Increasing resistance to currently available influenza antivirals highlights the need to develop alternate approaches for the prevention and/or treatment of influenza. DAS181 (Fludase), a novel sialidase fusion protein that enzymatically removes sialic acids on respiratory epithelium, exhibits potent antiviral activity against influenza A and B viruses. Here, we use a mouse model to evaluate the efficacy of DAS181 treatment against a highly pathogenic avian influenza H5N1 virus. When used to treat mice daily beginning 1 day before infection with A/Vietnam/1203/2004(H5N1) virus, DAS181 treatment at 1 mg/kg/day protected 100% of mice from fatal disease, prevented viral dissemination to the brain, and effectively blocked infection in 70% of mice. DAS181 at 1 mg/kg/day was also effective therapeutically, conferring enhanced survival of H5N1 virus–challenged mice when treatment was begun 72 h after infection. This notable antiviral activity underscores the potential utility of DAS181 as a new class of drug that is effective against influenza viruses with pandemic potential.

Since late 2003, highly pathogenic avian influenza (HPAI) H5N1 viruses have devastated poultry populations across Asia, Europe, and Africa and have caused >300 laboratory-confirmed human infections, with a fatality rate of ~60% [1]. These events highlight the potential for a pandemic strain, such as H5N1, to arise from avian species. In such an event, effective antiviral drugs will be an essential control measure, particularly in the first stages of a pandemic when antigenically well-matched vaccines are not yet available [2]. Currently licensed anti-influenza drugs consist of the adamantane M2 ion-channel blockers and the neuraminidase inhibitors, oseltamivir and zanamivir [3, 4]. Unfortunately, a majority of clade 1 and some clade 2 H5N1 viruses circulating in southeast Asia are resistant to the M2 blockers [5–8]. Isolation of quasispecies that include oseltamivir-resistant variants from H5N1 virus–infected patients has raised further concerns that existing antiviral modalities for H5N1 viruses are inadequate [9, 10] and emphasize the need to develop novel therapeutic agents that are effective against avian influenza viruses with pandemic potential [11].

Influenza viruses initiate infection by binding to terminal sialic acid receptors on glycoconjugates on the surface of susceptible host cells. Therefore, a drug with sialidase activity that can remove available receptors on human airway epithelium has the potential to prevent or reduce the infectivity of multiple influenza subtypes. DAS181 (Fludase) is a recombinant fusion protein consisting of a sialidase catalytic domain derived from Acti-
nomys viscosus fused with a respiratory epithelium–anchoring domain that tethers the drug to the site of influenza virus infection in humans and cleaves both α(2,6)-linked and α(2,3)-linked sialic acid receptors, which are preferentially recognized by human and avian/equine influenza viruses, respectively [12]. DAS181 has exhibited potent antiviral activity against both influenza A and B viruses in vitro and has reduced replication of H1N1 viruses in mice and ferrets [12]. However, the in vivo activity of the drug against avian influenza viruses with pandemic potential is not known. Here, we demonstrate that DAS181 can effectively protect mice from lethal disease, significantly reduce virus replication, and even prevent infection with an HPAI H5N1 virus.

MATERIALS AND METHODS

Virus. The HPAI A/Vietnam/1203/2004 (H5N1) virus (hereafter, VN/1203) was grown in the allantoic cavity of 10-day-old embryonated hens’ eggs at 37°C. Allantoic fluid was collected 26 h after inoculation and stored in aliquots at −70°C. The EID₅₀ titer was determined by serial titration of virus in eggs and was calculated by the method of Reed and Muench [13]. All experiments with highly pathogenic viruses were conducted under biosafety level 3 containment, including enhancements required by the US Department of Agriculture and the Select Agent Program [14]. Laboratory workers wore appropriate respiratory equipment at all times (RACAL Health and Safety). Animal research was approved by the Centers for Disease Control and Prevention’s Institutional Animal Care and Use Committee and was conducted in an Association for Assessment and Accreditation of Laboratory Animal Care International–accredited animal facility.

