Published in final edited form as:

Virus Res. 2014 August 30; 0: 14–23. doi:10.1016/j.virusres.2014.05.003.

Genome Rearrangement of Influenza Virus for Anti-Viral Drug Screening

Troy C. Sutton¹, Adebimpe Obadan¹, Johanna Lavigne¹, Hongjun Chen¹, Weizhong Li¹, and Daniel R. Perez^{1,*}

¹Virginia-Maryland Regional College of Veterinary Medicine, Maryland Campus, University of Maryland, College Park

Abstract

Rearrangement of the influenza A genome such that NS2 is expressed downstream of PB1 permits the insertion of a foreign gene in the NS gene segment. In this report, the genome rearranged strategy was extended to A/California/04/2009 (pH1N1), and *Gaussia* luciferase (GLuc) or GFP was expressed downstream of the full-length NS1 gene (designated GLucCa04 and GFPCa04, respectively). In growth kinetics studies, culture of amantadine sensitive GLucCa04 (Sens/ GlucCa04) in the presence of amantadine significantly decreased GLuc expression and viral titers for 48 h post-infection (hpi). When Sens/GLucCa04 was subsequently used in an *in vitro* anti-viral screening assay, amantadine treatment significantly decreased GLuc expression from amantadine sensitive compared to amantadine resistant GLucCa04 (Res/GLucCa04) as early as 16 hpi. In *in vivo* screening studies, DBA mice were treated daily with amantadine from 1 day prior to infection and inoculated with either Sens/GLucCa04 or Res/GLucCa04 alone or as a co-infection with the parental strain. On days 3 and 5 post-infection, lung samples were collected and amantadine treatment was shown to decrease GLuc expression by 2 orders of magnitude ($p < 0.05$) in Sens/ GlucCa04 infected mice. Furthermore, while both Sens and Res/GLucCa04 were highly attenuated, addition of the parental strain to the inoculum yielded clinical disease indicative of GLuc expression and pulmonary viral titers. These findings indicate that the use of GLucCa04 can potentially accelerate *in vitro* and *in vivo* anti-viral screening by shortening the time required for virus detection.

Keywords

influenza; anti-viral screening; amantadine; *Gaussia* luciferase

© 2014 Elsevier B.V. All rights reserved.

*Corresponding Author: Dr. Daniel Perez, Department of Veterinary Medicine, University of Maryland, College Park, MD, USA 20742, dperez1@umd.edu; Telephone: 301-314-6811.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

INTRODUCTION

Influenza A viruses cause annual epidemics and occasional pandemics (Jhung et al., 2011; Molinari et al., 2007; Morens et al., 2010). To control influenza, vaccines are administered prior to seasonal outbreaks, and anti-viral drugs are administered after the presentation of clinical signs. Influenza A belongs to the family *Orthomyxoviridae* and, as such, has an 8-segmented negative polarity single stranded RNA genome (Palese and Shaw, 2007). Using a genome rearrangement strategy, we have previously shown that an H9N2 vaccine strain could be modified to express a second HA protein (H5 HA) from segment 8 (NS gene segment). When this virus was administered as a vaccine, both mice and ferrets were protected against lethal highly pathogenic H5N1 challenge (Pena et al., 2013). According to this genome rearrangement strategy, NS1 was truncated to NS1(1–99) (Talon et al., 2000) and NS2 was deleted from segment 8. The foot-and-mouth-disease virus (FMDV) 2A protease was cloned downstream of NS1(1–99) and followed by the transgene of interest (i.e. H5 HA, GFP, or luciferase). To re-introduce NS2, segment 2 was modified such that FMDV 2A protease was cloned downstream of PB1 followed by the NS2 open-reading frame (ORF). In both segment 2 and 8, the segment specific packaging signals were reconstituted at the end of the inserted gene sequence (Fujii et al., 2005; Liang et al., 2005). Herein, the genome rearrangement was applied to an additional virus strain, A/California/04/2009 (pandemic H1N1), and *Gaussia* luciferase (GLuc) expressing variants maintaining the full-length NS1 gene were evaluated for anti-viral screening.

To date, only two groups of compounds have been licensed for treatment of influenza: the adamantanes (amantadine and rimantidine) and the neuraminidase (NA) inhibitors (oseltamivir and zanamivir). Unfortunately, most circulating strains of influenza are resistant to adamantanes, and oseltamivir-resistant virus strains continue to be isolated (Hurt et al., 2011; Lackenby et al., 2011; Ujike et al., 2011). Consequently, there is a need for rapid development of novel anti-viral compounds. For antiviral screening, there are two conventional assay systems: (1) cytopathic effect (CPE) assay or plaque reduction assay, and (2) NA inhibitor assays (Buxton et al., 2000; Hayden et al., 1980; Kao et al., 2010; Potier et al., 1979; Severson et al., 2008; Su et al., 2010). Each assay has specific drawbacks. For example, the CPE assay requires culturing the virus in the presence of a compound for 3–5 days, and NA assays are specific only to compounds that target the viral NA.

