu)	12	
Niid_H5D ¹⁾	OM-B	IgG2a	+++	-	++	-	57 kDa		+(mo)	12	
Niid_H5E ¹⁾	OM-C	IgG2a	+++	-	++	-	57 kDa			12 (Clade-dep)	
Niid_H5F	AY-2C2	IgG1	+++	-	++	-	ND	++		6	-
Niid_N1A ¹⁾	YH-2D3	IgG2a	+++	-	-	+	ND	++			
Niid_150KA ¹⁾	OM-D	IgG1	+++	-	-	-	150 kDa	++			-

¹⁾: Clones derived from mice immunized with UV-inactivated virus. The remaining clone is derived from a mouse immunized with formaldehyde-inactivated virus.

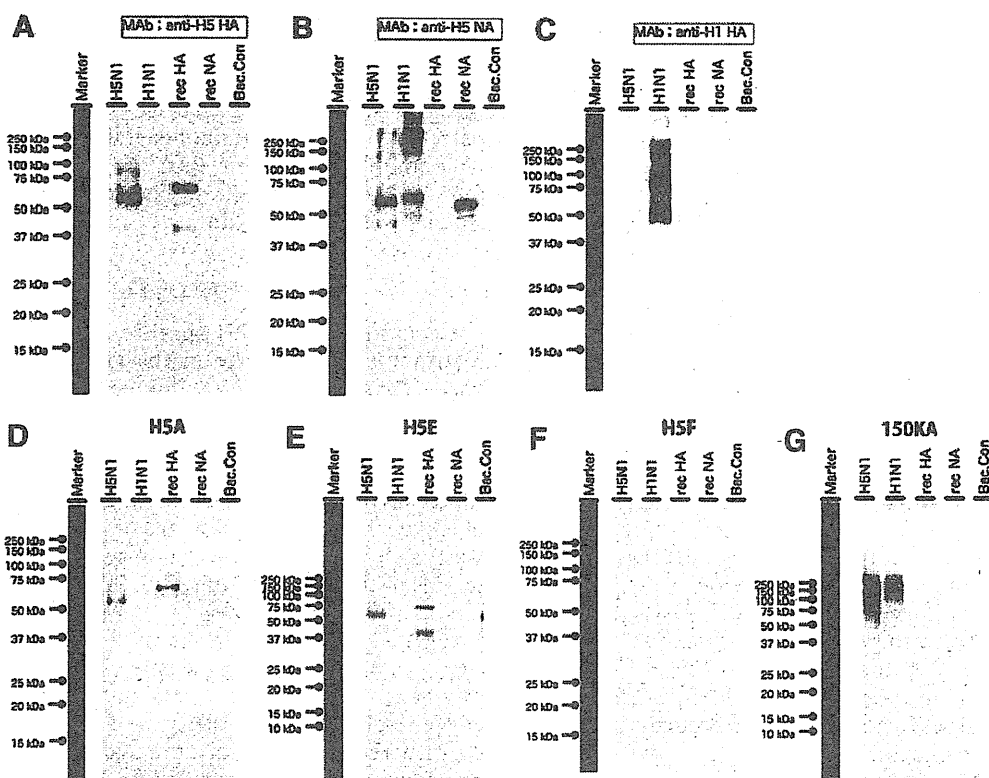


Fig. 1. Detection of influenza virus proteins in Western-blot analysis. Purified influenza virus proteins (0.5 $\mu\text{g}/\text{lane}$) were subjected to SDS-PAGE under reducing conditions. After blotting on a PVDF membrane, the proteins were detected by incubation with the eight monoclonal antibodies (mAbs), followed by incubation with the peroxidase-labeled F(ab')₂ fragment of donkey anti-mouse IgG. The mAbs were then visualized by chemiluminescent reaction. A, authentic anti-H5_{hemagglutinin} mAb; B, authentic anti-H5_{neuraminidase} mAb; C, authentic anti-H1_{hemagglutinin} mAb; D, Niid_H5A; E, Niid_H5E; F, Niid_H5F; G, Niid_150KA. The molecular weight markers are shown on the left.

Western blot analyses with the mAbs: Five mAbs (Niid_H5A, Niid_H5B, Niid_H5C, Niid_H5D, Niid_H5E) detected the 57-kDa H5_{H1} protein by Western blot analysis, which suggests that the antibodies detected the linear epitope(s) of a HA1 fragment of H5_{HA} (Table 1 and Fig. 1). These antibodies also detected the 60-kDa recombinant H5-HA containing the His-tag. One of these clones, Niid_H5E, detected a 40-kDa sub-fragment of recombinant HA1, which suggests that the antigenic footprint detected by the mAb differs from

that of the other four clones (Fig. 1). Niid-H5F, which reacted strongly to NIBRG-14 and rHA (H5) in ELISA, did not react to any proteins by Western blot analysis, presumably because the mAb detects a conformational epitope of H5-HA. The remaining clone, Niid_150KA, detected an unknown high molecular weight protein of approximately 150 kDa.

