Morpholino oligomers targeting the PB1 and NP genes enhance the survival of mice infected with highly pathogenic influenza A H7N7 virus

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INTRODUCTION

Influenza A viruses belong to the family Orthomyxoviridae and possess eight RNA genome segments (PB1, PB2, PA, NP, HA, M, NS and NA) of negative polarity, all of which are encapsidated by the nucleoprotein NP. All influenza virus RNA synthesis is carried out in the nucleus of the infected cell and is mediated by the viral polymerase subunits (PB1, PB2 and PA) and NP (Elton et al., 2006; Neumann et al., 2004). The influenza virus strain SC35 of the H7N7 subtype is highly pathogenic to chickens but has low pathogenicity in mice (Gabriel et al., 2005; Li et al., 1990). SC35 was passaged several times in mice to produce SC35M, which is highly pathogenic to mice (Gabriel et al., 2005; Scheiblauer et al., 1995). Recently, we showed that the high pathogenicity of SC35M is due primarily to mutations in the genes of the polymerase complex. Recombinant SC35 viruses with single SC35M-specific mutations in the PB1, PB2, PA and NP genes correlated with high polymerase activity in mammalian cells and high virulence in mice (Gabriel et al., 2005). Therefore, we considered it of interest to investigate the effects of reducing the levels of SC35M polymerase and NP components on SC35M pathogenicity to mice.

There is increasing evidence that strains of highly pathogenic avian influenza viruses (HPAIV) of the H5N1 and H7N7 subtypes have been transmitted directly from birds to humans (Claas et al., 1998; Fouchier et al., 2004; Subbarao et al., 1998), and pose a pandemic threat. As there is no vaccine currently available against these HPAIV subtypes, the development of a therapeutic strategy employing the targeting of viral gene sequence that is highly conserved between influenza A virus subtypes and strains is generally acknowledged as highly desirable.

Phosphorodiamidate morpholino oligomers (PMO) are antisense agents that are structurally similar to single-stranded DNA. Each base is joined to a novel backbone consisting of a morpholine ring and phosphorodiamidate internucleotide linkage (Summerton & Weller, 1997). PMO

Peptide-conjugated phosphorodiamidate morpholino oligomers (PPMO) are single-stranded nucleic acid-analogue antisense agents that enter cells readily and can reduce gene expression by steric blocking of complementary RNA (cRNA) sequences. Here, we tested a panel of PPMO designed to target conserved sequences in the RNA genome segments encoding polymerase subunits of a highly pathogenic mouse-adapted influenza A virus (SC35M; H7N7). Three PPMO, targeting the translation start site region of PB1 or NP mRNA or the 3′-terminal region of NP viral RNA (vRNA), potently inhibited virus replication in MDCK cells. Primer extension assays showed that treatment with any of the effective PPMO led to markedly reduced levels of mRNA, cRNA and vRNA. Initially, the potential toxicity of a range of intranasally administered PPMO doses was evaluated, by measuring their effect on body weight of uninfected mice. Subsequently, a non-toxic dosing regimen was used to investigate the effect of various PPMO on SC35M infection in a mouse model. Mice administered intranasal treatment of PPMO targeting the PB1-AUG region or NP vRNA, at 3 μg per dose, given once 3 h before and once 2 days after intranasal infection with 10×LD50 of SC35M, showed a 2 log10 reduction of viral titre in the lungs and 50% survival for the 16 day duration of the experiment, whereas the NP-AUG-targeted PPMO treatment resulted in 30% survival of an otherwise lethal infection. These data suggest that PPMO provide a useful reagent to investigate influenza virus molecular biology and may constitute a therapeutic strategy against highly pathogenic influenza viruses.

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Supplementary material is available with the online version of this paper.
are usually 20–25 subunits in length and can base pair with complementary RNA (cRNA) target sequence, forming a steric block (Stein et al., 1997; Summerton, 1999). Conjugation of PPMO to an arginine-rich cell-penetrating peptide (CPP) results in efficient delivery into cells without the need for additional transfection reagents or manipulations (Moulton et al., 2004; Yuan et al., 2006). CPP-PMO (PPMO) are water soluble, stable in cells and in human serum for several hours (Abes et al., 2006), and have recently been shown to successfully inhibit multiple subtypes of influenza A viruses in cell culture (Ge et al., 2006).