More recently, several high throughput-screening (HTS) assays have been developed. These assays are cell-based and include the generation of stable cell lines (MDCK, Hela, or 293T) expressing influenza driven *Renilla* luciferase (RLuc) or *Firefly* luciferase (FLuc) reporter constructs (Hossain et al., 2010; Martinez-Gil et al., 2012; Zhang et al., 2011), and a 293T cell line expressing the viral ribonucleoprotein genes (Ozawa et al., 2013). Importantly, these assays take advantage of luciferase expression that can be quickly assayed from cell lysates. With the introduction of secreted *Gaussia* luciferase (GLuc), luciferase activity can be assayed directly from cell culture supernatant (Tannous et al., 2005), and with a 1,000 fold increase in sensitivity of GLuc compared to RLuc or FLuc (Zhu et al., 2011), there is the potential to improve upon existing assays. Furthermore, as a result of the cell-based nature of existing assays, expression of the reporter gene is an indirect measure. In contrast, the use of a virus carrying the reporter gene has the potential to give a direct representation

of viral replication and anti-viral efficacy (Heaton et al., 2013; Manicassamy et al., 2010; Pena et al., 2013).

Thus, in this study, the genome rearrangement strategy was applied to A/California/04/2009 while maintaining full-length NS1. In initial studies, we characterized viral growth and luciferase expression from the rearranged viruses, and evaluated growth kinetics in the presence of amantadine and oseltamivir. As a means to develop an alternative anti-viral screening assay, amantadine sensitive and resistant GLuc expressing variants were further evaluated in the presence of amantadine. After pre-incubation and culture of virus with amantadine in MDCK cells, GLuc expression was indicative of drug efficacy from as early as 16 hpi. Subsequently, the stability of GLuc expression was evaluated over serial passage, and the use of GLucCa04 in MN assays was also explored. Lastly, *in vivo*, we demonstrate that when GLucCa04 is given alone or as a co-infection with its parental strain, GLuc expression can be used as an indicator of amantadine sensitivity and anti-viral efficacy

MATERIALS AND METHODS

Cells and viruses

MDCK cells were kindly provided by Dr. R. Webster (St. Jude Children's Research Hospital) and maintained in 10% fetal bovine serum (Cellgro, Corning) supplemented DMEM (Sigma). The parental virus for these studies was recombinant mouse-adapted A/California/04/2009 (H1N1) for which the 8-plasmid reverse genetics (RG) system has been described (Ye et al., 2010). Mouse-adapted A/California/04/2009 (H1N1), like the original human isolate, is amantadine resistant and for clarity is referred to as Res/Ca04.

To generate an amantadine sensitive strain, site-direct mutagenesis was performed using a QuikChange II XL Site-Directed Mutagenesis Kit (Agilent) and primers to change M2 N31S in segment 7. Successful mutagenesis was confirmed by sequencing and the virus was rescued in a co-culture of 293T:MDCK (Hoffmann et al., 2000). All viruses were stored at -80°C until use. Amantadine sensitive reverse genetics Ca04 was designated Sens/Ca04.

Cloning and Construction of Rearranged Viruses

To generate GLuc or GFP expressing Ca04 strains, both segments 2 and 8 were modified as previously described (Pena et al., 2013), with the exception that full-length NS1 was maintained (Fig. 1). To prevent aberrant splicing and prevent NS2 expression, the acceptor and donor splicing sites in NS1 were mutated by site-direct mutagenesis and a stop-codon was inserted out-of frame with NS1 but early in the NS2. Subsequently, NS2 was deleted using inverse PCR, and the resulting NS1 gene was designated NS1 splice. Using overlapping PCR, a fragment was generated containing BsmBI sites flanking NS1 splice followed by the FMDV 2A protease, the *Gaussia* luciferase (New England Biolabs) or GFP ORF, and the NS gene packaging signal (Fujii et al., 2005). This PCR fragment was subsequently digested with BsmBI and cloned into pDP-2002. To modify segment 2, a construct containing a C-terminal portion of the PB1 gene followed by the FMDV 2A protease, the NS2 ORF and the PB1 packaging signal (Liang et al., 2005) was produced. This fragment was subsequently sub-cloned into the PB1 gene segment using suitable

restriction sites. Upon completion of cloning, both plasmids were sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit and a 3500XL Sequencer (Applied Biosystems).

Amantadine resistant (i.e. wild-type) GFP variant (GFPCa04), and both amantadine sensitive (Sens/GLucCa04) and resistant (Res/GLucCa04) strains of the GLuc expressing viruses were rescued by incorporating the appropriate M gene plasmid.

Viral Growth Kinetics

Growth kinetics experiments were performed in MDCK cells in 6-well plates using an MOI of 0.01 as described previously (Pena et al., 2013). At 0, 6, 12, 18, 24, 48, and 72 h, 250 μ L of supernatant was collected from each well and replenished with OPTI-MEM (Invitrogen) containing 1 μ g/mL of TPCK-trypsin (Worthington). From the supernatant, 70 μ L was frozen at -20°C for later analysis of luciferase expression and 180 μ L was frozen at -80°C for viral titration by TCID₅₀. GLuc expression was quantified using a BioLux® *Gaussia* Luciferase Assay Kit (New England Biolabs). Briefly, the luciferase substrate was prepared immediately prior to use by performing a 1/1000 dilution of the substrate into the sample buffer. Next, 50 μ L of diluted luciferase substrate in sample buffer was added to 20 μ L of cell culture supernatant in a white flat bottom 96 well plate (Greiner). Immediately after addition of the substrate, the luminescence was measured over a 1 sec integration time on a Victor X4 Luminescence Plate Reader (Perkin Elmer).

Virus growth kinetics/luciferase expression experiments were performed in the presence of amantadine hydrochloride (Sigma) or oseltamivir carboxylate (Toronto Research Chemicals). The Sens/GLucCa04 virus (MOI 0.01) was pre-incubated for 30 min with amantadine hydrochloride or oseltamivir carboxylate at concentrations of 0, 0.1, 1, or 10 μ g/mL. After incubation, the virus-drug mixture was overlaid on MDCK cells in 6-well plates and incubated for 15 min at 4°C and 35°C for 45 min. Subsequently, the cells were rinsed three times with PBS, and overlaid with media containing the appropriate drug concentration and 1 μ g/mL TPCK-trypsin. At 1, 8, 16, 24, 48 and 72 h, supernatant was collected and processed as described above, and the media was replenished.

