Toll-like receptor 9 ligand D-type oligodeoxynucleotide D35 as a broad inhibitor for influenza A virus replication that is associated with suppression of neuraminidase activity

Hiroshi Yamada [a], Satoshi Nagase [b], Kazuo Takahashi [c], Yoshihiro Sakoda [d], Hiroshi Kida [d, e], Shigefumi Okamoto [b, *]

[a] Laboratory of Virology and Vaccinology, National Institute of Biomedical Innovation, Ibaraki, Osaka, Japan
[b] Department of Laboratory Sciences, Division of Health Sciences, Kanazawa University Graduate School of Medical Science, Kanazawa, Japan
[c] Department of Infectious Diseases, Osaka Prefectural Institute of Public Health, Osaka, Japan
[d] Laboratory of Microbiology, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Japan
[e] Research Center for Zoonosis Control, Hokkaido University, Sapporo, Japan

ARTICLE INFO

ABSTRACT

The most effective drugs available to treat influenza are neuraminidase (NA) inhibitors, which provide important additional measures for the control of influenza virus infections. However, since the emergence of NA inhibitor-resistant viruses may compromise the clinical utility of this class of anti-influenza agents, it is very important to develop new anti-influenza agents which target a different region in NA responsible for its sensitivity from that for NA inhibitors and could be used to treat NA inhibitors-resistant isolates. The oligodeoxynucleotide D35, multimerized and aggregated, suppressed replication of influenza A viruses except A/WSN/33 (WSN). The suppressive viral replication by D35 depended on G-terad and multimer formation. The range of the suppressive viral replication at the late stage, including virus assembly and release from infected cells, was much larger than that at the initial stage, viral attachment and entry. D35 suppressed NA activity of influenza A viruses. Furthermore, replacing the NA gene of A/Puerto Rico/8/34 (PR8), in which viral replication was inhibited by D35 at the late stage, with the NA gene from WSN, in which viral replication was not inhibited, eliminated the D35-dependent suppression. D35 showed an additive anti-influenza effect with oseltamivir. It was also effective *in vivo*. These results suggest that the influenza virus NA mainly contributse to the D35-suppressible virus release from infected cells at the late stage. In addition, because administration of D35 into the virus-infected mice suppressed viral replication and weight loss, clinical application of D35 could be considered.

1. Introduction

Influenza viruses cause annual epidemics and occasional pandemics in humans, resulting in high mortality primarily in the elderly and other high-risk populations (Hayden, 1997; Whitley and Monto, 2006). Vaccines and antiviral drugs are two available strategies for preventing and controlling influenza virus infections. It usually takes 3—6 months to prepare a vaccine in case of pandemic influenza. During this period, the pandemic can spread, infect humans and cause great damage to the economy (Neumann et al., 2009). In this lag phase, taking antiviral drugs is the only available approach to control and treat the influenza virus infections. Moreover, as influenza virus infection cannot be completely prevented by vaccination, antiviral drugs are still necessary for the therapeutic treatment of influenza (Nistal-Villan and Garcia-Sastre, 2009).

To date, the most effective drugs to treat influenza are neuraminidase (NA) inhibitors. NA inhibitors including oseltamivir and zanamivir provide an important additional measure for the control of influenza (Boltz et al., 2010). These antiviral drugs target the active center of the influenza virus NA molecule, in which amino acids are largely conserved among influenza A and B viruses (Colman et al., 1993). However, the emergence of NA inhibitor-resistant viruses, as a result of drug use or due to circulation of

* Corresponding author. Department of Laboratory Sciences, Division of Health Sciences, Kanazawa University Graduate School of Medical Science, Kanazawa, Ishikawa, 920-0942, Japan.
E-mail address: sokamoto@mhs.mp.kanazawa-u.ac.jp (S. Okamoto).

natural variants, may compromise the clinical utility of this class of anti-influenza agents (Hauge et al., 2009; van der Vries et al., 2010).

Polyanions, such as dextran sulfate (DS) and heparin, proved to have a broad spectrum of antiviral activity against enveloped viruses, such as human immunodeficiency virus (HIV), herpes viruses, and vesicular stomatitis virus (Baba et al., 1988; Hosoya et al., 1991). The mechanisms of inhibition of virus replication by polyanions are attributed to inhibition of virus adsorption to the cell membranes and/or virus envelope fusion with the endosomal membranes (Mitsuya et al., 1988; Arnberg et al., 2002; Luscher-Mattli et al., 1993). On influenza virus, several studies have demonstrated that the suppression of viral replication by DS is caused inhibition of hemagglutinin (HA) activity, which plays a role in viral fusion process at the early stage of infection (Luscher-Mattli and Gluck, 1990; Ramalho-Santos and de Lima, 2001). Recently we reported that DS also suppressed the NA activity and release from infected cells at the late stage of the replication, resulting in the suppressive viral replication (Yamada et al., 2012). Since DS is negatively charged, the change in the viral surface from a positive to negative charge by adding DS may play a role in the suppression of viral replication (Moulard et al., 2000; Yamada et al., 2014).

Bacterial and synthetic oligodeoxynucleotides (ODNs) containing unmethylated CpG dinucleotides in specific sequence contexts (CpG ODN9) trigger the vertebrate immune system through the activation of Toll-like receptor 9 (TLR9) (Krieg, 2006; Klinman, 2004). The resulting innate immune response limits the early spread of infectious organisms, while promoting the development of adaptive immunity. Optimal sequences for the activation of TLR9 vary among species (Bauer et al., 2001). There are at least four types of CpG ODNs, each of which has a different backbone, sequence, and immunostimulatory properties (Vollmer and Krieg, 2009). D-type (also called A) CpG ODNs typically comprise one palindromic CpG motif with a phosphodiester (PO) backbone and phosphorothioate (PS) poly(G) tail to increase resistance to nucleases, and activates plasmacytoid dendritic cells (pDCs) to produce a large amount of interferon (IFN)-α but fails to induce pDC maturation and B-cell activation (Krug 2001, Verthelyi et al., 2001). The three other types of ODNs consist of a PS backbone. K-type (also called B) CpG ODNs contain nonpalindromic multiple CpG motifs, and strongly activates B cells to produce interleukin (IL)-6 and pDCs to maturation but barely produces IFN-α (Hartmann and Krieg, 2000; Verthelyi et al., 2001). C and P-type CpG ODNs contain one and two palindromic CpG sequences, respectively, both of which can activate B cells like K-type and pDC like D-type, although C CpG ODN induces weaker IFN-α production compared with P-type CpG ODN (Hartmann and Krieg, 2000; Marshall et al., 2003, Samulowitz et al., 2010).

D-type CpG ODNs are characterized by having a core sequence with a single palindromic purine-pyrimidine-CpG-purine-pyrimidine motif flanked on both sides by 3–5 self-complementary bases on a PO backbone. Such a structure renders the sequence flexible allowing for the formation of a stem–loop conformation and/or forming dimers with other D-type ODN molecules (Verthelyi et al., 2001). In addition, D-type ODNs have a 3′ end poly(G) motif (Verthelyi et al., 2001), which is known to self-associate via Hoogsteen base-pairing to form parallel quadruplex structures called G-tetrads (Panyutin et al., 1990; Costa et al., 2004).

We reported that DS suppressed the replication of influenza A viruses by electrically interacting with viral proteins through its sulfate containing negative charge (Yamada et al., 2014). The result raised a hypothesis that ODNs, as well as DS, may suppress the viral replication, because it is well known that the existence of PO bond results in electrically negative charge of ODNs. Therefore, we examined synthetic ODNs as candidates for anti-influenza compounds in the present study and found that the D-type ODN D35 suppressed the NA activity and release from infected cells at the late stage of infection.

2. Materials and methods

2.1. Cells and viruses

Madin–Darby canine kidney (MDCK) cells were maintained in Minimum Essential Medium (MEM) (Nissui Pharmaceutical) supplemented with 0.3 mg/ml L-glutamine, 6% fetal calf serum (FCS) and 20 μg/ml gentamicin. Human embryonic kidney 293T cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Nissui Pharmaceutical) supplemented with 0.3 mg/ml L-glutamine, 8% FCS and 20 μg/ml gentamicin. Influenza A viruses, A/Panama/2007/1999 (H3N2) (Panama) and A/Wyoming/03/2003 (H3N2) (Wyoming), were obtained from Dr. T. Odagiri (National Institute of Infectious Diseases, Japan). The human 2009 pandemic H1N1 virus A/Osaka/164/2009 (Pdm09) was isolated in Osaka, Japan from a patient who exhibited mild influenza symptoms and recovered (Uraki et al., 2013). A/duck/Hokkaido/Vac-3/2007 (H5N1) (Vac-3) and A/duck/Hokkaido/Vac-2/2004 (H7N7) (Vac-2) were described previously (Soda et al., 2008; Sakabe et al., 2008). A/Puerto Rico/8/34 (H1N1) (PR8) and A/WSN/33 (H1N1) (WSN) were generated by plasmid-based reverse genetics (Neumann et al., 1999; Horimoto et al., 2007), and plasmids (pHH21) each containing a genomic segment of PR8 and WSN and four WSN protein expression plasmids encoding PB1, PB2, PA and NP were kindly provided by Dr. Y. Kawaoka (University of Wisconsin, USA). Reassorted viruses between PR8 and WSN and recombinant viruses of PR8 with mutated NA genes were described previously (Yamada et al., 2012, 2014).

2.2. Compounds

Synthetic ODNs, D35, D35 GpC, Core-12, 7-deaza D35, K3 and A151, were synthesized and purified by GeneDesign (Japan) and listed in Table 1. Diethylaminoethyl-dextran hydrochloride (DEAED) with a mean molecular weight of 500,000 was purchased from MP Biomedicals. Dextran with a mean molecular weight of 500,000 was purchased from Polysciences. Oseltamivir carboxylate was purchased from ChemScene.