In this report, we describe the evaluation of PPMO targeting the mRNA, cRNA and viral RNA (vRNA) of influenza virus polymerase subunit and NP genes in cell culture, and in the SC35M murine model. We found that treatment of mice with PPMO targeting the translation start site region of PB1 or the 3’ end of NP vRNA significantly suppressed virus titre in the lungs and protected 50 % of mice from an otherwise lethal infection.

**METHODS**

**Cells and viruses.** Madin-Darby canine kidney (MDCK) cells were grown in minimal essential medium (MEM) (Gibco) containing 10 % fetal calf serum (FCS) and antibiotics. SC35M is an influenza A virus of the H7N7 subtype (Gabriel et al., 2005, 2007). Virus stock was grown in 11-day-old embryonated chicken eggs. Allantoic fluid was used for determining virus titre by plaque assay. Virus stock was stored at −80 °C until use.

**PPMO synthesis.** PMO were synthesized at AVI BioPharma Inc. by previously described methods (Summerton, 1999; Summerton & Weller, 1997). All PMO for this study were covalently conjugated, at the 5’ end, to the CPP (RXR)4XB (R=arginine, X=6-aminohexanoic acid, B=beta alanine) to yield PPMO, by procedures described previously (Abes et al., 2006). The PPMO, of 21–23 subunits in length, were designed against SC35M (GenBank accession nos. DQ266094–DQ266101) and were intended to target, by complementary base pairing, sequence regions that were previously identified as productive PPMO target sites (Ge et al., 2006) or regions of the NP or PB2 gene segments not previously targeted with PPMO. All of the PPMO were designed to target either translation start site regions in mRNA or 3’–terminal regions of cRNA or vRNA. PPMO sequences, name designations, and target regions are specified in Table 1. A PPMO of random sequence having 50 % G/C content (named ‘Dscr’) was prepared in an identical manner for use as a control for non-sequence-specific activity. Antisense PPMO sequences were screened with BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) against primate and murine mRNA sequences, and the negative control was additionally screened against influenza A virus sequences in order to preclude unintentional hybridization events. Prior to use, lyophilized PPMO were resuspended with filter-sterilized distilled water to a concentration of 1–2 mM and stored at 4 °C.

**Growth curves of SC35M in MDCK cells treated with PPMO.** MDCK cells seeded in 6 cm dishes and grown to 80–90 % confluence were mock (PBS)-treated or treated with PPMO at concentrations of 5, 10 or 20 μM in 5 h in 2 ml serum-free medium. The cells were then washed with PBS and inoculated with SC35M at an m.o.i. of 10−1. After 30 min incubation, virus inoculum was removed, the cells were washed twice with PBS and then incubated in MEM containing 0.2 % BSA at 37 °C/5 % CO2 without further PPMO treatment. At time points 0, 24, 48 and 72 h post-infection (p.i.), supernatant was collected and plaque titres determined on MDCK cells as described previously (Gabriel et al., 2005).

**Cytotoxicity assay.** Cell viability was quantified using a colorimetric MTT cell proliferation assay kit (American type Culture Collection). Cells were plated at a density of 104 cells per well in a 96-well-culture plate and allowed to adhere overnight. MDCK cells were treated with PPMO at concentrations of 5, 10 and 20 μM, in triplicate, in 100 μl serum-free medium and incubated at 37 °C/5 % CO2 for 5 h. PPMO was then removed, the cells were washed two times with PBS and then incubated in MEM containing 0.2 % BSA at 37 °C/5 % CO2 for 24 h without further PPMO treatment, after which the MTT cell proliferation assay was performed according to the manufacturer’s protocol. The absorbance of each well was determined on a microplate spectrometer (BioTek Instruments EL311) at a wavelength of 570 nm. Cytotoxicity was calculated by dividing the mean of the optical density of PPMO-treated samples by the mean of the optical density of non-treated (Mock) samples.