Virus Titrations by Tissue-Culture-Infectious Dose-50 (TCID₅₀)

To titrate stock viruses and lung homogenates, TCID₅₀ titrations were performed using MDCK cells cultured in 96-well plates. MDCK cells (20,000/well) were seeded in 200 μ L of OPTI-MEM and incubated overnight at 37°C . Subsequently, serial 10-fold dilutions of virus samples were prepared in OPTI-MEM supplemented with 1 μ g/mL TPCK-trypsin. The media was then removed from the MDCK cells and 200 μ L of each dilution was overlaid on multiple wells (8 wells for virus stocks and 4 wells for animal samples) per dilution. The virus-overlaid cells were then incubated for 3 days at 35°C . After the incubation, 50 μ L of virus supernatant was removed from each well and added to a V-bottom plate (Corning). Next, 50 μ L of 0.05% turkey red blood cells (TyRBCs) in PBS was added to each well and the plates were incubated for 45 min at room temperature. Lastly, the plates were read for hemagglutination and the 50% infection dose was determined as described (Reed and Muench, 1938).

Anti-Viral Compounds

For *in vitro* assays, amantadine hydrochloride (Sigma) was prepared at a stock concentration of 100 mM in 30% DMSO, 50 mM HEPES (pH 7.9), and oseltamivir carboxylate (Toronto Research Chemicals) was prepared at a concentration of 1 mM in sterile distilled deionized water (Wagaman et al., 2002). Both compounds were aliquoted and stored at -20°C . Immediately prior to use, the stock solution was thawed and diluted to the desired concentration in OPTI-MEM. For animal studies, amantadine was prepared daily and was dissolved in sterile distilled water.

Anti-Viral Screening Assays

For screening assays, MDCK cells were seeded in OPTI-MEM in 96-well plates (20,000 cells/well) and incubated overnight. Sens/GLucCa04 and Res/GLucCa04 viruses at MOI 0.01, 0.001, and 0, were incubated at 37°C for 30 min with amantadine hydrochloride at concentrations of 0, 0.001, 0.0032, 0.01, 0.032, 0.1, 0.32, 1, 3.2, 10, 32, and 100 $\mu\text{g}/\text{ml}$ in OPTI-MEM containing 1 $\mu\text{g}/\text{ml}$ of TPCK trypsin. MDCK cells were then infected with 200 μl of the amantadine-virus mixture. At 16, 24, 48, and 72 hpi, 20 μl of tissue culture supernatant was collected from each well and subjected to a luciferase assay as described above. To control for variation between Sens and Res/GLucCa04, the relative luciferase activity was expressed as a percentage of the untreated (0 $\mu\text{g}/\text{mL}$ amantadine) virus infected control at each time point for each virus.

Serial Passage of GLucCa04

MDCK cells were seeded in 6-well plates, and incubated overnight at 37°C until 80–90% confluent. Amantadine resistant GLucCa04 was then serially diluted in triplicate to initiate three separate serial passage lineages. The virus was diluted in 10-fold dilutions in 1.5 mL of Opti-MEM (Life Technologies), and 1 mL of each dilution was overlaid on a single well of a 6-well plate. The virus was incubated on the plate for 1 h at 35°C , the wells were rinsed three times with PBS, and 2 mL of Opti-MEM containing 1 $\mu\text{g}/\text{mL}$ of TPCK-trypsin (Worthington) was added to each well. In parallel, a mock or sham infected well was also serially passaged. At 48 h post-passage, the supernatant from the wells was subject to an HA assay using 0.5% TyRBCs. The highest virus dilution that yielded the highest HA titer was then collected, serially diluted, and used to initiate the next passage for a total of five serial passages. The mock infected well was similarly diluted and also passaged. From each passage, the remaining virus supernatant was divided and stored at -20°C and -80°C for quantification of luciferase expression and viral titer by TCID₅₀, respectively.

Microneutralization (MN) using GLucCa04

MNs were performed as described previously (Rao et al., 2008) using Res/Ca04 and Res/GLucCa04 viruses. Three serum samples from de-identified individuals that have been vaccinated against pandemic H1N1 were subject to MN. Serum samples were treated overnight with receptor-destroying enzyme (Denka Seiken) and inactivated via incubation at 56°C for 30 min. PBS was then added to each sample to yield a dilution of 1:10 of input sample, and further 2-fold serial dilutions were performed. Diluted serum samples were incubated with 100 TCID₅₀ of virus for 2 h at 37°C , and overlaid on MDCK cells in 96-well

plates. The 96-well plates were then incubated at 4°C for 15 min and 35°C for 45 min. Next, the virus-serum mixture was aspirated and 200 µL of Opti-MEM containing 1 µg/mL of TPCK-trypsin was added to each well. For a given replicate of each virus, three replicate plates were prepared. At 24, 48, and 72 hpi, one plate for each virus was subject to an HA assay using 50 µL of supernatant and 50 µL of 0.05% TyRBCs, and 20 µL of supernatant from the GlucCa04 virus infected plate was subject to a luciferase assay. Each serum sample was assayed in triplicate on each plate. Neutralization titers were determined as the highest dilution of serum that inhibited infection, and for luciferase expression, 10-fold above background was considered an infected well.