We determined the efficacy of the compounds on viability of MDCK cells performed by a MTT assay (MTT Cell Viability Assay Kit; Biotium). In brief, confluent monolayers of MDCK cells in 96-well tissue culture plates were washed with PBS twice, and the 100 μl of virus dilution medium (VDM; MEM supplemented with 0.3 mg/ml L-glutamine, 20 μg/ml gentamicin and 0.1% bovine serum albumin (BSA)) with the several concentrations of compounds were applied to the monolayers and incubated at 35 °C. At every 24 h post infection (hpi), MTT assay performed in according to the manufacturer instruction. Cell viability was expressed as the percentage of MTT reduction, assigning the 100% value to the absorbance of the non-treated MDCK cells. We confirmed that the coumpounds had no ability to decrease viability of MDCK cells (Fig. 1A). Furthermore, adding D35 and oseltamivir did not change

Table 1
Synthetic ODNs used.

D35	g˄gtgcatcgatgcagggg˄g˄g
D35 GpC	g˄gtgcatgcatgcagggg˄g˄g
7-deaza D35	g7˄g7tgcatcgatgcag7g7g7g7˄g7˄g7
Core-12	tgcatcgatgca
K3	a˄t˄c˄g˄a˄c˄t˄c˄t˄c˄g˄a˄g˄c˄g˄t˄t˄c˄t˄c
A151	ttagggtttagggtttagggtttaggg

˄:phosphorothioated.
7:7-deazaguanine.

viability of MDCK cells (Fig. 1B).

2.3. Virus infection

For stock virus preparation, recombinant viruses collected from transfected 293T cells were grown in MDCK cells as described previously (Barman et al., 2004). Briefly, MDCK cells were infected with viruses at a multiplicity of infection (MOI) of 0.001 using VDM. The infected MDCK cells were incubated at 35 °C in virus growth medium (VGM; MEM containing MEM Vitamin Solution (Invitrogen), 10 mM HEPES (pH 7.4), 20 μg/ml gentamicin, 0.1% BSA and 0.7 μg/ml crystal trypsin (Sigma–Aldrich), and the supernatant was collected when cytopathic effects were observed.

2.4. Plaque assay

Ten-fold-diluted viruses in 300 μl of MEM with 0.1% BSA were applied to confluent monolayers of MDCK cells in 6-well plates, and incubated at 35 °C for 1 h. Unbound viruses were removed, and the cells were washed with MEM. The cells were then overlaid with 2 ml VGM containing 0.7% agarose (Sigma–Aldrich) and 0.7 μg/ml crystal trypsin (Sigma–Aldrich). After a 72-h incubation at 35 °C, the cells were fixed with 10% formaldehyde, and stained with 0.1% crystal violet solution.

2.5. Negative-stain electron microscopy (EM)

Before staining, ODN samples diluted in PBS (1 mg/ml) were dropped on formvar-carbon-coated grids. For negative staining, a drop of 2% (wt/vol) uranyl acetate (pH 4.0) was placed on the grid and left to air dry. The grids were examined at a magnification of 20,000 on an electron microscope H-7650 (Hitachi, Japan).

2.6. NA enzyme activity assay

NA activity was measured using the NA-Star Influenza Neuraminidase Inhibitor Resistance Detection Kit (Applied Biosystems) according to the manufacturer's instructions. Viruses from the cell culture supernatant were diluted appropriately, pre-incubated with various concentrations of D35 for 20 min at room temperature, and then incubated with NA-Star substrate for 30 min. After the addition of NA-Star accelerator, the chemiluminescent signals were measured at a rate of 1 s/well by the GloMax-Multi + Detection System (Promega). The relative NA activity was determined as the mean percent for triplicate wells, and calculated in % of control value obtained in the absence of D35.

2.7. Thin section EM

MDCK cells were infected with PR8 or WSN at an MOI of 5 at 37 °C. At eight hpi, the cultures were washed three times, and VDM with or without 50 μg/ml D35 was added to the cells. At thirteen hpi, the cells were fixed in 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer (PB) (pH 7.2), postfixed with 1% OsO$_4$ in 0.1 M PB (pH 7.2), and embedded in Epon 812 (Electron Microscopy Sciences). Ultrathin sections were cut with an EM UC6 ultramicrotome (Leica Microsystems) and observed at a magnification of 30,000 with an electron microscope H-7650.
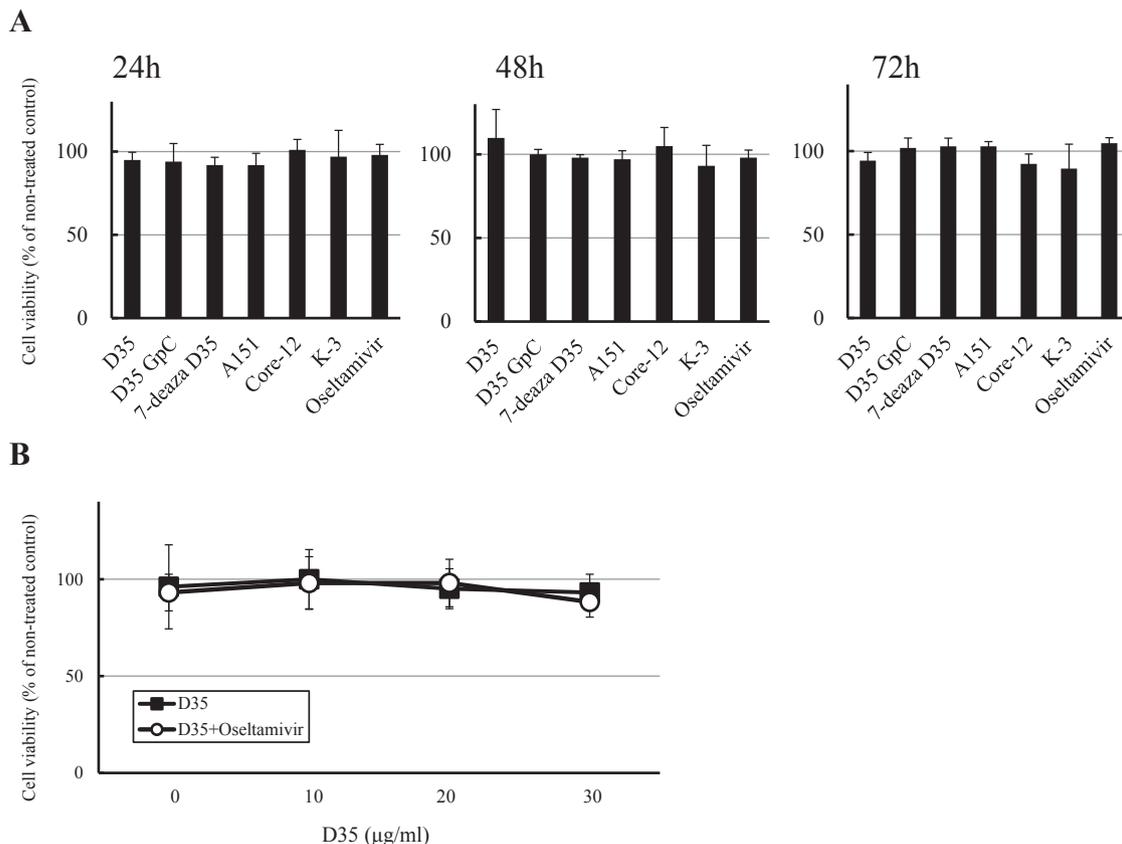

Fig. 1. Efficacy of compounds on cell viability. (A) MDCK cells were treated with 30 μg/ml of D35, GpC, Core-12, 7-deaza D35, K3, A151, and 10 nM of oseltamivir for 24, 48, and 72 h, followed by measurement of viability of the cells by MTT assay. Bars represent the mean ± standard deviation of three independent samples. All groups were $P > 0.05$ versus the control group. (B) MDCK cells were treated with 0, 10, 20, and 30 μg/ml of D35 with or without 10 nM of oseltamivir for 72 h, followed by measurement of viability of the cell by MTT assay. Plots represent the mean ± standard deviation of three independent samples.
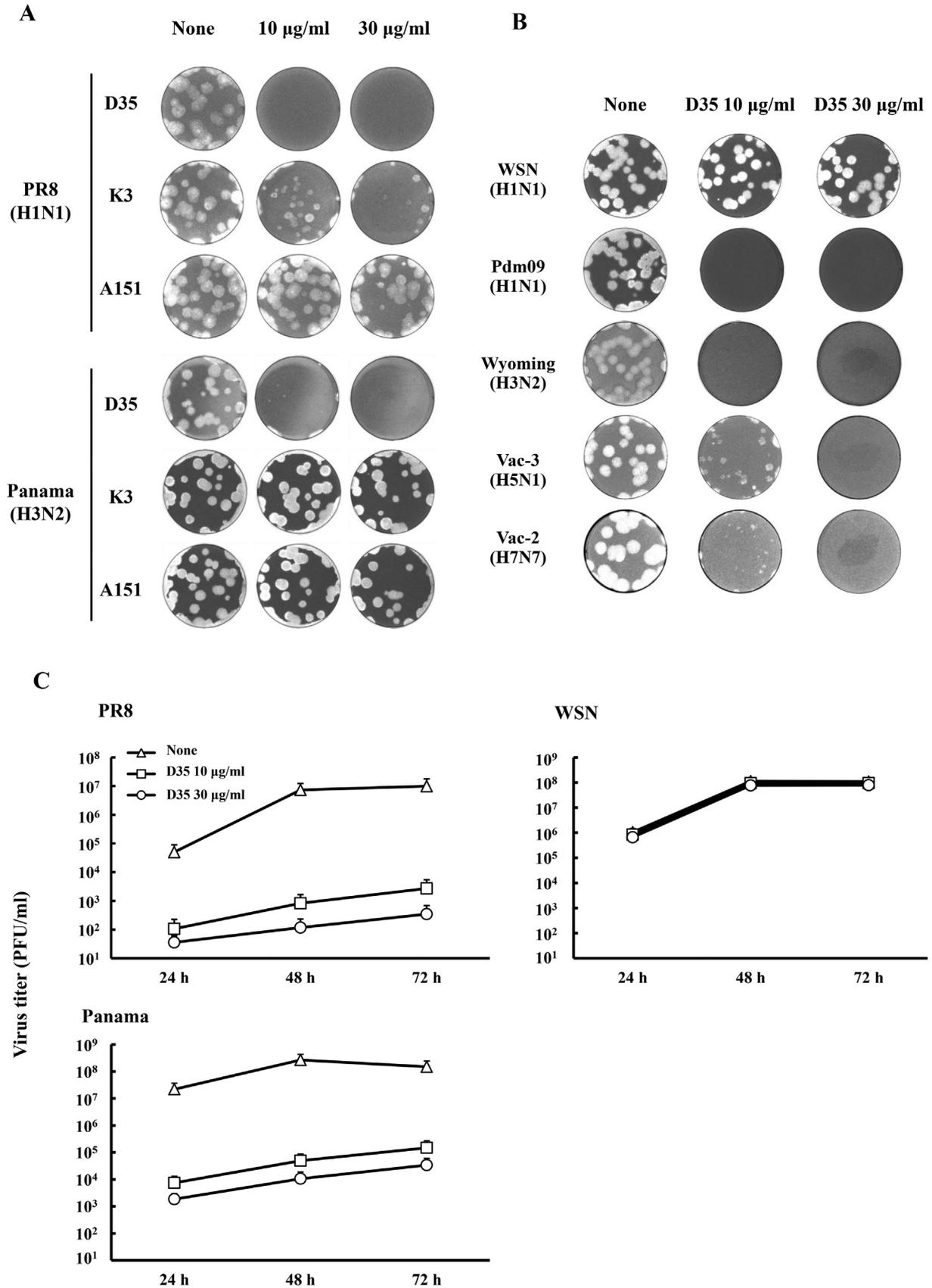
Fig. 2. Efficacy of suppression of viral replication by synthetic ODNs. (A) Plaque formation of PR8 or Panama in the presence of D35, K3 or A151. MDCK cells were infected with PR8 or Panama in the absence of ODNs and incubated in agarose with or without 10 or 30 μg/ml D35, K3 or A151 at 35 °C. (B) Plaque formation of influenza A viruses in the presence of D35. MDCK cells were infected with WSN, Pdm09, Wyoming, Vac-3 or Vac-2 in the absence of D35 and incubated in agarose with or without 10 or 30 μg/ml D35 at 35 °C. (C) Continuous D35-dependent suppression of virus growth. MDCK cells were infected with PR8, WSN, or Panama with or without 10 or 30 μg/ml D35 at 35 °C at an MOI of 0.001 and