**Analysis of vRNA, mRNA and cRNA by primer extension assay.** Cells were mock- or PPMO-treated for 5 h, washed with PBS, then infected with SC35M at an m.o.i. of 0.1. At 14 h p.i., cells were harvested and total RNA was isolated using TRIzol reagent (Gibco-BRL). After spectrophotometric quantification and normalization, RNA primer extensions were carried out as described previously (Fodor et al., 2002; Fodor & Smith, 2004; Gabriel et al., 2007), with minor modifications. Briefly, 1 pmol DNA primer was 5’-end labelled with [γ-32P]ATP using T4 polynucleotide kinase, it was mixed with 1 μg total RNA in 6 μl water and denatured at 95 °C for 3 min. The mixture was then cooled on ice and reverse transcriptase SuperScriptII (Invitrogen) and its reaction buffer were added. Primer extensions were performed at 45 °C for 1.5 h. The PB1, NP and NA primer sequences are available upon request. Transcription products were

| Table 1. PPMO names, sequences and target locations in SC35M |
|-----------------|----------------------|-----------------|
| **PPMO name** | **PPMO sequence (5’ → 3’)** | **PPMO target region in SC35M** |
| PB1-AUG | GACATCCTACATTCAAATGGTTTTG | Translation start site of PB1 mRNA |
| PB2-AUG | TCTTTTATTTCTCCATATGCAA | Translation start site of PB2 mRNA |
| PB2-v3’ | AGCCAGACGGTCAAATATAT | 3’ terminus of PB2 cRNA |
| PB2-c3’ | AGTAGAAAAGGGTCTTTTAA | 3’ terminus of PB2 mRNA |
| NP-AUG | GAGAGAGCATATGATGGATGTC | Translation start site of NP mRNA |
| NP-v3’ | AGCAAGAGGATGATAATCT | 3’ terminus of NP vRNA |
| NP-c3’ | AGTAGAAACAGGATTTCTT | 3’ terminus of NP cRNA |
| Dscr | AGTCTCGACTTGCTACCCTA | ‘nonsense’ negative control |
analysed on 6% polyacrylamide gels containing 7 M urea in TBE buffer and detected by autoradiography.

**Mice.** Female, 4–6-week-old BALB/c mice were purchased from Harlan Winkelmann, Borchen, Germany. All studies followed Philipps University Institutional Animal Care Guidelines.

**Evaluation of PPMO toxicity in uninfected mice.** Groups of five mice were anaesthetized with isofluorane and then treated intranasally with 0.5 μg (25 μg kg⁻¹), 1 μg (50 μg kg⁻¹), 1.5 μg (75 μg kg⁻¹) or 3 μg (150 μg kg⁻¹) per dose of Dscr, PB1-AUG, NP-AUG or NP-v3’ PPMO in a volume of 50 μl PBS, or 50 μl PBS alone, daily for 4 days. All mice were weighed daily and monitored for signs of illness.

**PPMO treatment of SC35M-infected mice.** Groups of 10 mice were anaesthetized with isofluorane and then treated intranasally with 50 μl PBS or 50 μl PBS containing 0.5, 1, 1.5 or 3 μg per dose of Dscr, PB1-AUG, NP-AUG or NP-v3’ PPMO. Three hours after the initial treatment, mice were infected intranasally with 10, 25 or 50 × 10⁵ pfu of SC35M (mouse LD₅₀ of SC35M is 10².₈ pfu.) in a volume of 50 μl PBS. Mice were treated again intranasally at 48 h p.i. Non-treated and non-infected mice were included as further control. When 10 × 10⁵ pfu was used, mice were weighed daily for 14 days, and two mice from each group were sacrificed on days 5, 10 and 16 p.i., for organ (lung, spleen and brain) removal. Organs were homogenized in 1 ml PBS, centrifuged at 1000 r.p.m. (rotor with 4 cm radius) for 10 min and the lung homogenate. After incubation, each membrane was washed three times with 0.5 M PBS containing 0.5, 1, 1.5 or 3 M NaCl.