Animal Studies

Six-week old, female, DBA/2 mice (National Cancer Institute, Frederick, MD) were divided into groups (n=24/group except PBS group with n=12) as follows: 1) PBS, 2) Sens/GlucCa04, 3) Res/GlucCa04, 4) Sens/GlucCa04 + Sens/Ca04, 5) Res/GlucCa04 + ResCa04, 6) Sens/GlucCa04 + Res/Ca04. For all virus-infected groups, half of the animals were treated daily with 80 mg/kg of amantadine given as two 40 mg/kg doses and separated by a minimum of 6 h. Mice were treated with amantadine beginning 24 h prior to infection, and treatment was continued for the duration of the experiment (i.e. until day 14). On the day of inoculation, mice were treated with amantadine 1 h prior to infection and again at 5 hpi. For virus inoculation, mice were anesthetized with 3% isoflurane and intranasally inoculated with 4,000 TCID₅₀ of Sens/GlucCa04 or Res/GlucCa04 viruses. For mice receiving a mixture of viruses, 20 TCID₅₀ of the appropriate Sens or Res/Ca04 virus was added to the inoculum. On day 3 and 5 post-infection (pi), 4 mice/group with or without amantadine treatment were euthanized. One lung lobe was homogenized and clarified, and the supernatant was aliquoted for luciferase assays (stored at -20°C) and virus titration (stored at -80°C). The remaining mice were maintained to day 14 to evaluate survival and weight loss. Mice showing >20% weight loss were humanely euthanized. All animal studies were approved by the University of Maryland Institutional Animal Care and Use Committee protocols R-09-63 and R-12-100.

Statistical Analysis

All data analysis was performed using GraphPad Prism Software Version 5.00 (GraphPad Software Inc., San Diego, CA). All assays were performed a minimum of two times in triplicate. To calculate the inhibitory concentration-50% (IC₅₀), the normalized data points were fit to a nonlinear regression curve. For multiple comparisons, two-way ANOVA was performed followed by a post-hoc Bonferroni test. $p < 0.05$ was considered significant.

RESULTS

Foreign gene expression and viral growth kinetics of rearranged viruses

To evaluate foreign or transgene expression, a rearranged A/California/04/2009 (H1N1) virus carrying GFP in segment 8 was produced (GFPCa04). In MDCK cells infected with GFPCa04 (Fig. 2A), GFP was readily visualized at 16 and 24 hpi. Subsequently, amantadine sensitive and resistant GLucCa04 viruses (Sens/GlucCa04 and Res/GlucCa04, respectively) were rescued and evaluated in multi-cycle growth kinetics experiments (Fig. 2B and C).

Rearrangement of the viral genome significantly reduced the peak titers compared to the recombinant Sens/GlucCa04 and Res/GlucCa04 (Fig. 2B). Both the Sens/GlucCa04 and Res/GlucCa04 grew to similar titers, although the sensitive virus appears to grow to slightly higher titers than the resistant virus. In parallel, GLuc expression was also assayed (Fig. 2C). In comparing Fig. 2B and C, it is evident that GLuc expression and viral titers of Sens/GlucCa04 and Res/GlucCa04 increased in parallel and that GLuc-expression reflects viral growth. Furthermore, both viruses had similar levels of GLuc expression (Fig. 2C) with Res/GlucCa04 showing slightly elevated expression at 18 and 24 hpi. These differences may be the result of the enhanced sensitivity of luciferase expression relative to TCID₅₀, or the resistance mutation in M2 may influence protein expression kinetics of segment 8.

Growth kinetics of rearranged Sens/GlucCa04 in the presence of amantadine or oseltamivir

To determine if anti-viral compounds could inhibit viral growth and luciferase expression, growth kinetics experiments were performed using Sens/GlucCa04 in the presence of amantadine or oseltamivir. Amantadine treatment significantly decreased both luciferase expression and viral titers from 16 to 48 hpi (Fig 3A and C). With respect to the effect of oseltamivir, treatment did not result in a significant decrease in luciferase expression (Fig. 3B), but was able to significantly decrease virus titers at 16 and 24 hpi (Fig. 3D). These findings indicate that GLucCa04 can distinguish between compounds that affect early versus late phases of the viral life cycle.

In vitro anti-viral drug screening using amantadine resistant and sensitive GLucCa04 viruses

Given that most methods of anti-viral screening use cell-based assays (Dai et al., 2012; Hossain et al., 2010; Martinez-Gil et al., 2012; Ozawa et al., 2013; Zhang et al., 2011), and that amantadine treatment decreased both luciferase expression and viral titers of Sens/GlucCa04, Sens and Res/GlucCa04 were evaluated in the context of an anti-viral screening assay. Thus, *in vitro* screening assays were performed in which Sens and Res/GlucCa04 (MOI 0.01 and 0.001) were pre-incubated with increasing concentrations of amantadine and then overlaid on MDCK cells. Subsequently, cell culture supernatants were collected over time and the samples were assayed for GLuc activity. Significant differences could be distinguished between Sens and Res/GlucCa04, as early as 16 hp, at both MOI (Fig. 4A), and these differences were maintained at all time points examined. At later time points of 48 and 72 h (Fig. 4C and D) the difference in GLuc expression between sensitive and resistant viruses was reduced; however, there was still a greater than 80% reduction between virus strains. For comparison to published IC₅₀ values determined by CPE reduction, the IC₅₀ values for Sens/GlucCa04 and Res/GlucCa04 were calculated based on GLuc expression (Table 1). The IC₅₀ values for the Sens/GlucCa04 and Res/GlucCa04 viruses were between 0.007 – 0.28 µg/mL, and 5.1 – 48.8 µg/mL, respectively. The values for the Res/GlucCa04 virus are similar to published values between 5–150 µg/mL for H1N1 isolates and pandemic H1N1 (Brooks et al., 2012; Nguyen et al., 2010; Nguyen et al., 2009; Zhan et al., 2012). In contrast, the values for Sens/GlucCa04 were dramatically reduced, indicative of the sensitivity of the virus to amantadine, and demonstrating the utility of rearranged viruses for anti-viral screening. Importantly, given anti-viral efficacy could be demonstrated as early as

16 hpi, and that luciferase assays can be performed in less than 10 min, the use of rearranged viruses dramatically decreased anti-viral screening time.