Infection of mice and challenge experiments. Six-to-eight-week-old female BALB/c mice (Charles River Laboratories) were lightly anesthetized with CO₂, and 50 μL of infectious virus diluted in PBS was inoculated intranasally (inl). The MLD₅₀ of VN/1203 was determined as described elsewhere [15]. To evaluate the degree of drug efficacy, mice were challenged inl with 3 MLD₅₀ (1 × 10⁻².₅ EID₅₀) or 1.5 MLD₅₀ (1 × 10⁻².₀ EID₅₀) of VN/1203. Three to five mice per group were killed 6 or 9 days after infection. Lung and brain tissues were collected and titrated for virus as described elsewhere [15]. Briefly, whole lungs and brains were collected and homogenized in 1 mL of PBS, clarified by centrifugation, and serially titrated in eggs. Virus titers were expressed as the mean ± SE log₁₀ EID₅₀/mL. The remaining 12–20 mice in each group were observed daily for weight loss and survival for 21 days.

Treatment of mice with DAS181. BALB/c mice were anesthetized by intraperitoneal injection with 100 mg/kg ketamine before treatment with DAS181 or placebo (sterile PBS); 10 U (8 μg) or 30 U (23 μg) of DAS181 or PBS was delivered inl to respiratory mucosa in a 50-μL volume once or twice daily for a total of 7 or 8 days. One unit of DAS181 is equivalent to 0.77 μg of protein. To evaluate the prophylactic and therapeutic efficacy of DAS181 against an HPAI H5N1 virus, mice were treated with DAS181 starting 24 h before or 24, 48, or 72 h after inl infection with VN/1203.

Serum collection and antibody assays. Serum samples were isolated from blood collected from the orbital plexus of mice and treated with receptor-deleting enzyme from Vibrio cholerae (Denka-Seiken) before testing for the presence of H5 hemagglutinin (HA)–specific antibodies [16]. The hemagglutination-inhibition (HI) assay was performed using 4 hemagglutinating units of VN/1203 and 1% horse red blood cells, as described elsewhere [17]. Serum samples with titers ≥40 (≥log₂ 5.32), indicating a ≥4-fold increase in antibody compared with preinfection titers, were considered to be positive for H5 antibody. H5 HA–specific IgG antibody was detected by ELISA. The ELISA was performed as described elsewhere [18], except that 1 μg/mL of purified baculovirus-expressed H5 recombinant VN/1203 HA protein was used to coat plates (Protein Sciences). The ELISA end-point titers were expressed as the highest dilution that yielded an optical density >2 times the mean plus SD of similarly diluted negative control samples. A titer >100 was considered to be positive. Because the ELISA is generally more sensitive than the HI in the detection of influenza virus–sensitive antibody, a positive result by ELISA was considered to be evidence of productive H5N1 virus infection in mice; untreated and uninfected (naïve) mice have HI antibody titers ≤10 and IgG titers <100.

Statistical analysis. Mean time-to-death values were calculated by the SAS life test; mice that survived the observation period were assigned a value 21 days for this life test. The survival function was calculated by the Kaplan-Meier method, and hazard ratios were established by the Cox proportional hazards regression model. The significance of survival in test groups versus the placebo group was determined by Fisher’s exact test. The significance of weight loss in mice was determined by analysis of variance. The significance of viral titers was determined by a 2-tailed Student’s t test; P < .05 was considered to indicate significance. All statistical analyses were performed with SAS for Windows software (version 9.1; SAS Institute).

RESULTS

Prophylactic efficacy of DAS181 against a highly virulent H5N1 virus in mice. A previous study determined that DAS181 at a dose range of 0.3 to 1 mg/kg/day was effective in inhibiting replication of a neurotropic H1N1 influenza virus in BALB/c mice [12]. To evaluate the prophylactic efficacy of DAS181 against a highly virulent H5N1 virus, BALB/c mice were treated inl with 1 mg/kg DAS181 once daily or 0.33 mg/kg DAS181 once or twice (0.66 mg/kg/day, or ∼0.7 mg/kg/day) daily for 7 days beginning 1 day before challenge with 3 or 1.5
MLD50 of VN/1203. Mice were monitored daily for 21 days for weight loss and survival. Mice treated with DAS181 at 1 mg/kg/day and challenged with either virus dose were completely protected from death and effectively lost no weight \((P < .0001)\), whereas 100% of mock–treated mice (PBS) died of infection, with a mean weight loss of 16% on day 5 after infection and a mean time to death of 7 days (table 1 and figure 1A and 1B). Mice that received DAS181 at 0.3 or 0.7 mg/kg/day also exhibited a significant reduction in weight loss \((P < .05)\) and enhanced survival, compared with mock-treated mice. In particular, at 0.7 mg/kg/day, DAS181 reduced mortality by 70% and 94% \((P < .05)\) in mice challenged with 3 or 1.5 MLD50, respectively.