**Cytokine assay.** A mouse cytokine antibody array (RayBiotech Inc.) was used according to the manufacturer’s protocols to assay the levels of 21 cytokines and chemokines from the lung homogenates of the PPMO-treated, SC35M-infected mice. For these assays, we pooled the lungs from both mice receiving each treatment. The array detects the following: GCSF, GM-CSF, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12-p40p70, IL-12-p70, IL-13, IL-17, IFN-γ, MCP-1, MCP-5, RANTES, SCF, TNF-α, thrombopoietin and VEGF. Briefly, membranes with bound cytokine antibodies were incubated with 200 μl horseradish peroxidase-conjugated streptavidin. Cytokine levels and incubated with biotin-conjugated anti-cytokine antibody and then with horseradish peroxidase-conjugated streptavidin. Cytokine levels were then detected by chemiluminescence and revealed on X-ray film. Intensity of signals was quantified by TINA 2.0 software.

**RESULTS**

**Design of PPMO**

Past research results with PPMO as well as with SC35M guided the design of PPMO for this study. Ge et al. (2006) showed that two PPMO (PB1-AUG and NP-v3’) produced considerable efficacy against several influenza A virus subtypes. Both, the PB1-AUG and NP-v3’ PPMO target sequences are both highly conserved across H1N1, H5N1 and H7N7 influenza A virus strains (Ge et al., 2006), including SC35M. In the present study, we therefore sought to evaluate those same compounds against the highly pathogenic SC35M in cell cultures and in vivo.

Ge et al. (2006) found that the translation start site regions of mRNA, and the terminal ends of influenza A virus gene segments can provide productive PPMO target sites (Ge et al., 2006). The 3’ ends of vRNA and cRNA are thought to participate as promoter regions in influenza RNA synthesis (Flick et al., 1996). Also, it is well established that the NP and PB2 genetic segments are integral in the process of RNA synthesis, and that variations in PB2 and NP RNA sequences can have a profound effect on the pathogenicity of SC35M in mice (Gabriel et al., 2005). We therefore designed PPMO to target the AUG translation start site regions of mRNA, and the 3’-terminal sequence regions of the cRNA and vRNA, of SC35M PB2 and NP (see Table 1).

**Effect of PPMO on influenza virus growth in cell culture**

Initially, we evaluated the panel of PPMO in a dose–response format over time against SC35M in MDCK cells. At a concentration of 5 μM, only two PPMO significantly inhibited virus growth. At 48 h p.i., NP-v3’ inhibited virus growth by 3 log₁₀ and NP-AUG by over 6 log₁₀ (to below 1 log₁₀ the minimum level of detection by our plaque assay) (Fig. 1a). At 10 μM, NP-v3’ generated a 4 log₁₀ PB1-AUG a 5 log₁₀ and NP-AUG complete inhibition of virus growth (Fig. 1b). At 20 μM, the highest concentration used, PB1-AUG, NP-AUG and NP-v3’ all inhibited virus growth to undetectable levels at all three time points (Fig. 1c). The other PPMO produced moderate to no inhibition at early time points, and little to no inhibition at later time points (Fig. 1c).

In cell viability assays carried out under conditions similar to those of the antiviral experiments described above, PB1-AUG, NP-AUG and NP-v3’ revealed no cytotoxicity at 5 or 10 μM, and minimal cytotoxicity (less than 15 %) at 20 μM compared to mock- or 20 μM Dscr-treated cells (Supplementary Fig. S1 available in JGV Online). Likewise, 20 μM Dscr PPMO treatment produced negligible cytotoxicity. These data indicate that virus growth inhibition by PPMO in the antiviral cell culture experiments below was not due to cytotoxicity.