2.8. Virus challenge in mice

Eight-week old female BALB/c mice (Japan SLC) were anesthetized and inoculated intranasally with 90 plaque forming units (PFU) of PR8 diluted in 30 μl PBS. At indicated times, mice were anesthetized and inoculated intranasally (inoculation volume, 40 μl) with compounds diluted in PBS. At 55 h after infection, the lungs were excised and homogenized using Multi-Beads Shocker (Yasui Kikai, Japan) according to the manufacturer's protocol. The viral titer in the lung homogenate was estimated using a plaque assay. The mortality status and weight loss of the mice were assessed daily for up to 14 days thereafter. National Institute of Biomedical Innovation authorized this animal study (approval number DS21-21), and all experiments were performed according to the guidelines of this committee.

2.9. Statistical analysis

Fisher's exact test was performed using the Statcel2 software (OMS, Tokyo, Japan), to evaluate the differences between groups in the mortality experiments. To analyze the data in the other experiments, nonparametric Student's t-tests were used. A p value of <0.05 was considered significant.

3. Results

3.1. Efficacy of suppression of viral replication by synthetic ODNs

We first examined whether plaque formation by infection of MDCK cells with influenza A viruses was inhibited by ODNs, D35, K3 or A151 (Table 1). ODNs, D35 and K3, which contain a CpG motif, activate TLR9-mediated immune response (Puig et al., 2006), while A151 comprised of the immunosuppressive motif TTAGGG blocks TLR9 signaling (Kaminski et al., 2013). Influenza virus strain H1N1 PR8 or H3N2 Panama was inoculated onto MDCK cells, and after 1-h incubation, the cells were washed three times with MEM and then further overlaid with VGM containing 0.8% agarose in the presence or absence of ODNs. As shown in Fig. 2A, the number of plaque were remarkably decreased in the presence of D35 in PR8 and Panama-infected cells. In contrast, A151 did not inhibit significantly plaque formation of both strains, and K3 was only moderately effective against PR8. Therefore, we focused on D35 and examined its inhibitory effect against other influenza A viruses. As shown in Fig. 2B, D35 suppressed plaque formation by infection of MDCK cells with H1N1 Pdm09, H3N2 Wyoming, H5N1 Vac-3, and H7N7 Vac-2. On the other hand, the plaque formation of H1N1 WSN was not significantly suppressed.

We next determined whether D35 also suppressed the continuous growth of virus strains whose plaque formation was inhibited by D35. MDCK cells were infected with PR8, WSN or Panama in the presence or absence of D35, and then further continuously incubated. In the presence of D35, the titer of PR8 and Panama, whose plaque formation was suppressed by D35, was less than 0.1% of those without D35 at 24 and 48 hpi (Fig. 2C). In contrast, D35 did not significantly affect viral growth of WSN, whose plaque formation was not suppressed. These results indicated that virus strains whose plaque formation was suppressed by D35 also showed growth suppression by D35 in infected cells without agarose.

3.2. Effect of electric charge and G-tetrad formation on the inhibition of plaque formation by D35

Since D35 has negative charge by PO backbone, we examined whether adding DEAED, which has positive charge, affected suppression of influenza virus replication by D35. MDCK cells infected with PR8 were incubated with VGM containing agarose in the presence of 20 μg/ml D35 and various concentrations of DEAED. As shown in Fig. 3A, DEAED dose-dependently recovered suppressive plaque formation by D35. In contrast, adding 20 μg/ml dextran, which has neutral charge, did not restore the plaque formation suppressed by D35, suggesting that neutralization of negative charge of D35 by DEAED disabled D35 from inhibiting influenza virus replication.

Since D35 containing unmethylated CpG dinucleotides trigger the vertebrate immune response through the activation of TLR9 (Klinman, 2004; Krieg, 2006), we examined whether a CpG motif in D35 would affect the suppressive effect on the plaque formation by using D35 GpC, which does not induce activation of TLR9. Fig. 3B shows that D35 GpC also suppressed influenza virus replication, indicating that immune response triggered by D35 was not involved in D35's suppressive effect.

D35 has a 3′ end poly(G) motif, which is known to self-associate via Hoogsteen base-pairing to form parallel quadruplex structures called G-tetrads (Verthelyi et al., 2001). It has been reported that the formation of multimers is necessary for D-type ODNs to localize to early transferring receptor-positive endosomes to signal through TLR9 and induce IFNα, and that monomeric sequences behave as competitive antagonists (Wu et al., 2004; Kerkmann et al., 2005; Guiducci et al., 2006). Thus we examined whether the G-tetrad formation would affect D35's suppressive effect on the plaque formation. We used two ODNs, Core-12 which removed 5′ and 3′ end poly(G) motifs and 7-deaza D35 which replaced 5′ and 3′ end guanines with 7-deazaguanines to impede G-tetrad formation (Table 1). Fig. 3B shows that the plaque formation of PR8 was not suppressed by Core-12 or 7-deaza D35. The results suggest that the G-tetrad formation of D35 is required for its suppressive effect.

Although A151 comprised of telomeric TTAGGG repeats assumes an antiparallel G-tetrad structure by intramolecular folding (Balagurumoorthy and Brahmachari, 1994), D-type ODNs have the ability to form secondary structures and aggregates, and multimerization was promoted by the presence of a guanine-rich 3′-terminus (Wu et al., 2004). Next, to examine whether D35 aggregated, ODNs diluted in PBS were observed by negative-stain EM. As shown in Fig. 3C, D35 and D35 GpC self-assembled and formed a network structure. However, 7-deaza D35 and A151 formed globular structures and did not form aggregates. These results indicated that aggregation by formation of multimers was necessary for D35 and D35 GpC to inhibit influenza virus replication.

3.3. D35 mainly inhibited the late step of influenza virus infection

To determine which stage(s) of viral replication was inhibited by D35, we performed a time course study of inhibitory effects of D35. Since it takes 8–12 h for influenza viruses to produce its progeny viruses after virus absorption, four treatment intervals covering the whole life cycle of influenza viruses from absorption to progeny production were used to disclose the time frame when D35 exerted the maximal effect. D35 was applied during each of these intervals and the cells were washed three times with MEM at specific time points. Following the treatment, fresh compound-free VDM was