To assess the effect of DAS181 treatment on H5N1 virus replication and systemic spread, we next determined viral titers in

Table 1. Survival in mice prophylactically administered DAS181 and lethally challenged with A/Vietnam/1203/2004(H5N1) virus (VN/1203).

<table>
<thead>
<tr>
<th>Total dose per daya</th>
<th>Mean weight loss on day 5 after infection, %</th>
<th>No. infected/total no. (%)b</th>
<th>No. of survivors/total no. (%)c</th>
<th>Time to death, mean ± SE, daysd</th>
<th>Hazard ratio for death</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 MLD50 challenge</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 mg/kg</td>
<td>0.0e</td>
<td>4/12 (33)</td>
<td>12/12 (100)e</td>
<td>&gt;21.0 ± 0.0e</td>
<td>0.000e</td>
</tr>
<tr>
<td>0.7 mg/kgf</td>
<td>2.5a</td>
<td>14/16 (88)</td>
<td>11/16 (69)</td>
<td>9.4 ± 0.3a</td>
<td>0.096e</td>
</tr>
<tr>
<td>0.3 mg/kg</td>
<td>3.7a</td>
<td>13/15 (87)</td>
<td>6/15 (40)</td>
<td>10.7 ± 0.7e</td>
<td>0.203e</td>
</tr>
<tr>
<td>PBS</td>
<td>16.0</td>
<td>14/14 (100)</td>
<td>0/14 (0)</td>
<td>6.7 ± 0.3</td>
<td>1.000</td>
</tr>
<tr>
<td>1.5 MLD50 challenge</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 mg/kg</td>
<td>0.0e</td>
<td>4/14 (29)</td>
<td>14/14 (100)e</td>
<td>&gt;21.0 ± 0.0e</td>
<td>0.000e</td>
</tr>
<tr>
<td>0.7 mg/kgf</td>
<td>6.2a</td>
<td>13/16 (81)</td>
<td>15/16 (94)e</td>
<td>20.2 ± 1.1e</td>
<td>0.013e</td>
</tr>
<tr>
<td>0.3 mg/kg</td>
<td>2.3a</td>
<td>12/17 (71)</td>
<td>8/17 (47)</td>
<td>12.3 ± 0.6e</td>
<td>0.137e</td>
</tr>
<tr>
<td>PBS</td>
<td>16.2</td>
<td>16/16 (100)</td>
<td>0/16 (0)</td>
<td>7.3 ± 0.4</td>
<td>1.000</td>
</tr>
</tbody>
</table>

a Once- or twice-daily dosing of DAS181 or vehicle only (PBS) for 8 days commencing 24 h before virus challenge. Challenge was with 3 or 1.5 MLD50 of VN/1203; mice were challenged intranasally with VN/1203 after 2 or 3 treatments. 
b No. of mice that became infected or that were positive for antibody to VN/1203 as detected by hemagglutination-inhibition assay or ELISA per the total no. of mice. 
c No. of survivors per the total no. of mice; mice were monitored daily for survival and weight loss for 21 days after challenge. 
d Mean day of death for all mice in each group; mice that survived the observation period (21 days) were given a value of 21. 
 e \(P < .0001\), compared with the PBS control group. 
f Mice received 0.3 mg/kg DAS181 twice daily. 
g \(P < .05\), compared with the PBS control group.

Figure 1. Prophylactic efficacy of DAS181 against a highly virulent H5N1 virus in mice. Mice were administered 1 mg/kg DAS181 once daily, 0.3 mg/kg DAS181 once or twice (0.7 mg/kg/day) daily, or PBS from 1 day before challenge to 6 days after infection. Mice were challenged with 3 or 1.5 MLD50 of A/Vietnam/1203/2004(H5N1) virus and monitored daily for survival (A and B). Mean ± SE virus titers in the lungs (black bars) and brains (white bars) of mice are shown on day 6 after infection (C and D). The limit of detection is \(1 \times 10^1.5\) EID50/mL for lungs and \(1 \times 10^2.8\) EID50/mL for brains (horizontal line). *\(P < .05\) and **\(P < .005\), compared with PBS-treated mice (Student’s t test).
the lungs and brains of mice 6 days after infection, when virus replication in these organs peaks in untreated mice [15, 19]. Treatment of mice with any dose of DAS181 reduced virus titers in the lungs by at least 10,000-fold, compared with those in mock-treated mice (figure 1C and 1D); similar results were observed with either challenge dose of VN/1203. Strikingly, virus was not detected in the brains of mice receiving DAS181 at any dose.