Serial passage of GLucCa04

To evaluate the stability of the transgene in Res/GLucCa04, serial passage experiments were performed. As shown in Fig. 5, three separate lineages were maintained, and GLuc activity and viral titers were determined over 5 passages. In lineages 1 and 3, GLuc expression was relatively stable over three passages but diminished over the fourth and fifth passage (Fig. 5A). In contrast, for lineage 2, GLuc expression significantly increased upon serial passage (Fig. 5A). For all lineages, viral titer decreased gradually and was not different between lineages over serial passage, regardless of luciferase expression (Fig. 5B).

MN comparing Ca04 and GLucCa04 viruses

Given that luciferase expression from GLucCa04 can be used as an index of viral infection, we evaluated the use of Res/GLucCa04 in MN. Thus, MN assays were performed using Res/GLucCa04 in parallel with Res/Ca04, and both hemagglutination and luciferase expression were assayed to determine inhibition of infection. As shown in Fig. 6, for all three serum samples both Res/Ca04 and Res/GLucCa04 yielded similar neutralization titers, with the exception of Serum 2 at 48 h. This finding indicates that while genome rearrangement results in diminished replication/attenuation of the virus, GLucCa04 is still suitable for MN. Furthermore, the use of GLuc expression as the readout yielded similar micro neutralization titers at all time points to those generated using hemagglutination for both Ca04 and GLucCa04.

In vivo screening using amantadine resistant and sensitive strains of GLucCa04

To potentially decrease *in vivo* anti-viral screening time by using luciferase expression as an index of virus titers, mouse studies using Sens/GLucCa04 and Res/GLucCa04 combined with amantadine treatment were performed. Based on the viral growth kinetics studies showing reduced replication of the rearranged viruses (Fig. 2) and preliminary mouse studies (data not shown), we predicted that the Sens/GLucCa04 and Res/GLucCa04 viruses would be attenuated. Thus, additional groups consisting of the GLuc viruses co-infected with the Sens/Ca04 or Res/Ca04 to permit observation of clinical disease (i.e. weight loss and survival) were included. For clarity the results were divided into mice given Sens/GLucCa04 or Res/GLucCa04 alone (Fig. 7), and mice co-infected with Sens/GLucCa04 or Res/GLucCa04 and Sens/Ca04 or Res/Ca04 (Fig. 8).

All mice infected with Sens/GLucCa04 or Res/GLucCa04 alone had GLuc detected in their lung homogenates (Fig. 7A). On both days 3 and 5 pi, mice that received the Sens/GLucCa04 virus in combination with amantadine treatment had a significant decrease in GLuc expression, while all other groups showed high levels of expression (Fig. 7A). This is consistent with the amantadine sensitive and resistant phenotypes. As shown in Fig. 7B, only low levels of virus were isolated from a limited number of mice per group. This demonstrates that GLuc expression can be used to assay anti-viral efficacy even in the absence of detectable virus replication. Furthermore, all mice had minimal body weight

changes (Fig. 7C), did not develop clinical disease, and survived the challenge (data not shown).

Next, we evaluated co-infection of Sens/GlucCa04 or Res/GlucCa04 with Sens/Ca04 or Res/Ca04. Four groups of mice were evaluated: 1) PBS, 2) Sens/GlucCa04 + Sens/Ca04, 3) Res/GlucCa04 + Res/Ca04, and 4) Sens/GlucCa04 + Res/Ca04. The fourth group was included to determine if anti-viral efficacy could be evaluated in the presence of a resistant virus. As above, half of the animals in each group were treated with amantadine, and tissues were collected on day 3 and 5 pi. Luciferase was expressed from all groups of infected animals, and when Sens/GlucCa04 + Sens/Ca04 inoculated mice were treated with amantadine there was a significant decrease in relative luciferase expression (Fig 8A). For the mice infected with Res/GlucCa04 + Res/Ca04 amantadine treatment did not decrease luciferase expression. In contrast, in mice inoculated with Sens/GlucCa04 + Res/Ca04, amantadine treatment significantly reduced GLuc expression although to a lesser extent than in the amantadine treated Sens/GLucCa04 + Sens/Ca04 group. Nevertheless, high levels of replicating virus were detected in the lungs of mice inoculated with Sens/GlucCa04 + Res/Ca04 regardless of amantadine treatment (Fig. 8B). This indicates that even in the presence of a resistant virus, GLuc expression can be used to evaluate anti-viral efficacy. Further examination of Fig 8B shows that all infected animals had replicating virus in the lungs. Given that mice inoculated with Sens/GlucCa04 or Res/GlucCa04 alone had minimal pulmonary virus titers, this virus is most likely the corresponding strain of Sens/Ca04 or Res/Ca04. Importantly, mice infected with Sens/GlucCa04 + Sens/Ca04 had significantly decreased levels of replicating virus in the lungs on day 3 ($p < 0.01$) and had a trend towards a decrease on day 5, while all other groups did not show decreased replication. All of the mice in the Sens/GlucCa04 + Sens/Ca04 group that were treated with amantadine showed minimal weight loss (Fig. 8C) and survived the infection (Fig. 8D). All other mice, excluding the PBS controls, lost weight and displayed 100% mortality. Collectively, our results demonstrate that GLucCa04 alone or in combination with wt Ca04 can be used to screen anti-viral compounds *in vivo*.