---

then continuously maintained in VGM with or without 10 or 30 μg/ml D35 at 35 °C. At every 24 hpi, the culture supernatants were collected, and the virus titers in the supernatant were assessed by plaque assay. Bars represent the mean ± standard deviation of three independent samples.
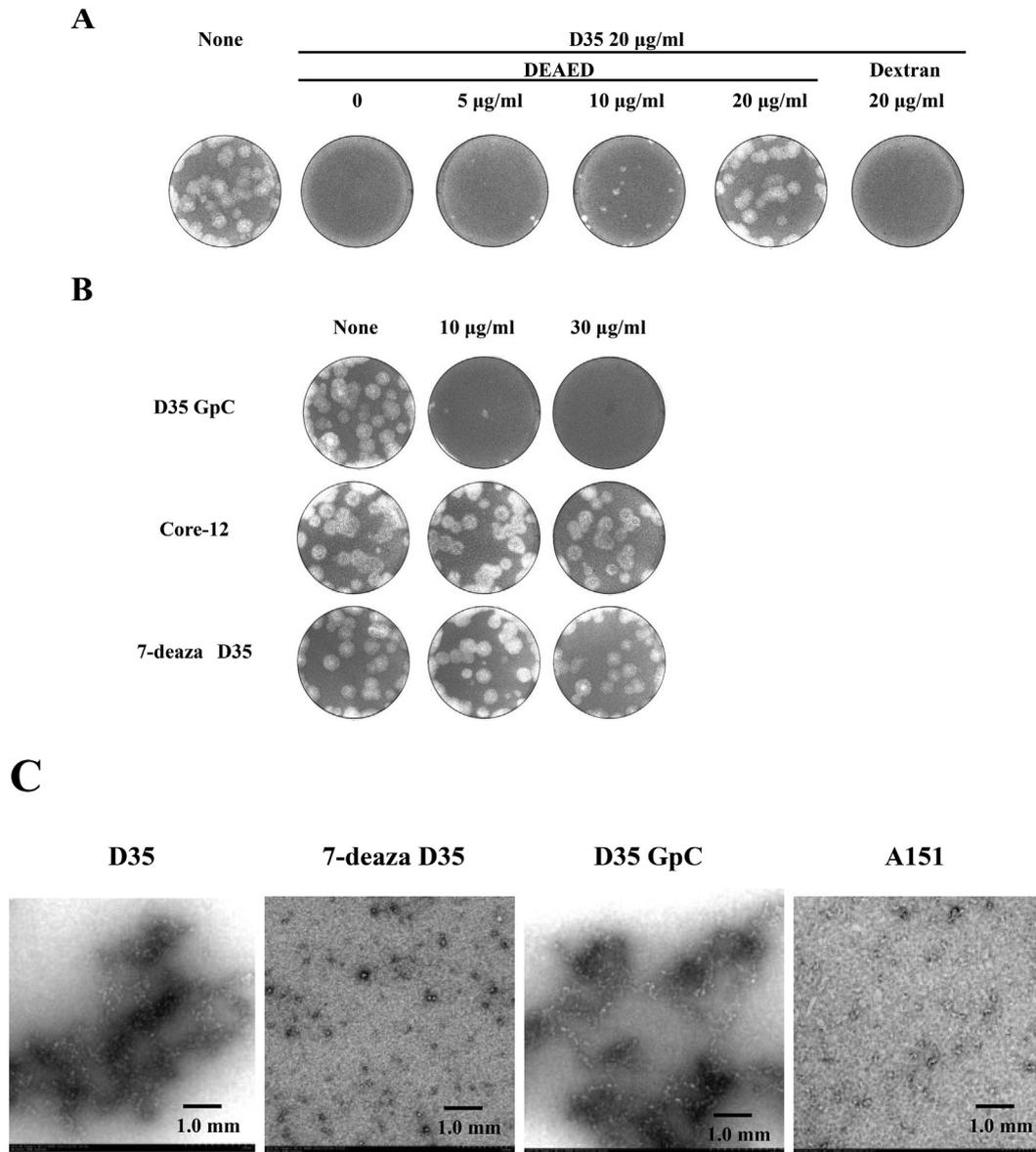
Fig. 3. Characterization of the mechanism of D35 on the inhibition of virus replication. (A) Effect of negative charge on the inhibition of plaque formation by D35. MDCK cells were infected with PR8 in the absence of D35 and incubated in agarose with or without 20 μg/ml D35 and indicated concentrations of DEAED or 20 μg/ml dextran at 35 °C. (B) Effect of a CpG motif and a 3′ end poly(G) motif on the inhibition of plaque formation by D35. MDCK cells were infected with PR8 in the absence of compounds and incubated in agarose with or without 10 or 30 μg/ml D35 GpC, Core-12 or 7-deaza D35 at 35 °C. (C) D35 and D35 GpC self-assembled forming a network structure. One mg/ml of D35, D35 GpC, 7-deaza D35 or A151 diluted in PBS was examined by negative-stain EM at a magnification of 30,000.

added. All treated cells were harvested at 11.5 hpi, and the virus yield was determined by measuring virus titers of every culture. As shown in Fig. 4A, apparent inhibition of virus replication by D35 was observed when infected cells were treated with D35 only at the interval of 9–11.5 hpi, which corresponded to the late step of influenza virus replication such as budding or release from infected cells. In case of treatment during an interval of 0–1 hpi, a moderate inhibition (ca 30%) was observed in infected cells. However, D35 treatment during an interval of 1–6 or 4–9 hpi did not inhibit virus yield when compared to control groups. These results suggest that D35 inhibits the early and late steps of viral replication. Thus we next examined the suppressive effect of D35 against several strains at early and late steps of viral replication. A severe decrease of viral replication was observed in PR8, Pdm09, Panama, Wyoming, H5N1 and H7N7-infected cells when D35 was present only at the late stage, whereas little reduction in the viral growth of WSN was

observed (Fig. 4B and C). The results suggested that D35 mainly suppressed the late stage of viral growth, which includes viral assembly and release from infected cells.

3.4. Suppression of NA enzymatic activity of influenza viruses by D35

NA promotes the release of progeny viruses from infected host cells by destroying the sialic acid receptors on the host cells and on the viruses themselves, contributing to the release of progeny viruses from infected cells at the late stage of infection (Palese et al., 1974; Palese and Compans, 1976). Thus we examined whether NA activity was suppressed by D35 of late-stage-sensitive influenza virus strains (Fig. 5A). The concentrations of D35 that inhibited the NA activity of D35-sensitive PR8 and Panama were much lower than those required to inhibit NA activity of D35-resistant WSN.
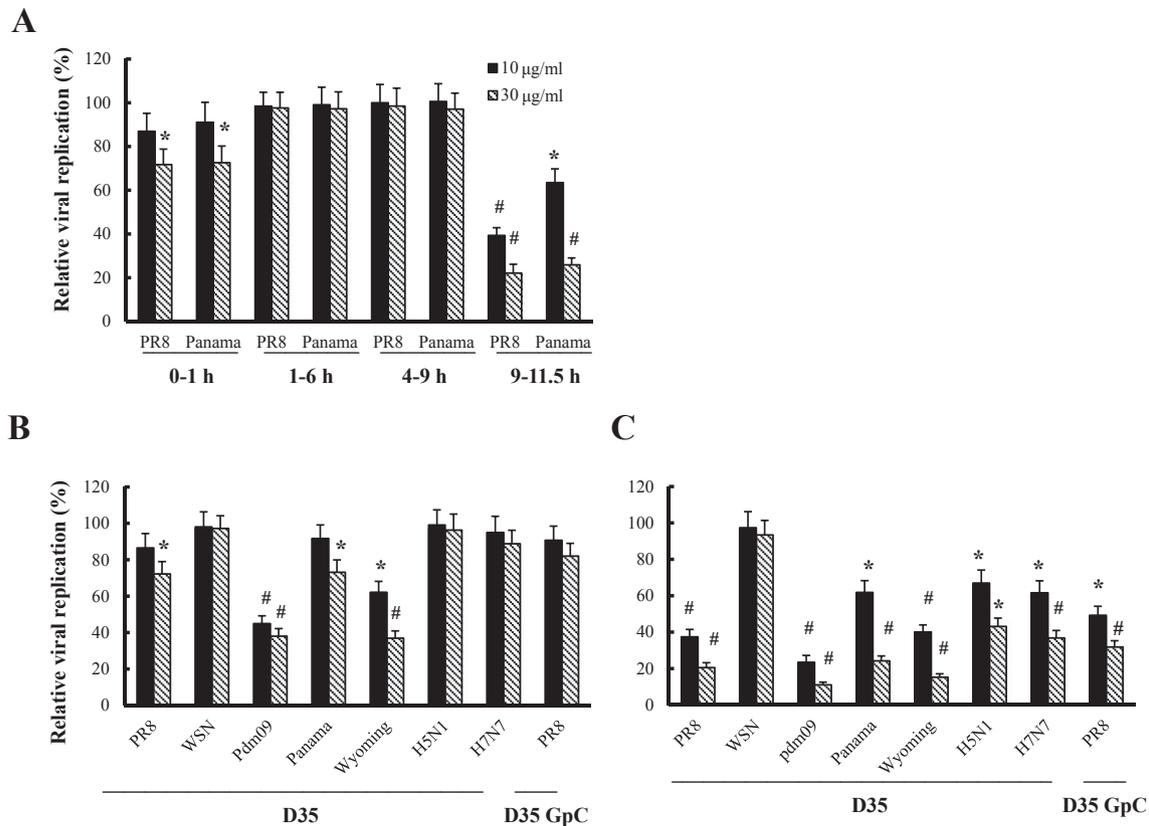
Fig. 4. The inhibitory effect of D35 in one cycle of influenza virus replication. (A) D35 was present only during indicated intervals. MDCK cells were infected with PR8 and Panama at an MOI of 1 at 37 °C. After viral adsorption with (0−1 h) or without D35, VDM was added. At indicated intervals after infection, the cultures were washed three times, and VDM with or without 10 or 30 μg/ml D35 was added to the cells. At 11.5 hpi, the culture supernatants were collected for virus titration by plaque assays. Relative viral replication was determined as the mean percent, given in % of the control values. Bars represent the mean ± standard deviation of three independent samples. (B) D35 was present only during adsorption for 0 to 1 hpi. (C) D35 was present only for 10 to 11.5 hpi. *; $P < 0.05$, #; $P < 0.01$ versus the control group.

The result suggest that D35 suppresses the enzymatic activity of NA in D35-sensitive influenza viruses. In addition, an EM experiment showed that D35 treatment induced viral aggregation on the cell surface, whereas such aggregation was not observed in the WSN-infected cells (Fig. 5B). These results indicate that D35 causes viral aggregation on infected cell surface by inhibiting the enzymatic activity of NA and thus the release of viruses from infected cells.

3.5. K432 and K435 of NA are responsible for the inhibition of viral replication by D35

Previously we reported that the NA was mainly responsible for the inhibition by DS, using a series of constructed reassortants between DS-sensitive PR8 and DS-resistant WSN strains (Yamada et al., 2012). We examined their sensitivity to D35 by plaque assay in MDCK cells (Fig. 6A). Plaque formation of the reassortant containing NA from PR8 and all other proteins from WSN (WSN-PR8 NA), but not that containing HA from PR8 and all other proteins from WSN (WSN-PR8 HA), was inhibited by D35. Furthermore, plaque formation of the reassortant containing NA from WSN and all other proteins from PR8 (PR8-WSN NA) was not inhibited by D35, but that containing HA from WSN and all other proteins from PR8 (PR8-WSN HA) was inhibited. These results indicated that the NA was the main determinant of the difference in D35 sensitivity between PR8 and WSN strains.