**Effect of PPMO on viral mRNA, cRNA and vRNA synthesis**

Primer extension assays are able to distinguish between the three species of RNA produced by an individual gene segment, and were used to determine the effect of PB1-AUG, NP-AUG and NP-v3’ treatment on the relative abundance of vRNA, cRNA and mRNA of both PPMO-targeted and non-targeted SC35M genes. Cells were treated with PPMO at several concentrations, total RNA isolated, and primer extension assays performed using PB1-, NP- and NA-specific primers. The levels of RNA were identical with PPMO treatment little to no RNA synthesis was detectable with 10 or 20 μM Dscr-treated cells (Supplementary Fig. S1 available in JGV Online). Likewise, 20 μM Dscr PPMO treatment produced negligible cytotoxicity. These data indicate that virus growth inhibition by PPMO in the antiviral cell culture experiments below was not due to cytotoxicity.
NP were significantly reduced, and at higher concentrations (10 or 20 μM) abolished (Fig. 2a–b). After treatment with 5 μM NP-v3′, no PB1 RNA synthesis could be detected (Fig. 2c). Treatment of cells with 5 μM NP-AUG dramatically inhibited NP and NA RNA synthesis, and completely inhibited PB1 RNA synthesis (Fig. 2a–c). These data indicate that PPMO inhibited SC35M RNA production in a sequence-specific and dose-dependent manner.

Toxicity assessment of PPMO in non-infected mice

Before undertaking antiviral in vivo experiments we sought to evaluate potential PPMO toxicity by measuring weight loss in PPMO-treated non-infected mice treated with a PPMO regimen. Groups of five mice were intranasally administered 0.5–3 μg per dose of the PB1-AUG, NP-AUG, NP-v3′ or Dscr PPMO each day for 4 days, and observed for weight loss and signs of illness for a total of 10 days after the first dose. A group of PBS-treated mice was included for comparison. None of the PPMO-treated groups showed any weight loss or illness compared with the PBS-treated group. Fig. 3 shows body weight data from groups receiving PBS or the highest PPMO dose used (3 μg). These data indicate that all dosing regimens of all PPMO used in the studies of this report were sub- or non-toxic to mice.

PPMO treatment and SC35M infection of mice

We next carried out a series of experiments to evaluate the effect of the various PPMO on a lethal SC35M infection of mice. In all the experiments, we treated groups of mice with PB1-AUG, NP-AUG, NP-v3′, a combination of the preceding three PPMO, Dscr PPMO or PBS alone, and 3 h later infected them with 10, 25 or 50 × LD50 of SC35M, then treated them again at 48 h p.i. with the same compound(s) at the same dose. A PBS-treated and non-infected group was included in each experiment and uniformly showed no weight loss. With 50 × LD50, single doses of 0.5, 1 or 1.5 μg PPMO were used, with groups of four mice, and the NP-AUG and NP-v3′ groups each had one survivor at day 16, with no survivors in any of the other groups. With 10 × LD50, doses of 3 μg PPMO were used, and all animals in the PBS and Dscr PPMO control groups lost over 40% of their body weight and died by day 13, while mice treated with PB1-AUG, NP-AUG, NP-v3′ or a combination of these three PPMO lost about 20–30% body weight by day 9 (Fig. 4a; Table 2 and Supplementary Fig. S2 available in JGV Online). However, 50% of the mice in the PB1-AUG, NP-v3′ or three-PPMO combination groups regained almost all their lost weight between days 11 and 16 and survived the duration of the experiment. The NP-AUG-treated mice had 30% survivorship (Fig. 4b; Table 2).

Except for the three-PPMO combination treatment group, two mice from each of the groups receiving 10 × LD50 were sacrificed on days 5 and 10 p.i. (and likewise from the groups with survivors on day 16 p.i.), and lung, spleen and brain were removed for the determination of viral titres and lung cytokine levels. Viral titre data from tissues taken during the course of the above experiment were reflective of the survival data. PBS-treated non-infected control mice showed no virus titre in lung, brain or spleen at any of the three time points (Fig. 5). The control groups of PBS- or Dscr-treated and infected mice showed virus titres of 10^6 p.f.u. in the lung, and virus at detectable levels in the spleen and brain, at 5 and 10 days p.i., indicating that SC35M...
Fig. 2. Primer extension in MDCK cells. Cells were treated for 5 h with 5, 10 or 20 μM PB1-AUG, NP-AUG or NP-v3′ PPMO. As controls non-treated and non-infected, PBS-treated and infected (Mock) and Dscr PPMO-treated (20 μM) and infected cells were used. The treatment was then removed and the cells were infected with an m.o.i. 0.1 of SC35M. No PPMO was added after infection. Total RNA was isolated 14 h p.i. and primer extensions targeting the NA (a), NP (b) or PB1 (c) genes carried out as described in Methods.