DISCUSSION

In this study, we characterized GLuc expressing strains of A/California/04/2009 (H1N1) with a rearranged genome, and performed *in vitro* and *in vivo* anti-viral screening studies. The genome rearrangement strategy used to express GLuc from segment 8 is an extension of our previous work (Pena et al., 2013). In agreement with these studies, rearrangement of the A/California/04/2009 genome resulted in diminished growth characteristics (Fig. 2). This is most likely a result of impaired PB1 activity by the FMDV 2A protease fused to its C-terminus (Pena et al., 2013). Given that NS1 also has the 2A protease fused to its C-terminus and NS2 is no longer expressed from segment 8, these modifications may also contribute to decreased viral replication (Chua et al., 2013). Despite the reduced growth characteristics, GLuc expression paralleled viral growth (Fig. 2), and growth kinetics experiments using Sens/GlucCa04 and increasing concentrations of amantadine resulted in decreased GLuc expression and viral titers (Fig. 3A and C). This finding suggested that Sens/GlucCa04 could be utilized for antiviral screening. Thus, we performed additional growth kinetics experiments using oseltamivir carboxylate, and found that while oseltamivir was capable of

reducing virus titers in the supernatant, GLuc expression was not decreased (Fig. 3B and D). This finding is not unexpected as oseltamivir is virustatic and prevents viral release but has minimal effect on viral infection and replication. Furthermore, *in vitro*, oseltamivir treatment may not prevent lateral cell-to-cell infection and this might account for the high levels of GLuc expression. In contrast, amantadine has virucidal properties as it inhibits the M2 ion channel and prevents replication. Thus, the rearranged virus assay system is best suited for screening compounds that inhibit the early stages of infection and/or replication of viral genes.

Based on the findings with amantadine, Sens and Res/GlucCa04 were evaluated in an anti-viral screening assay. From this assay, the IC₅₀ values determine via luciferase expression for Res/GlucCa04 were consistent with published values for existing assays, indicating that the rearranged GLucCa04 viruses could be used for anti-viral screening. Furthermore, given that anti-viral screening with Sens/GlucCa04 permitted identification of anti-viral efficacy as early as 16 hpi, this assay dramatically decreased screening time compared to conventional CPE assays. Thus, the use of Sens/GlucCa04 has the potential to accelerate *in vitro* anti-viral screening.

Recently, a novel strategy of GLuc expression from downstream of PB2 has been described in the A/Puerto Rico/1/1934 (H1N1) backbone (Heaton et al., 2013). This strategy offers many similar benefits to our strategy; however, the PR/8 backbone is an early influenza isolate and does not necessarily represent current circulating influenza strains. Thus anti-viral compounds identified using this approach would still require additional screening with recent isolates. In this context, our *in vivo* system, using a combination of GLucCa04 and Ca04, may be advantageous as GLuc expression could be used to screen for anti-viral efficacy and viral titers of the unmodified virus could be determined if required. A key feature of the studies using GLuc expressed from the PB2 segment is that *in vivo* imaging of virus replication can be performed (Heaton et al., 2013). We are similarly exploring imaging techniques using rearranged viruses expressing GLuc and other fluorescent proteins.

To further characterize Res/GlucCa04, serial passage experiments were performed to evaluate stability of the foreign gene (Fig. 5). In these studies, GLuc expression was maintained for at least 5 passages; however, in two lineages, expression decreased after the third passage, while one lineage had increased GLuc expression. Previously, when the dual H5-H9 rearranged vaccine (Pena et al., 2013), was evaluated for stability, the H5 transgene was maintained. As the dual H5-H9 vaccine has a truncated NS1(1–99), while GLucCa04 maintained full-length NS1, these findings suggest that duplicated packaging signals in full-length NS1 may lead to instability possibly via recombination. Future experiments should be performed to sequence lineage 2 from the serial passage experiment, and subsequent generations of rearranged reporter viruses should incorporate silent mutations to eliminate duplicate packaging signals (Heaton et al., 2013) and enhance stability.

As an additional application of GLuc expressing viruses, MN were performed to compare ResGLucCa04 and Res/Ca04 using both hemagglutination and GLuc expression as a readout. Both Res/Ca04 and Res/GlucCa04 yielded similar neutralization titers by hemagglutination at all time points, and neutralization titers determined via GLuc expression

were similar to hemagglutination titers (Fig 5). This finding was unexpected, as the enhanced sensitivity of GLuc was anticipated to yield representative titers at early time points. However, it is likely that the low levels of virus that escaped neutralization required at least 48 h to yield sufficient luciferase expression. In future studies, the amount of input virus could be optimized and this would likely lead to enhanced sensitivity. Importantly, the use of GLucCa04 for micro neutralization also offers the potential for automation or use in high-throughput systems as the need for visual interpretation by hemagglutination is not required.