We also reported that the critical amino acids of PR8 NA for DS-sensitivity were basic amino acids, R430, K432 and K435, in the

430-loop which corresponds to amino acids from R430 to T439 (Supplementary Fig. 1) (Yamada et al., 2014). We examined the plaque formation of a series of recombinant viruses in which NA had a single mutation found in DS-resistant PR8 viruses. Plaques were undetectable in MDCK cells infected with recombinant viruses with R95C, K389E or R430L on the NA, in the presence of D35. However, in MDCK cells infected with recombinant viruses, K220E or K432E on the NA, small plaques were detected, and K435E NA virus-infected MDCK cells, plaques of moderate sizes were detected in the presence of D35 (Fig. 6B). These results suggested that basic amino acids, specifically K432 and K435, in the 430-loop of PR8 NA were important for D35-sensitivity, similarly to DS-sensitivity.

3.6. The additive effect of D35 with oseltamivir

D35 mainly targeted the 430-loop of PR8 NA, which was largely different from the target site of oseltaminir, a NA inhibitor that binds to the active site of the viral NA molecule. Thus we examined whether oseltamivir was effective against D35-resistant recombinant viruses or whether the oseltamivir-resistant virus was sensitive to D35. We used the recombinant virus H274Y, which is resistant to oseltamivir (Fig. 7A) (Carr et al., 2002). Plaques were undetectable in MDCK cells infected with the oseltamivir-resistant H274Y in the presence of D35 or in the cells infected with K435E in the presence of oseltaminir. Next we found that D35 and oseltamivir had additive effect against PR8 by the result that adding 2 μg/ml of D35 or 2 nM oseltamivir decreased the size of plaques, and adding both compounds decreased greater (Fig. 7B). Moreover, D35
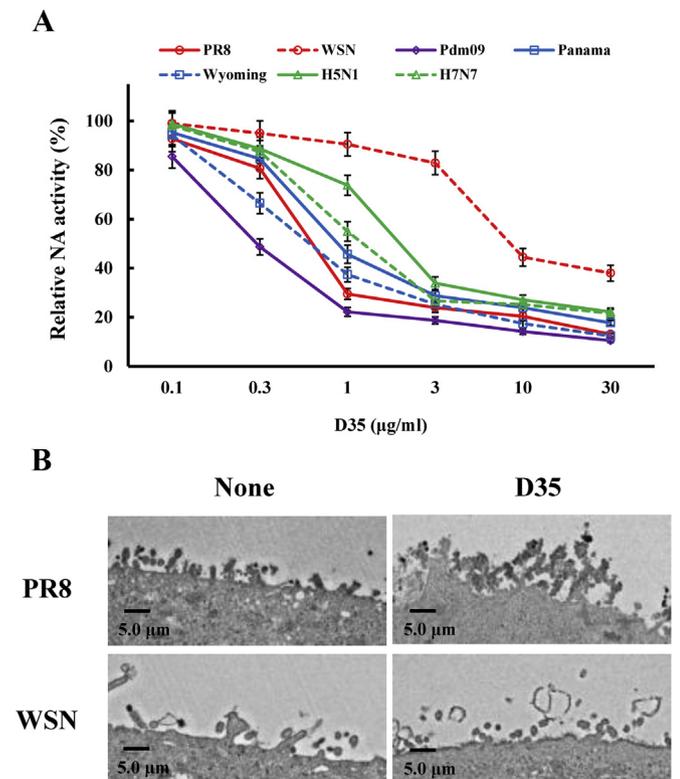
A

B

Fig. 5. D35-dependent suppression of NA activity of influenza A viruses. (A) The culture supernatants from MDCK cells containing each influenza A virus were treated with or without D35, followed by the measurement of NA activity in the samples as described in Materials and Methods. The relative NA activity was determined as the mean percent, given in % of the control values. Lines represent the mean ± standard deviation of three independent samples. (B) MDCK cells were infected with PR8 or WSN at a MOI of 5 at 37 °C. After viral adsorption, VDM without D35 was added to the cells. At 8 hpi, the cultures were washed three times, and VDM with or without 50 μg/ml D35 was added to the cells. At 12 hpi, the cells were processed for EM at a magnification of 3000.

and oseltamivir showed additive effect against PR8 NA by *in vitro* NA activity assay (Fig. 7C). These results suggest that the mechanism of D35 action differs from that of oseltamivir and that D35 shows the additive antiviral effect with oseltamivir.

3.7. In vivo anti-influenza activity of D35

To examine whether D35 inhibits virus replication *in vivo*, we infected mice intranasally with a sublethal dose of PR8. We pre-treated mice intranasally with D35 or D35 GpC at 18 h before infection, or treated with D35, D35 GpC, oseltamivir or PBS at 6 hpi. As shown in Fig. 8A, the mean weight loss of mice treated with D35 GpC after PR8 infection was significantly less than that of the PBS-treated mice and its effect was similar to the case of oseltamivir treatment. Since mice that received D35 after infection exhibited weight loss at early times after infection (1—3 dpi), which was not observed in mice that received PBS or D35 GpC after infection, we supposed that D35 activated immune response, together with activated immune response by influenza virus infection, led to specific weight loss. In contrast, treatment of D35 before infection significantly inhibited weight loss of mice, but treatment of D35 GpC before infection was less efficient. Consistent with improved outcomes, D35, D35 GpC, and oseltamivir significantly reduced PR8 titers in lung homogenates examined at 55 hpi (Fig. 8B).

4. Discussion

In this study, we showed that a short ODN D35 containing negative charge by PO backbone, but not K3 or A151, significantly suppressed replication of several influenza virus strains. We also found that D35 mainly inhibited replication of influenza A viruses at the late step which was dependent on NA activity.

We previously reported that the 430-loop of PR8 NA was the main target for DS (Yamada et al., 2014), which was similar pattern of suppressive influenza virus replication by D35. Here we found that the K435E mutation in the 430-loop of D35-sensitive PR8 NA changed it to D35-resistant. In addition, PR8 with NA containing the K432E mutation in the 430-loop was partially resistant to D35 (Fig. 6B). The results suggest that the 430-loop of PR8 NA is the main target for D35, as well as DS. Using molecular dynamics simulations of influenza NA, extended conformational shifts of the 150-loop and 430-loop in NA were observed (Amaro et al., 2007, 2009). It has been demonstrated that these NA regions can open to a much larger extent than was anticipated from prior X-ray structure analyses, which can be systematically explored for the development of more potent, chemically diverse inhibitors. It has been reported that a novel small-molecule inhibitor can inhibit viral replication of both the H1N1 and H5N1 viruses and the compound is predicted to establish hydrogen bonds with residues V116 and R156 of the 150-loop and T439 of the 430-loop (An et al., 2009). They also suggested that another binding site within the pocket formed by the 430-loop could be accessed by the new inhibitors. Therefore, D35 which targets the 430-loop should be a novel anti-influenza drug.

Oseltamivir carboxylate is an NA inhibitor that binds to the active site of the viral NA and is frequently used to treat influenza. However, a marked increase in oseltamivir-resistant isolates has been reported and these strains have become prevalent worldwide (Hauge et al., 2009; Hurt et al., 2009). We showed that the recombinant virus H274Y, which is resistant to oseltamivir, was sensitive to D35, K435E was sensitive to oseltamivir, and D35 and oseltamivir had additive effect against PR8 (Fig. 7). These results indicate that the region of the NA responsible for oseltamivir-sensitivity differs from that for D35-sensitivity, and that D35 might be useful for the treatment of infection with oseltamivir-resistant virus. However, since we have not determined whether D35 reduces replication of recent oseltamivir-resistant virus strains, it should be studied in future. In case of treating AIDS, combination therapy, which uses two or more drugs simultaneously to inhibit HIV replication, has been developed to lower toxicity by decreasing the dosage of individual compounds. This approach reduces the risk of developing drug resistance and maintains synergistic antiviral activity (De Clercq, 1995), and would be a major advance in the treatment of influenza virus infection.