Blocking of influenza virus infection in mice after prophylactic administration of DAS181. To assess the extent to which mice administered DAS181 were actually infected with the H5N1 virus, we determined the levels of H5-specific HI or IgG antibody in serum samples collected from surviving mice (table 1 and figure 2). H5-specific antibody was detected in a majority of survivors that received DAS181 at 0.3 or 0.7 mg/kg/day but in only one-third of survivors that received DAS181 at 1 mg/kg/day, indicating that a majority of mice receiving this dose were not productively infected with VN/1203. This effect was seen at either challenge dose of VN/1203. These results demonstrate that daily inl delivery of DAS181 significantly reduced

Table 2. Survival in mice therapeutically administered DAS181 after lethal challenge with A/Vietnam/1203/2004(H5N1) virus (VN/1203).

<table>
<thead>
<tr>
<th>Dose per treatmenta</th>
<th>Dosing scheduleb</th>
<th>Mean weight loss on day 5 after infection, %</th>
<th>No. of survivors/total no. (%)c</th>
<th>Time to death, mean ± SE, daysd</th>
<th>Hazard ratio for death</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mg/kg 24 h before</td>
<td>5.6#</td>
<td>19/20 (95)#</td>
<td>20.7 ± 0.4#</td>
<td>0.007#</td>
<td></td>
</tr>
<tr>
<td>1 mg/kg 24 h after</td>
<td>18.2</td>
<td>14/20 (70)#</td>
<td>11.4 ± 0.4#</td>
<td>0.046#</td>
<td></td>
</tr>
<tr>
<td>1 mg/kg 48 h after</td>
<td>17.1</td>
<td>7/20 (35)#</td>
<td>10.2 ± 0.4#</td>
<td>0.133#</td>
<td></td>
</tr>
<tr>
<td>1 mg/kg 72 h after</td>
<td>13.3</td>
<td>3/20 (15)#</td>
<td>9.5 ± 0.3#</td>
<td>0.209#</td>
<td></td>
</tr>
<tr>
<td>PBS 24 h after</td>
<td>22.0</td>
<td>0/20 (0)</td>
<td>6.9 ± 0.3</td>
<td>1.000</td>
<td></td>
</tr>
</tbody>
</table>

a Once-daily dosing of DAS181 or vehicle only (PBS) for 8 days (24 h before) or 7 days. Challenge was with 3 MLD50 VN/1203.
b Dosing commenced 24 h before or 24, 48, or 72 h after virus challenge.
c No. of survivors per the total no. of mice; mice were monitored daily for survival and weight loss for 21 days after challenge.
d Mean day of death for all mice in each group; mice that survived the observation period (21 days) were given a value of 21.
# P < .0001, compared with the PBS control group.
† P < .05, compared with the PBS control group.
H5N1 virus replication and spread and, at a dose of 1 mg/kg/day, protected 100% of mice from death and a majority of mice from infection with a highly virulent H5N1 virus.

**Therapeutic efficacy of DAS181 against a highly virulent H5N1 virus in mice.** We next evaluated the therapeutic activity of DAS181 at 1 mg/kg/day when treatment was initiated 24, 48, or 72 h after challenge with 3 MLD$_{50}$ of A/Vietnam/1203/2004(H5N1) virus. As a control for antiviral efficacy, one group of mice received drug at 1 mg/kg/day starting 24 h before challenge; this group exhibited a 95% survival rate, similar to that observed in the previous experiment. Therapeutic administration of DAS181 was unable to protect mice from infection with VN/1203, as demonstrated by substantial weight loss between groups that was not statistically significant regardless of the day treatment was initiated. When treatment was delayed until 24 h after infection, mice exhibited substantial weight loss but had a significantly delayed mean time to death compared with the mock-treated group ($P < .0001$), and, remarkably, 70% of treated mice survived the lethal chal-