Fig. 3. Evaluation of PPMO toxicity in uninfected mice. Groups of five mice were treated intranasally with 3 mg PBS, Dscr, PB1-AUG, NP-AUG or NP-v3′ once a day for 4 consecutive days, and weighed daily for 10 days. The mean values from each group are reported.

Fig. 4. Mice treated with PPMO and infected with SC35M. Groups of 10 mice were administered PBS or 3 μg per dose of the indicated PPMO intranasally, 3 h before infection and 48 h after intranasal infection with 10×LD₅₀ of SC35M. A group of PBS-treated and non-infected mice were also included. Mean body weight per group charted daily for 16 days (a). Number of surviving animals in each group over time p.i. (b).

Table 2. Survival of mice in experiments described in Fig. 4 and Supplementary Fig. S2

<table>
<thead>
<tr>
<th>PPMO treatment</th>
<th>Survival at day 16</th>
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<tbody>
<tr>
<td>PBS</td>
<td>0/6</td>
</tr>
<tr>
<td>Dscr</td>
<td>0/6</td>
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<tr>
<td>PB1-AUG</td>
<td>3/6</td>
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<tr>
<td>NP-AUG</td>
<td>2/6</td>
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<tr>
<td>NP-v3’</td>
<td>3/6</td>
</tr>
<tr>
<td>Three-PPMO combination (PB1-AUG, NP-AUG and NP-v3’)</td>
<td>3/6</td>
</tr>
<tr>
<td>Non-infected</td>
<td>6/6</td>
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produces a systemic infection in mice. By day 16 p.i., all mice in the PBS- or Dscr-treated and infected control groups had died (Fig. 5b). All three antisense PPMO-treated groups showed reduced virus titres in spleen and brain at 10 days p.i., suggesting a reduction in systemic infection (Fig. 5b). At day 16 p.i., surviving animals had no virus detectable in lung, spleen or brain, indicating a recovery from SC35M infection (Fig. 5c). We note that the only groups to achieve 50% survivorship at day 16 (PB1-AUG- or NP-v3'-treated) were the same groups that showed markedly reduced viral titres in the lungs on day 10 p.i.

**Cytokine response in mice after PPMO treatment and virus infection**

To evaluate the level of cytokine and chemokine response over time in PPMO-treated and SC35M-infected mice, we assayed homogenates of the same lung tissues evaluated above, for various cytokines and chemokines. In addition, we assayed for four relevant cytokines (see below) in non-infected animals 1 day after treatment with PBS or 3 μg with each of the PPMO, and observed no detectable expression, suggesting that PPMO itself does not engender rapid immune stimulation (Fig. 6a). In infected animals, differences in measurable quantity over time could be observed only for IL-4, IL-12-p70, MCP-1 and thrombopoietin (Fig. 6b–e). No expression of any of the cytokines was detectable at day 5 p.i. in any of the groups. At day 10 p.i., all groups of infected mice had similarly low levels of IL-4 expression (Fig. 6b), and the NP-v3’ group had an elevated thrombopoietin level compared to all other groups (Fig. 6e). On day 16 p.i., surviving mice showed high levels of IL-4, IL-12-p70, MCP-1 and thrombopoietin compared with any of the readings at earlier time points (Fig. 6b–e), suggesting that surviving mice had mounted an immune response against SC35M.