K3, a K-type ODN, consists of PS backbones. Amphipathic and polyanionic PS-oligonucleotides (ONs) have a sequence-independent antiviral activity (Gao et al., 1990; Yao et al., 1993; Fennewald et al., 1995). PS-ONs prevent viral attachment or entry via interaction with viral glycoproteins such as HIV-1, herpes simplex virus (HSV) and hepatitis C virus (Vaillant et al., 2006; Guzman et al., 2007; Matsumura et al., 2009). However, the antiviral activity of PS-ONs is dependent on the length and hydrophobicity of the ONs. It has been known that phosphorothioation increases the hydrophobicity of ONs (Agrawal et al., 1990). The increased hydrophobicity of PS-ONs contributes to their inhibitory activity against HIV-1 fusion and entry, because longer PS-ONs ($\geq$30 bases) which have a greater hydrophobicity are more potent in blocking the hydrophobic interactions involved in the gp41 six-helix bundle formation and inhibiting the HIV-1-mediated cell—cell fusion than shorter PS-ONs (<30 bases) (Vaillant et al., 2006). The antiviral
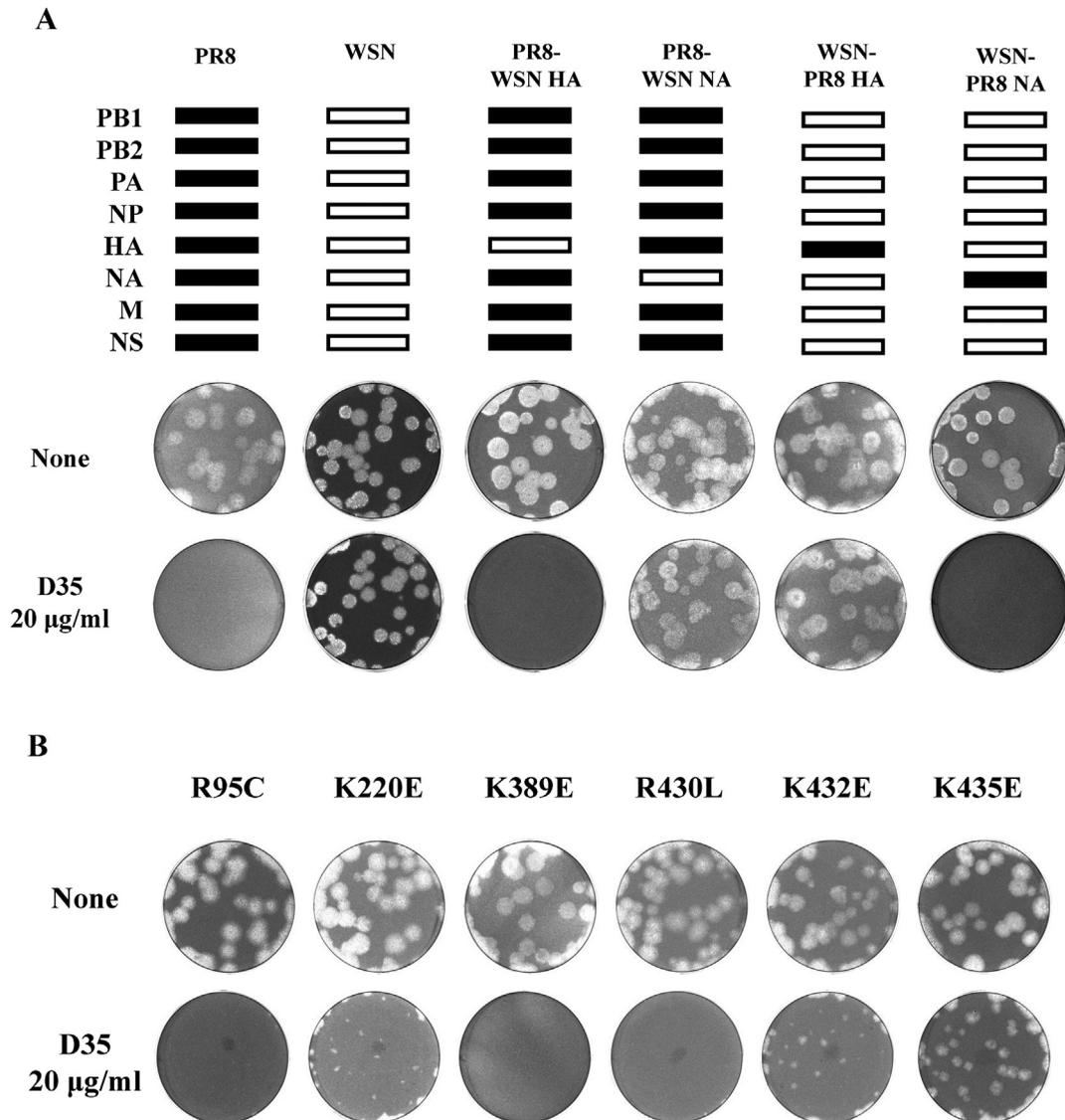
Fig. 6. The suppressive effects of viral replication by D35 on reassortant viruses between PR8 and WSN and recombinant viruses with a mutation in NA of PR8. (A) MDCK cells were infected with reassortants between PR8 and WSN at 35 °C in the absence of D35, and incubated in agarose with or without 20 μg/ml D35. Black and white bars represent genes from PR8 and WSN, respectively. (B) MDCK cells were infected with recombinant viruses in the absence of D35, and maintained in agarose with or without 20 μg/ml D35 at 35 °C.

activity of PS-ONs in duck hepatitis B virus infectionis also size dependent, with PS-ONs ≥40 bases in length displaying the highest activity (Noordeen et al., 2013). Thus we speculated that longer PS-ODNs than K3 (20 bases) would have an anti-influenza activity.

Short and GT rich-ODNs which assume a G-tetrad structure by intramolecular folding are potent inhibitors against HIV (Rando et al., 1995; Bishop et al., 1996) and they inhibit HIV-1 integrase (Jing et al., 2000). Moreover, a GT rich 20 base PS-ODN has a potent antiviral activity against HSV which is thought to be mediated by a conformational change in the viral glycoprotein gB (Shogan et al., 2006). D35 inhibited significantly the replication of influenza viruses, but A151, which did not multimerize and form aggregates, did not (Figs. 1A and 2C). Thus our results suggest that in contrast to ODNs with an ability to inhibit HIV-1 or HSV, for inhibiting influenza A viruses ODNs need to mutimerize and aggregate. It has been reported that D19 (gˆgtgcatcgatgcaggˆgˆgˆgˆg, ˆ: phosphorothioated), which has same sequence as D35 and two more PS linkage at the 3′ end poly(G) track than D35, consists an extremely heterogeneous mixture of secondary structures in DPBS and a portion of the D19 molecules exists as very high-order aggregates containing more than 30 D19 strands (Marshall et al., 2003). We do not know why spontaneous formation of nanoparticles was responsible for inhibiting influenza A viruses; however, the antiviral activity of DS against a influenza A virus increases when the molecular weight is increased from 1000 to 10,000 and levels off when the molecular weight was further increased to 70,000 (Hosoya et al., 1991). The result may suggest that the size of multimerized ODNs is important.

It has been reported that intranasal pretreatment with both synthetic TLR2/6 and TLR9 agonists protects mice against lethal influenza pneumonia but intranasal pretreatment with a TLR9 agonist alone results in no protection (Tuvim et al., 2012). It has also been reported that intranasal pretreatment with TLR3 agonist poly(I·C) and, to a lesser extent, a synthetic TLR9 agonist protects mice against lethal coronavirus infection and intranasal pretreatment with IFN-α or IFN-β protects mice against lethal influenza pneumonia (Zhao et al., 2012). Thus it was plausible that TLR9 agonist D35, but not D35 GpC, induced cytokines such as type I IFN by TLR9 activation and protecred mice against influenza virus infection. However, in mice treated with D35 GpC without
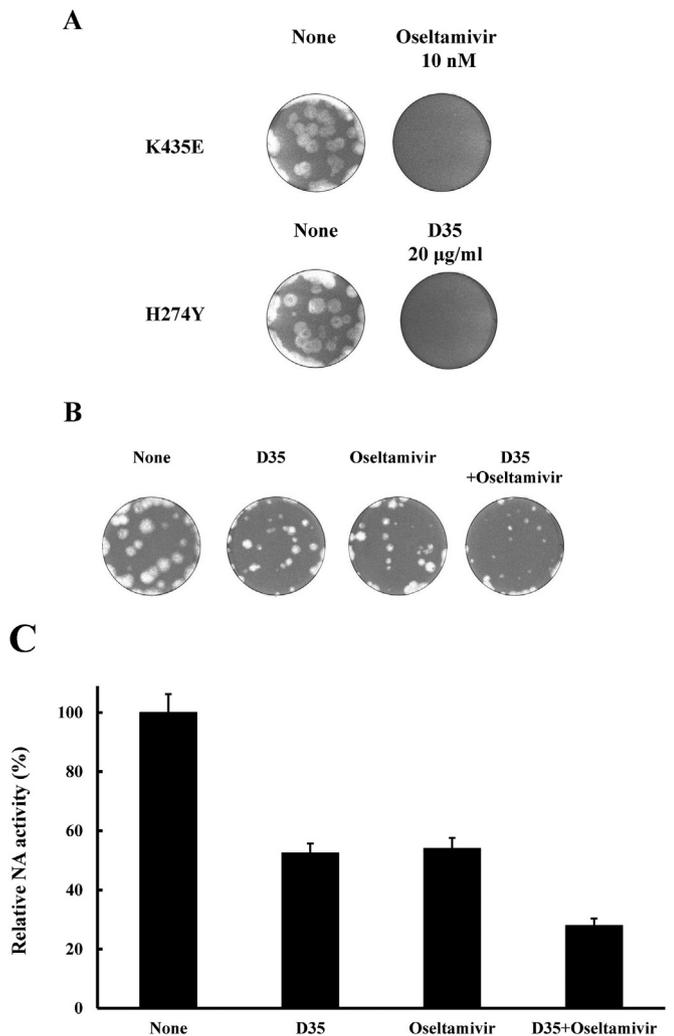
Fig. 7. The additive effect of D35 with oseltamivir. (A) MDCK cells were infected with K435E or H274Y in the absence of compounds and maintained at 35 °C in agarose with or without 10 nM oseltamivir or 20 μg/ml D35, respectively. (B) MDCK cells were infected with PR8 in the absence of compounds and incubated in agarose with 2 μg/ml D35, 2 nM oseltamivir, or both compounds at 35 °C. (C) The culture supernatant from PR8-infected MDCK cells was treated with or without 1 μg/ml D35, 1 nM oseltamivir, or both, followed by the measurement of NA activity in the samples. The relative NA activity was determined as the mean percent, given in % of the control values. Lines represent the mean ± standard deviation of three independent samples.

Fig. 8. The effect of D35 in mouse. Eight-week-old female BALB/c mice were treated with 10 mg/kg D35 or D35 GpC 18 h before, or 10 mg/kg D35, D35 GpC, oseltamivir or PBS 6 h after intranasal infection with 100 PFU of PR8 virus. (A) Weight loss was monitored daily for 14 days. n = 10 mice/group. (B) To obtain virus titers, lungs were isolated at 55 hpi, homogenized and virus titers determined on MDCK cells. Titers are expressed as PFU/lung homogenate. n = 5 mice/group.

unmethylated CpG after infection, viral titers in lung homogenates were reduced and body weight losses were minimal, suggesting that immune response through TLR9 leading to cytokine expression were not involved in protective effect of D35 GpC. Although D35 treatment before or after infection reduced viral titers in lung homogenates more than D35 GpC, mice that received D35 after infection exhibited larger weight loss than D35 GpC-treated mice at early times after infection (Fig. 7). The consequences of inappropriate TLR9 activation can be detrimental for the host, contributing to the pathogenesis of bacterial septic shock or autoimmune diseases, such as systemic lupus erythematosus (Ehlers and Ravetch, 2007). Thus we think that D35 GpC without a CpG motif would be a better candidate for influenza virus treatment than D35 containing a CpG motif.