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**Figure 3.** Therapeutic efficacy of DAS181 against a highly virulent H5N1 virus in mice. Mice were administered DAS181 at 1 mg/kg/day or PBS daily for 7–8 days as indicated. Mice were challenged with 3 MLD$_{50}$ of A/Vietnam/1203/2004(H5N1) virus and monitored daily for survival (A). Mean ± SE virus titers in lungs (B) or brains (C) of mice on day 6 (black bars) or day 9 (white bars) after infection are shown. The limit of detection (horizontal line) is $1 \times 10^{1.5}$ EID$_{50}$/mL for lungs and $1 \times 10^{0.8}$ EID$_{50}$/mL for brains. †Control mice treated with PBS did not survive to day 9. *$P < .05$ and **$P < .005$, compared with PBS-treated mice (Student’s $t$ test).
challenge (P < .0001). Delaying treatment until 48 or 72 h after infection reduced overall survival to 35% and 15%, respectively (table 2 and figure 3A).

We next determined the effect of postchallenge treatment on viral titers on days 6 and 9 after infection, to establish whether viral load increased after cessation of treatment. When treatment was begun 1 day before challenge and completed on day 6 after infection, day 6 and 9 lung viral titers remained at least 5 logs lower than those detected in mock-treated mice; day 9 titers were not significantly higher than those detected on day 6 after infection (figure 3B). As expected, delaying treatment until after challenge resulted in substantially more virus replication in mouse lungs, but, nevertheless, titers were lower than those in mock-treated mice in all groups. Furthermore, in mice that began treatment 1 day after challenge and that ceased treatment on day 8 after infection, day 9 lung virus titers were dropping and not rebounding. Postchallenge treatment with DAS181 was unable to prevent virus spread to the brain (figure 3C); however, treatment started as late as 72 h after infection significantly reduced viral titers in the brain on day 6 after infection. These results suggest that DAS181 treatment, even when initiated early after infection, was able to control virus replication until late in infection, providing time for adaptive immune responses to come into play.

**DISCUSSION**

High viral burden has been implicated as a critical feature of H5N1 virus pathogenesis in humans [20], suggesting that clinical management of human H5N1 disease should focus on suppression of virus replication through the use of effective antivirals. In the present study, we have demonstrated that the novel sialidase fusion protein DAS181 has dose-dependent antiviral activity in mice against a highly virulent H5N1 virus isolated from a human with a fatal case of influenza. When delivered before challenge at a dose of 1 mg/kg/day, DAS181 not only protected mice from lethal disease but completely blocked infection in ~70% of mice. DAS181 was also effective when administered after challenge. Most importantly, after a 7-day treatment regimen, there was no significant increase in viral titers after cessation of treatment.

The mouse model has been widely used to evaluate the efficacy of neuraminidase inhibitors against both human and avian influenza viruses [21–24]. The efficient replication and systemic spread of VN/1203 in the mouse without the need for prior adaptation made this an appropriate choice to evaluate the antiviral efficacy of DAS181 here. Yen et al. [25] demonstrated that an 8-day prophylactic oseltamivir regimen delivered twice daily was required for optimal survival of mice after infection with VN/1203, whereas a previous study found that a 5-day oseltamivir regimen was effective against a less virulent 1997 H5N1 strain [24]. At doses of 1 or 10 mg/kg oseltamivir, the 8-day regimen protected 60% and 80% of mice from death, respectively, with the higher dose required to prevent viral replication in the brain [25]. The present study, using a challenge dose of VN/1203 that elicited similar if not higher levels of virus replication than in the study by Yen et al. [25], found that 1 mg/kg DAS181 conferred complete survival by significantly reducing lung viral titers and preventing dissemination of H5N1 virus to the brain. Further assessment of these 2 antiviral strategies awaits a side-by-side comparison. Even so, the prophylactic prevention of infection and significant therapeutic antiviral activity demonstrated by DAS181 in the present study against a highly virulent H5N1 virus is unprecedented.

Whereas currently available influenza antiviral treatments target viral gene products, DAS181 targets host cellular receptors, which may offer a decreased opportunity for the emergence of resistance. Nevertheless, it will be important to evaluate carefully the potential for selection of receptor-binding variants after DAS181 treatment. The remarkable in vivo efficacy against a highly virulent avian H5N1 virus highlights the potential of DAS181 as an effective antiviral agent for both seasonal and pandemic influenza. A phase 1 clinical trial of DAS181 will begin in late 2007.

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