**DISCUSSION**

In this study, we evaluated seven PPMO designed against RNA sequence in the gene segments encoding the polymerase components PB1, PB2 and NP of SC35M, a highly pathogenic mouse-adapted influenza A virus of the H7N7 subtype. Three of the seven PPMO tested (PB1-AUG, NP-AUG and NP-v3’) produced potent and specific inhibition of virus growth in MDCK cell culture and we pursued testing these PPMO against SC35M infection in mice. Oddly, the PPMO that had generated the highest efficacy in cell culture studies, NP-AUG, was the least effective of the three antisense PPMO tested in vivo, producing only 30% survivorship, compared with 50% survivorship for the NP-v3’- or PB1-AUG-treated animals. As noted in Results, PB1-AUG or NP-v3’ treatment produced considerably higher titre suppression in lung tissue at 10 days p.i. compared with NP-AUG (Fig. 5b). Although PB1-AUG and NP-v3’ generated identical in vivo survivorship rates, treatment with NP-v3’ resulted in lower viral titre in the spleen and brain at 5 days p.i. compared with treatments with PB1-AUG (Fig. 5a).
Treatment of mice with a combination of three PPMO PB1-AUG, NP-AUG and NP-v3' also produced a 50% survival rate (Supplementary Fig. S2 available in JGV Online). Our results indicate that treatment with a combination of PPMO does not necessarily result in improved in vivo efficacy over that of an individual PPMO. However, treatment with a combination of PPMO would be expected to be less likely to produce resistant viruses compared with treatment with an individual PPMO.

Primer extension analysis revealed that the three antisense PPMO tested all caused profound reductions in the levels of vRNA, cRNA and mRNA of three different gene segments (PB1, NA and NP) during SC35M infection in cell culture (Fig. 2). The global reduction of SC35M RNA levels observed at 14 h p.i. indicates that each of the antisense PPMO interfered with events critical to vRNA synthesis, and that both NP and PB1 RNA and/or protein are critical to all influenza A vRNA production. We can infer from these results that the NP-v3' PPMO interferes with transcription of NP cRNA or mRNA from vRNA. The NP-AUG and PB1-AUG PPMO are complementary to both the cRNA and mRNA of their respective target genes, and it is not possible to conclude from this data if the NP-AUG and PB1-AUG PPMO interfere with the conversion of cRNA to vRNA, or with mRNA translation. Perhaps primer extension analysis of the relative abundance of vRNA, cRNA and mRNA at various time points p.i., in PPMO-treated and non-treated samples, would help to address this question.

![Graphs showing cytokine response in mice](http://vir.sgmjournals.org)
It has been documented that several highly pathogenic influenza A viruses stimulate a ‘cytokine-storm’, which apparently contributes to pathogenicity in host animals (de Jong et al., 2006; Kobasa et al., 2007). In contrast, the in vivo study here indicates that highly pathogenic SC35M infection caused little cytokine response in mice, at least up to 10 days p.i. Interestingly, Tumpey et al. (2000) reported similar observations with HPAIV of the H5N1 subtype. They compared the mouse-lethal A/HK/483/97 with the non-lethal A/HK/486/97 for their relative effects on the murine immune system, and found that lung and lymphoid tissue of A/HK/483/97-infected mice demonstrated a reduction in CD4+ and CD8+ T cells and reduced synthesis of the cytokine IFN-γ and the chemokine macrophage inflammatory protein compared with A/HK/486/97-infected mice (Tumpey et al., 2000). Their data and the results presented here, suggest that destructive effects on the immune system may be one important factor contributing to the pathogenicity of some HPAIV in the mammalian host.

At day 16 p.i., surviving mice from SC35M-infected groups showed much higher levels of IL-12-p70, IL-4, MCP-1 and thrombopoietin compared with any of the mice at earlier time points (Fig. 6e), suggesting that surviving mice mounted an immune response sufficient to permit survival of an otherwise lethal infection. IL-12-p70 is important for the differentiation of T helper cells and cell-mediated immunity (Hofmann et al., 2002). Indeed, IL-12 was previously shown to be important for resistance to influenza virus infection in mice (Monteiro et al., 1998). IL-4 has an important role in regulating antibody production, inflammation and the development of effector T-cell responses (Brown & Hural, 1997). Further, IL-4 stimulates the production of MCP-1, which recruits macrophages to eliminate infected cells (Winsor et al., 2000). Thrombopoietin is the primary regulator of platelet production and important in wound healing (Verbeek et al., 2000; Wolber & Jelkmann, 2002).