In conclusion, the TLR9 ligand D35 inhibited NA activity and replication of influenza A viruses. It was suggested that the basic amino acids in the 430-loop of PR8 NA were involved in D35-sensitivity. D35 showed additive effect with oseltamivir. D35 and
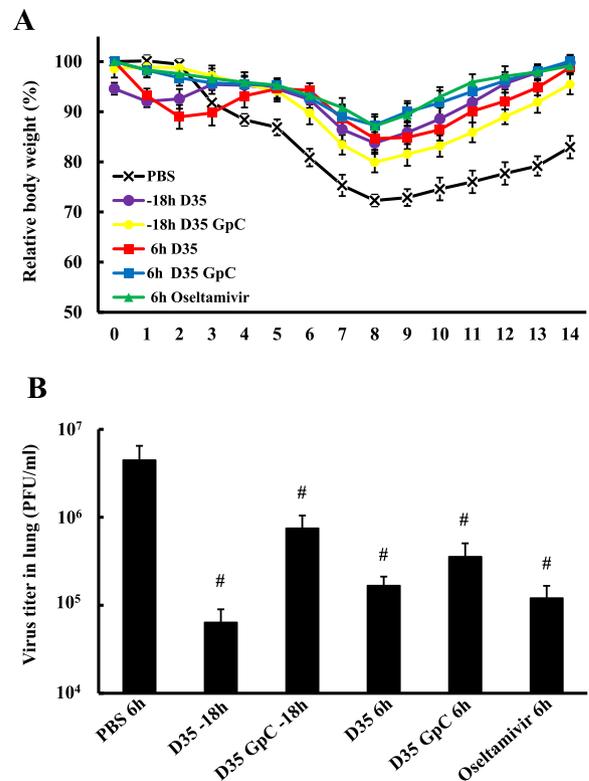
D35 GpC reduced viral replication in lungs of infected mice. Thus D35 could be used in combination with available NA inhibitors to treat influenza virus infections. Since D35 is well tolerated in clinical trials in nonhuman primates for Leishmania or malaria vaccine (Verthelyi et al., 2002; Tougan et al., 2013), further *in vivo* and clinical studies are warranted.

Acknowledgments

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.antiviral.2016.02.012.

References

Agrawal, S., Tang, J.Y., Brown, D.M., 1990. Analytical study of phosphorothioate analogues of oligodeoxynucleotides using high-performance liquid chromatography. J. Chromatogr. 509, 396–399.

Amaro, R.E., Cheng, X., Ivanov, I., Xu, D., McCammon, J.A., 2009. Characterizing loop dynamics and ligand recognition in human- and avian-type influenza neuraminidases via generalized born molecular dynamics and end-point free energy calculations. J. Am. Chem. Soc. 131, 4702–4709.

Amaro, R.E., Minh, D.D., Cheng, L.S., Lindstrom Jr., W.M., Olson, A.J., Lin, J.H., Li, W.W., McCammon, J.A., 2007. Remarkable loop flexibility in avian influenza N1 and its implications for antiviral drug design. J. Am. Chem. Soc. 129, 7764–7765.

An, J., Lee, D.C., Law, A.H., Yang, C.L., Poon, L.L., Lau, A.S., Jones, S.J., 2009. A novel small-molecule inhibitor of the avian influenza H5N1 virus determined through computational screening against the neuraminidase. J. Med. Chem. 52, 2667–2672.

Arnberg, N., Kidd, A.H., Edlund, K., Nilsson, J., Pring-Akerblom, P., Wadell, G., 2002. Adenovirus type 37 binds to cell surface sialic acid through a charge-dependent interaction. Virology 302, 33–43.

Baba, M., Snoeck, R., Pauwels, R., de Clercq, E., 1988. Sulfated polysaccharides are potent and selective inhibitors of various enveloped viruses, including herpes simplex virus, cytomegalovirus, vesicular stomatitis virus, and human immunodeficiency virus. Antimicrob. Agents Chemother. 32, 1742–1745.

Balagurumoorthy, P., Brahmachari, S.K., 1994. Structure and stability of human telomeric sequence. J. Biol. Chem. 269, 21858–21869.

Barman, S., Adhikary, L., Chakrabarti, A.K., Bernas, C., Kawaoka, Y., Nayak, D.P., 2004. Role of transmembrane domain and cytoplasmic tail amino acid sequences of influenza a virus neuraminidase in raft association and virus budding. J. Virol. 78, 5258–5269.

Bauer, S., Kirschning, C.J., Hacker, H., Redecke, V., Hausmann, S., Akira, S., Wagner, H., Lipford, G.B., 2001. Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. Proc. Natl. Acad. Sci. U. S. A. 98, 9237–9242.

Bishop, J.S., Guy-Caffey, J.K., Ojwang, J.O., Smith, S.R., Hogan, M.E., Cossum, P.A., Rando, R.F., Chaudhary, N., 1996. Intramolecular G-quartet motifs confer nuclease resistance to a potent anti-HIV oligonucleotide. J. Biol. Chem. 271, 5698–5703.

Boltz, D.A., Aldridge Jr., J.R., Webster, R.G., Govorkova, E.A., 2010. Drugs in development for influenza. Drugs 70, 1349–1362.

Carr, J., Ives, J., Kelly, L., Lambkin, R., Oxford, J., Mendel, D., Tai, L., Roberts, N., 2002. Influenza virus carrying neuraminidase with reduced sensitivity to oseltamivir carboxylate has altered properties in vitro and is compromised for infectivity and replicative ability in vivo. Antivir. Res. 54, 79–88.

Colman, P.M., Hoyne, P.A., Lawrence, M.C., 1993. Sequence and structure alignment of paramyxovirus hemagglutinin-neuraminidase with influenza virus neuraminidase. J. Virol. 67, 2972–2980.

Costa, L.T., Kerkmann, M., Hartmann, G., Endres, S., Bisch, P.M., Heckl, W.M., Thalhammer, S., 2004. Structural studies of oligonucleotides containing G-quadruplex motifs using AFM. Biochem. Biophys. Res. Commun. 313, 1065–1072.

De Clercq, E., 1995. Toward improved anti-HIV chemotherapy: therapeutic strategies for intervention with HIV infections. J. Med. Chem. 38, 2491–2517.

Ehlers, M., Ravetch, J.V., 2007. Opposing effects of Toll-like receptor stimulation induce autoimmunity or tolerance. Trends Immunol. 28, 74–79.

Fennewald, S.M., Mustain, S., Ojwang, J., Rando, R.F., 1995. Inhibition of herpes simplex virus in culture by oligonucleotides composed entirely of deoxyguanosine and thymidine. Antivir. Res. 26, 37–54.

Gao, W.Y., Hanes, R.N., Vazquez-Padua, M.A., Stein, C.A., Cohen, J.S., Cheng, Y.C., 1990. Inhibition of herpes simplex virus type 2 growth by phosphorothioate oligodeoxynucleotides. Antimicrob. Agents Chemother. 34, 808–812.

Guiducci, C., Ott, G., Chan, J.H., Damon, E., Calacsan, C., Matray, T., Lee, K.D., Coffman, R.L., Barrat, F.J., 2006. Properties regulating the nature of the plasmacytoid dendritic cell response to Toll-like receptor 9 activation. J. Exp. Med. 203, 1999–2008.

Guzman, E.M., Cheshenko, N., Shende, V., Keller, M.J., Goyette, N., Juteau, J.M., Boivin, G., Vaillant, A., Herold, B.C., 2007. Amphipathic DNA polymers are candidate vaginal microbicides and block herpes simplex virus binding, entry and viral gene expression. Antivir. Ther. 12, 1147–1156.

Hartmann, G., Krieg, A.M., 2000. Mechanism and function of a newly identified CpG DNA motif in human primary B cells. J. Immunol. 164, 944–953.

Hauge, S.H., Dudman, S., Borgen, K., Lackenby, A., Hungnes, O., 2009. Oseltamivir-resistant influenza viruses A (H1N1), Norway, 2007-08. Emerg. Infect. Dis. 15, 155–162.

Hayden, F.G., 1997. Prevention and treatment of influenza in immunocompromised patients. Am. J. Med. 102, 55–60 discussion 75–76.

Horimoto, T., Murakami, S., Muramoto, Y., Yamada, S., Fujii, K., Kiso, M., Iwatsuki-Horimoto, K., Kino, Y., Kawaoka, Y., 2007. Enhanced growth of seed viruses for H5N1 influenza vaccines. Virology 366, 23–27.

Hosoya, M., Balzarini, J., Shigeta, S., De Clercq, E., 1991. Differential inhibitory effects of sulfated polysaccharides and polymers on the replication of various myxoviruses and retroviruses, depending on the composition of the target amino acid sequences of the viral envelope glycoproteins. Antimicrob. Agents Chemother. 35, 2515–2520.

Hurt, A.C., Ernest, J., Deng, Y.M., Iannello, P., Besselaar, T.G., Birch, C., Buchy, P., Chittaganpitch, M., Chiu, S.C., Dwyer, D., Guigon, A., Harrower, B., Kei, I.P., Kok, T., Lin, C., McPhie, K., Mohd, A., Olveda, R., Panayotou, T., Rawlinson, W., Scott, L., Smith, D., D'Souza, H., Komadina, N., Shaw, R., Kelso, A., Barr, I.G., 2009. Emergence and spread of oseltamivir-resistant A(H1N1) influenza viruses in Oceania, South East Asia and South Africa. Antivir. Res. 83, 90–93.

Jing, N., Marchand, C., Liu, J., Mitra, R., Hogan, M.E., Pommier, Y., 2000. Mechanism of inhibition of HIV-1 integrase by G-tetrad-forming oligonucleotides in Vitro. J. Biol. Chem. 275, 21460–21467.

Kaminski, J.J., Schattgen, S.A., Tzeng, T.C., Bode, C., Klinman, D.M., Fitzgerald, K.A., 2013. Synthetic oligodeoxynucleotides containing suppressive TTAGGG motifs

inhibit AIM2 inflammasome activation. J. Immunol. 191, 3876–3883.

Kerkmann, M., Costa, L.T., Richter, C., Rothenfusser, S., Battiany, J., Hornung, V., Johnson, J., Englert, S., Ketterer, T., Heckl, W., Thalhammer, S., Endres, S., Hartmann, G., 2005. Spontaneous formation of nucleic acid-based nanoparticles is responsible for high interferon-alpha induction by CpG-A in plasmacytoid dendritic cells. J. Biol. Chem. 280, 8086–8093.