IFA with mAbs: Upon IFA, the HA-specific mAbs Niid-H5A and Niid_H5F, the NA-specific mAb Niid-N1A, and the Niid_150KA mAb that detects an

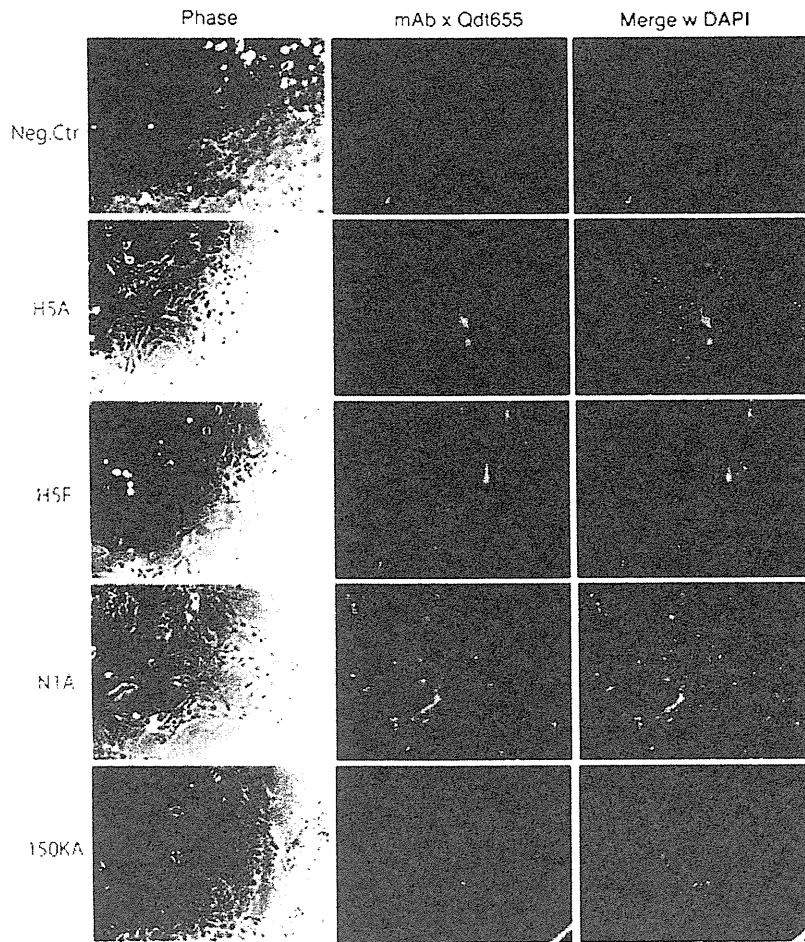


Fig. 2. Fluorescent immunostaining of H5N1 virus-infected MDCK cells with monoclonal antibodies (mAbs). Paraformaldehyde-fixed, H5N1 virus-infected MDCK cells were permeabilized by TBS-Tween and incubated with biotinylated mAbs. The mAbs were detected with Qdot655-conjugated streptavidin (red). Shown are representative staining patterns with Niid_H5A, Niid_H5F, Niid_N1A, and Niid_150KA. The negative control staining without mAb is shown on top. The nuclei were counterstained with DAPI (blue).

unknown 150-kDa protein bound to NIBRG-14-infected MDCK cells (Fig. 2). With the exception of Niid_H5F, these mAbs detected both the perinuclear region and the cell surface of NIBRG-14-infected MDCK cells. Niid_H5F did not detect the perinuclear region (presumably the Golgi body), which suggests that the antigenic footprint detected by this mAb differs from those of the other mAbs.

Immunohistochemistry: The Niid_H5C and Niid_H5D mAbs detected influenza virus antigens in the epithelial cells of the bronchioles and alveoli of 4% formaldehyde-fixed, paraffin-embedded lung tissue sections from mice infected with A/Vietnam/1194/2004 (NIBRG-14) (Fig. 3a). However, none of the mAbs detected influenza virus antigen in lung tissue sections from mice infected with A/HongKong/483/97 (HK483) (Fig. 3). In contrast, a polyclonal antibody against type A influenza nucleoprotein detected type A influenza virus nucleoprotein in the tissue sections from both the NIBRG-14- and HK483-infected mice (Fig. 3b, d). Thus, Niid_H5C and Niid_H5D specifically detected the HA antigen of A/Vietnam/1194/2004 (NIBRG-14). The specificity of these mAbs was then examined by using autopsied lung tissue sections from patients infected

with seasonal influenza virus (H1N1) or 2009 pandemic influenza virus (2009H1N1pdm). Niid_H5C did not exhibit any crossreactivity, but the Niid_H5D mAb did show non-specific staining with the human lung section. Two other mAbs, Niid_H5B and Niid_N1A, were also subjected to such immunohistochemical analysis but did not show any reaction.

Neutralization assay with mAbs: The ability of the mAbs to neutralize several H5N1 influenza strains was then tested (Table 2). The four purified H5N1 virus strains, NIBRG-14, Indo-RG2, NIBRG-23, and Anhui-RG5, were diluted to $2-3 \times 10^2$ TCID₅₀/0.05 mL (Table 2, lower panel) and incubated with titrated amounts of anti-H5_HA mAbs. The remaining infectivity was then noted (Table 2, upper panel). Niid_H5A most potently neutralized the NIBRG-14 strain; it completely neutralized influenza virus infectivity at a concentration of 78 ng/mL. However, Niid_H5A was less potent in neutralizing the Indo-RG2 and Anhui-RG5 strains, which indicates that the neutralizing ability of this mAb was clade-dependent. In contrast, Niid_H5F and Niid_H5D exhibited relatively broad neutralizing abilities, since they neutralized all of the strains that were tested. Niid_H5C and Niid_H5E also showed characteristic clade-