Seasonal influenza A causes tens of millions of cases of severe illness, and approximately 250 000–500 000 deaths each year worldwide (WHO, Fact Sheet 211). Recent cases of high-pathogenic avian influenza in humans have heightened the level of concern regarding the possibility of pandemic influenza. The currently available influenza drugs provide only partial therapeutic or prophylactic protection, and viral drug-resistance is a worsening problem (de Jong et al., 2005; Kiso et al., 2004; Le et al., 2005; Ludwig et al., 2003; McKimm-Breschkin, 2000). A variety of nucleic-acid based approaches have been reported as promising against influenza A virus (Abe et al., 2001; Ge et al., 2003, 2004a; b; McKimm-Breschkin, 2000; Plehn-Dujovich & Altman, 1998; Tado et al., 2001; Takahashi et al., 2004; Tompkins et al., 2004) and it appears that sequence-specific intervention in the influenza virus life cycle represents a promising avenue for drug development. Here, we show that PPMO targeting viral polymerase genes provide a useful strategy to inhibit highly pathogenic influenza virus infection in vivo. The PB1-AUG PPMO targets viral sequence that is highly conserved across influenza A virus strains. The conservation of the NP-v3’ PPMO target site is high across strains of the H1N1, H5N1 and H7N7 subtypes, but less so against H3N2 strains (see Table 2; Ge et al. 2006).

Importantly, PPMO were found to be non-toxic both in cell culture and in vivo at dose levels shown to be effectively antiviral. In cell culture, several of the PPMO generated little or no reduction of viral titre, even at the highest doses used, indicating their low impact on cell viability (Fig. 1). Likewise, MTT assays on several positively antiviral PPMO gave no indication of adverse effect on the health of cultured cells (Supplementary Fig. S1). Monitoring of weight in PPMO-treated and non-infected mice yielded no indication of toxicity, even when a regimen consisting of twice the number of doses as that employed in the in vivo antiviral experiments was tested. The low cytokine level at 1 day p.i. of PPMO-treated non-infected mice (Fig. 6a) suggests that the PPMO dosing regimen used here does not cause rapid immuno-stimulation.

Here, remarkably low intranasal dosing of 3 μg (equivalent to 150 μg kg−1) PPMO produced significant in vivo efficacy. This dose level is far less than the 3–10 mg PPMO kg−1 reported as effectively antiviral when delivered intraperitoneally to address infections with murine coronavirus (Burrer et al., 2007) or West Nile virus (Deas et al., 2007), or intravenously against coxsackievirus B3 (Yuan et al., 2006). Our results suggest that the PPMO delivered directly into the lungs may be a useful therapeutic strategy against respiratory viruses. We note, however, that the PPMO in this study were effective against only the lowest inoculating dose of virus (10 × LD50), and not against the higher inoculations (25 × and 50 × LD50) (Supplementary Table S1). We speculate that higher PPMO dosing or improved delivery of PPMO to vRNA within lung tissue through delivery enhancement strategies could further improve in vivo efficacy. More efficient delivery of PPMO to lung tissue in general and into the lower lungs in particular than that provided by intranasal administration could likely be achieved through the development of a small solid particle or fine liquid aerosol formulation of PPMO, and mechanized dispensation through an atomizer.

The in vivo efficacy studies here employed both pre- and post-infection PPMO treatments. The efficacy of PPMO treatment given only at various times p.i. holds considerable interest to us and will be explored in future studies. We note with optimism, reports in the literature documenting antiviral efficacy of PPMO treatments administered only p.i. against Ebola virus (Enterlein et al., 2006) or West Nile virus (Deas et al., 2007) in mouse models.

In summary, the low toxicity and considerable efficacy of the PB1-AUG and NP-v3’ PPMO delivered via intranasal instillation to mice in this study suggests that administration of these compounds via a respiratory route to larger
animals to treat influenza infection may be feasible and is worth further exploration.

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