Klinman, D.M., 2004. Immunotherapeutic uses of CpG oligodeoxynucleotides. Nat. Rev. Immunol. 4, 249–258.

Krieg, A.M., 2006. Therapeutic potential of Toll-like receptor 9 activation. Nat. Rev. Drug Discov. 5, 471–484.

Luscher-Mattli, M., Gluck, R., 1990. Dextran sulfate inhibits the fusion of influenza virus with model membranes, and suppresses influenza virus replication in vivo. Antivir. Res. 14, 39–50.

Luscher-Mattli, M., Gluck, R., Kempf, C., Zanoni-Grassi, M., 1993. A comparative study of the effect of dextran sulfate on the fusion and the in vitro replication of influenza A and B, Semliki Forest, vesicular stomatitis, rabies, Sendai, and mumps virus. Arch. Virol. 130, 317–326.

Marshall, J.D., Fearon, K., Abbate, C., Subramanian, S., Yee, P., Gregorio, J., Coffman, R.L., Van Nest, G., 2003. Identification of a novel CpG DNA class and motif that optimally stimulate B cell and plasmacytoid dendritic cell functions. J. Leukoc. Biol. 73, 781–792.

Matsumura, T., Hu, Z., Kato, T., Dreux, M., Zhang, Y.Y., Imamura, M., Hiraga, N., Juteau, J.M., Cosset, F.L., Chayama, K., Vaillant, A., Liang, T.J., 2009. Amphipathic DNA polymers inhibit hepatitis C virus infection by blocking viral entry. Gastroenterology 137, 673–681.

Mitsuya, H., Looney, D.J., Kuno, S., Ueno, R., Wong-Staal, F., Broder, S., 1988. Dextran sulfate suppression of viruses in the HIV family: inhibition of virion binding to CD4+ cells. Science 240, 646–649.

Moulard, M., Lortat-Jacob, H., Mondor, I., Roca, G., Wyatt, R., Sodroski, J., Zhao, L., Olson, W., Kwong, P.D., Sattentau, Q.J., 2000. Selective interactions of polyanions with basic surfaces on human immunodeficiency virus type 1 gp120. J. Virol. 74, 1948–1960.

Neumann, G., Noda, T., Kawaoka, Y., 2009. Emergence and pandemic potential of swine-origin H1N1 influenza virus. Nature 459, 931–939.

Neumann, G., Watanabe, T., Ito, H., Watanabe, S., Goto, H., Gao, P., Hughes, M., Perez, D.R., Donis, R., Hoffmann, E., Hobom, G., Kawaoka, Y., 1999. Generation of influenza A viruses entirely from cloned cDNAs. Proc. Natl. Acad. Sci. U. S. A. 96, 9345–9350.

Nistal-Villan, E., Garcia-Sastre, A., 2009. New prospects for the rational design of antivirals. Nat. Med. 15, 1253–1254.

Noordeen, F., Vaillant, A., Jilbert, A.R., 2013. Nucleic acid polymers inhibit duck hepatitis B virus infection in vitro. Antimicrob. Agents Chemother. 57, 5291–5298.

Palese, P., Compans, R.W., 1976. Inhibition of influenza virus replication in tissue culture by 2-deoxy-2,3-dehydro-N-trifluoroacetylneuraminic acid (FANA): mechanism of action. J. Gen. Virol. 33, 159–163.

Palese, P., Tobita, K., Ueda, M., Compans, R.W., 1974. Characterization of temperature sensitive influenza virus mutants defective in neuraminidase. Virology 61, 397–410.

Panyutin, I.G., Kovalsky, O.I., Budowsky, E.I., Dickerson, R.E., Rikhirev, M.E., Lipanov, A.A., 1990. G-DNA: a twice-folded DNA structure adopted by single-stranded oligo(dG) and its implications for telomeres. Proc. Natl. Acad. Sci. U. S. A. 87, 867–870.

Puig, M., Grajkowski, A., Boczkowska, M., Ausin, C., Beaucage, S.L., Verthelyi, D., 2006. Use of thermolytic protective groups to prevent G-tetrad formation in CpG ODN type D: structural studies and immunomodulatory activity in primates. Nucleic Acids Res. 34, 6488–6495.

Ramalho-Santos, J., de Lima, M.C., 2001. Fusion and infection of influenza and Sendai viruses as modulated by dextran sulfate: a comparative study. Biosci. Rep. 21, 293–304.

Rando, R.F., Ojwang, J., Elbaggari, A., Reyes, G.R., Tinder, R., McGrath, M.S., Hogan, M.E., 1995. Suppression of human immunodeficiency virus type 1 activity in vitro by oligonucleotides which form intramolecular tetrads. J. Biol. Chem. 270, 1754–1760.

Sakabe, S., Sakoda, Y., Haraguchi, Y., Isoda, N., Soda, K., Takakuwa, H., Saijo, K., Sawata, A., Kume, K., Hagiwara, J., Tuchiya, K., Lin, Z., Sakamoto, R., Imamura, T., Sasaki, T., Kokumai, N., Kawaoka, Y., Kida, H., 2008. A vaccine prepared from a non-pathogenic H7N7 virus isolated from natural reservoir conferred protective immunity against the challenge with lethal dose of highly pathogenic avian influenza virus in chickens. Vaccine 26, 2127–2134.

Samulowitz, U., Weber, M., Weeratna, R., Uhlmann, E., Noll, B., Kreig, A.M., Vollmer, J., 2010. A novel class of immune-stimulatory CpG oligodeoxynucleotides unifies high potency in type I interferon induction with preferred structural properties. Oligonucleotides 20, 93–101.

Shogan, B., Kruse, L., Mulamba, G.B., Hu, A., Coen, D.M., 2006. Virucidal activity of a GT-rich oligonucleotide against herpes simplex virus mediated by glycoprotein B. J. Virol. 80, 4740–4747.

Soda, K., Sakoda, Y., Isoda, N., Kajihara, M., Haraguchi, Y., Shibuya, H., Yoshida, H., Sasaki, T., Sakamoto, R., Saijo, K., Hagiwara, J., Kida, H., 2008. Development of vaccine strains of H5 and H7 influenza viruses. Jpn. J. Vet. Res. 55, 93–98.

Tougan, T., Aoshi, T., Coban, C., Katakai, Y., Kai, C., Yasutomi, Y., Ishii, K.J., Horii, T., 2013. TLR9 adjuvants enhance immunogenicity and protective efficacy of the SE36/AHG malaria vaccine in nonhuman primate models. Hum. Vaccin Immunother. 9, 283–290.

Tuvim, M.J., Gilbert, B.E., Dickey, B.F., Evans, S.E., 2012. Synergistic TLR2/6 and TLR9

activation protects mice against lethal influenza pneumonia. PLoS One 7, e30596.

Uraki, R., Kiso, M., Shinya, K., Goto, H., Takano, R., Iwatsuki-Horimoto, K., Takahashi, K., Daniels, R.S., Hungnes, O., Watanabe, T., Kawaoka, Y., 2013. Virulence determinants of pandemic A(H1N1)2009 influenza virus in a mouse model. J. Virol. 87, 2226–2233.

Vaillant, A., Juteau, J.M., Lu, H., Liu, S., Lackman-Smith, C., Ptak, R., Jiang, S., 2006. Phosphorothioate oligonucleotides inhibit human immunodeficiency virus type 1 fusion by blocking gp41 core formation. Antimicrob. Agents Chemother. 50, 1393–1401.

van der Vries, E., Stelma, F.F., Boucher, C.A., 2010. Emergence of a multidrug-resistant pandemic influenza A (H1N1) virus. N. Engl. J. Med. 363, 1381–1382.

Verthelyi, D., Ishii, K.J., Gursel, M., Takeshita, F., Klinman, D.M., 2001. Human peripheral blood cells differentially recognize and respond to two distinct CPG motifs. J. Immunol. 166, 2372–2377.

Verthelyi, D., Kenney, R.T., Seder, R.A., Gam, A.A., Friedag, B., Klinman, D.M., 2002. CpG oligodeoxynucleotides as vaccine adjuvants in primates. J. Immunol. 168, 1659–1663.

Vollmer, J., Krieg, A.M., 2009. Immunotherapeutic applications of CpG oligodeoxynucleotide TLR9 agonists. Adv. Drug Deliv. Rev. 61, 195–204.

Whitley, R.J., Monto, A.S., 2006. Prevention and treatment of influenza in high-risk groups: children, pregnant women, immunocompromised hosts, and nursing home residents. J. Infect. Dis. 194 (Suppl. 2), S133–S138.

Wu, C.C., Lee, J., Raz, E., Corr, M., Carson, D.A., 2004. Necessity of oligonucleotide aggregation for toll-like receptor 9 activation. J. Biol. Chem. 279, 33071–33078.

Yamada, H., Moriishi, E., Haredy, A.M., Takenaka, N., Mori, Y., Yamanishi, K., Okamoto, S., 2012. Influenza virus neuraminidase contributes to the dextran sulfate-dependent suppressive replication of some influenza A virus strains. Antivir. Res. 96, 344–352.

Yamada, H., Nagao, C., Haredy, A.M., Mori, Y., Mizuguchi, K., Yamanishi, K., Okamoto, S., 2014. Dextran sulfate-resistant A/Puerto Rico/8/34 influenza virus is associated with the emergence of specific mutations in the neuraminidase glycoprotein. Antivir. Res. 111, 69–77.

Yao, G.Q., Grill, S., Egan, W., Cheng, Y.C., 1993. Potent inhibition of Epstein-Barr virus by phosphorothioate oligodeoxynucleotides without sequence specification. Antimicrob. Agents Chemother. 37, 1420–1425.

Zhao, J., Wohlford-Lenane, C., Zhao, J., Fleming, E., Lane, T.E., McCray Jr., P.B., Perlman, S., 2012. Intranasal treatment with poly(I*C) protects aged mice from lethal respiratory virus infections. J. Virol. 86, 11416–11424.