To potentially decrease *in vivo* screening time, we performed studies in mice using either Sens or Res/GLucCa04 alone or in a co-infection with the corresponding Sens or Res/Ca04 strain. With both approaches, when amantadine treatment was used in mice given amantadine sensitive viruses there was a significant decrease in luciferase expression. Importantly, when mice were co-infected with Sens/GLucCa04 and Res/Ca04, amantadine treatment still dramatically decreased luciferase expression despite high levels of replicating virus. This demonstrates that GLucCa04 can be used for *in vivo* screening even in the presence of a resistant subpopulation. Furthermore, given that GLuc was assayed directly from lung homogenates, virus titrations are not required, and *in vivo* screening time is reduced.

CONCLUSIONS

The influenza genome rearrangement strategy can be used to express transgenes from A/California/04/09 (pH1N1). With anti-viral resistant influenza isolates being isolated with increasing frequency, the need for systems that accelerate anti-viral screening is clear. To meet this need, we extended our rearranged virus strategy to evaluate GLucCa04 for anti-viral screening. *In vitro*, screening time was reduced to 16 h and the use of a 96-well plate format permitted screening of multiple concentrations of a compound. *In vivo*, GLucCa04 could also be used for anti-viral screening, and the use of GLuc expression to determine efficacy limited the need to culture virus from tissue samples. Given the combined utility of GLucCa04 for both *in vitro* and *in vivo* screening, the use of GLucCa04 has the potential to accelerate anti-viral drug discovery.

Acknowledgments

We thank Matthew Angel for his advice on cloning and Andrea Ferrero, Yonas Araya, and Qiong Chen for technical assistance. This research was made possible through funding by NIAID-NIH contract (HHSN266200700010C).

GLOSSARY

<i>Gaussia</i> luciferase (GLuc)	secreted form of luciferase
Hemagglutinin (HA)	Influenza virus surface glycoprotein
Neuraminidase (NA)	influenza virus surface glycoprotein

Segment 2	influenza A RNA gene segment carrying the PB1 viral polymerase gene
Segment 8	influenza A RNA gene segment carrying the NS1 and NS2 genes
M2	viral protein ion channel expressed from viral RNA segment 7

REFERENCES

- Brooks MJ, Burtseva EI, Ellery PJ, Marsh GA, Lew AM, Slepishkin AN, Crowe SM, Tannock GA. Antiviral activity of arbidol, a broad-spectrum drug for use against respiratory viruses, varies according to test conditions. *J Med Virol.* 2012; 84(1):170–181. [PubMed: 22028179]
- Buxton RC, Edwards B, Juo RR, Voyta JC, Tisdale M, Bethell RC. Development of a sensitive chemiluminescent neuraminidase assay for the determination of influenza virus susceptibility to zanamivir. *Analytical biochemistry.* 2000; 280(2):291–300. [PubMed: 10790313]
- Chua MA, Schmid S, Perez JT, Langlois RA, Tenoever BR. Influenza A virus utilizes suboptimal splicing to coordinate the timing of infection. *Cell Rep.* 2013; 3(1):23–29. [PubMed: 23333274]
- Dai J, Wang G, Li W, Zhang L, Yang J, Zhao X, Chen X, Xu Y, Li K. High-throughput screening for anti-influenza A virus drugs and study of the mechanism of procyanidin on influenza A virus-induced autophagy. *J Biomol Screen.* 2012; 17(5):605–617. [PubMed: 22286278]
- Fujii K, Fujii Y, Noda T, Muramoto Y, Watanabe T, Takada A, Goto H, Horimoto T, Kawaoka Y. Importance of both the coding and the segment-specific noncoding regions of the influenza A virus NS segment for its efficient incorporation into virions. *Journal of virology.* 2005; 79(6):3766–3774. [PubMed: 15731270]
- Hayden FG, Cote KM, Douglas RG Jr. Plaque inhibition assay for drug susceptibility testing of influenza viruses. *Antimicrobial agents and chemotherapy.* 1980; 17(5):865–870. [PubMed: 7396473]
- Heaton NS, Leyva-Grado VH, Tan GS, Eggink D, Hai R, Palese P. In vivo bioluminescent imaging of influenza A virus infection and characterization of novel cross-protective monoclonal antibodies. *J Virol.* 2013; 87(15):8272–8281. [PubMed: 23698304]
- Hoffmann E, Neumann G, Kawaoka Y, Hobom G, Webster RG. A DNA transfection system for generation of influenza A virus from eight plasmids. *Proc Natl Acad Sci U S A.* 2000; 97(11):6108–6113. [PubMed: 10801978]
- Hossain MJ, Perez S, Guo Z, Chen LM, Donis RO. Establishment and characterization of a Madin-Darby canine kidney reporter cell line for influenza A virus assays. *J Clin Microbiol.* 2010; 48(7):2515–2523. [PubMed: 20504984]
- Hurt AC, Deng YM, Ernest J, Caldwell N, Leang L, Iannello P, Komadina N, Shaw R, Smith D, Dwyer DE, Tramontana AR, Lin RT, Freeman K, Kelso A, Barr IG. Oseltamivir-resistant influenza viruses circulating during the first year of the influenza A(H1N1) 2009 pandemic in the Asia-Pacific region, March 2009 to March 2010. *Euro surveillance : bulletin europeen sur les maladies transmissibles = European communicable disease bulletin.* 2011; 16(3)
- Jhung MA, Swardlow D, Olsen SJ, Jernigan D, Biggerstaff M, Kamimoto L, Kniss K, Reed C, Fry A, Brammer L, Gindler J, Gregg WJ, Bresee J, Finelli L. Epidemiology of 2009 pandemic influenza A (H1N1) in the United States. *Clin Infect Dis.* 2011; 52(Suppl 1):S13–S26. [PubMed: 21342884]
- Kao RY, Yang D, Lau LS, Tsui WH, Hu L, Dai J, Chan MP, Chan CM, Wang P, Zheng BJ, Sun J, Huang JD, Madar J, Chen G, Chen H, Guan Y, Yuen KY. Identification of influenza A nucleoprotein as an antiviral target. *Nat Biotechnol.* 2010; 28(6):600–605. [PubMed: 20512121]
- Lackenby A, Moran Gilad J, Pebody R, Miah S, Calatayud L, Bolotin S, Vipond I, Muir P, Guiver M, McMenamin J, Reynolds A, Moore C, Gunson R, Thompson C, Galiano M, Birmingham A, Ellis J, Zambon M. Continued emergence and changing epidemiology of oseltamivir-resistant influenza

- A(H1N1)2009 virus, United Kingdom, winter 2010/11. Euro surveillance : bulletin europeen sur les maladies transmissibles = European communicable disease bulletin. 2011; 16(5)
- Liang Y, Hong Y, Parslow TG. cis-Acting packaging signals in the influenza virus PB1, PB2, and PA genomic RNA segments. *Journal of virology*. 2005; 79(16):10348–10355. [PubMed: 16051827]
- Manicassamy B, Manicassamy S, Belicha-Villanueva A, Pisanelli G, Pulendran B, Garcia-Sastre A. Analysis of in vivo dynamics of influenza virus infection in mice using a GFP reporter virus. *Proceedings of the National Academy of Sciences of the United States of America*. 2010; 107(25):11531–11536. [PubMed: 20534532]
- Martinez-Gil L, Ayllon J, Ortigoza MB, Garcia-Sastre A, Shaw ML, Palese P. Identification of small molecules with type I interferon inducing properties by high-throughput screening. *PLoS One*. 2012; 7(11):e49049. [PubMed: 23145065]
- Molinari NA, Ortega-Sanchez IR, Messonnier ML, Thompson WW, Wortley PM, Weintraub E, Bridges CB. The annual impact of seasonal influenza in the US: measuring disease burden and costs. *Vaccine*. 2007; 25(27):5086–5096. [PubMed: 17544181]
- Morens DM, Taubenberger JK, Harvey HA, Memoli MJ. The 1918 influenza pandemic: lessons for 2009 and the future. *Critical care medicine*. 2010; 38(4 Suppl):e10–e20. [PubMed: 20048675]
- Nguyen JT, Hoopes JD, Le MH, Smee DF, Patick AK, Faix DJ, Blair PJ, de Jong MD, Prichard MN, Went GT. Triple combination of amantadine, ribavirin, and oseltamivir is highly active and synergistic against drug resistant influenza virus strains in vitro. *PloS one*. 2010; 5(2):e9332. [PubMed: 20179772]
- Nguyen JT, Hoopes JD, Smee DF, Prichard MN, Driebe EM, Engelthaler DM, Le MH, Keim PS, Spence RP, Went GT. Triple combination of oseltamivir, amantadine, and ribavirin displays synergistic activity against multiple influenza virus strains in vitro. *Antimicrob Agents Chemother*. 2009; 53(10):4115–4126. [PubMed: 19620324]
- Ozawa M, Shimojima M, Goto H, Watanabe S, Hatta Y, Kiso M, Furuta Y, Horimoto T, Peters NR, Hoffmann FM, Kawaoka Y. A cell-based screening system for influenza A viral RNA transcription/replication inhibitors. *Sci Rep*. 2013; 3:1106. [PubMed: 23346363]
- Palese, P.; Shaw, ML. *Orthomyxoviridae: The Viruses and Their Replication*. In: Knipe, DM.; Howley, PM., editors. *Fields Virology*. 5th ed.. Vol. 1. 2 s. Philadelphia: Lippincott Williams and Wilkins; 2007. p. 1648-1692.
- Pena L, Sutton T, Chockalingam A, Kumar S, Angel M, Shao H, Chen H, Li W, Perez DR. Influenza viruses with rearranged genomes as live-attenuated vaccines. *J Virol*. 2013; 87(9):5118–5127. [PubMed: 23449800]
- Potier M, Mameli L, Belisle M, Dallaire L, Melancon SB. Fluorometric assay of neuraminidase with a sodium (4-methylumbelliferyl- α -D-N-acetylneuraminate) substrate. *Analytical biochemistry*. 1979; 94(2):287–296. [PubMed: 464297]
- Rao S, Kong WP, Wei CJ, Yang ZY, Nason M, Styles D, DeTolla LJ, Panda A, Sorrell EM, Song H, Wan H, Ramirez-Nieto GC, Perez D, Nabel GJ. Multivalent HA DNA vaccination protects against highly pathogenic H5N1 avian influenza infection in chickens and mice. *PLoS One*. 2008; 3(6):e2432. [PubMed: 19293944]
- Reed L, Muench H. A simple method of estimating fifty percent endpoints. *American Journal of Hygiene*. 1938; 27:493–497.
- Severson WE, McDowell M, Ananthan S, Chung DH, Rasmussen L, Sosa MI, White EL, Noah J, Jonsson CB. High-throughput screening of a 100,000-compound library for inhibitors of influenza A virus (H3N2). *J Biomol Screen*. 2008; 13(9):879–887. [PubMed: 18812571]
- Su CY, Cheng TJ, Lin MI, Wang SY, Huang WI, Lin-Chu SY, Chen YH, Wu CY, Lai MM, Cheng WC, Wu YT, Tsai MD, Cheng YS, Wong CH. High-throughput identification of compounds targeting influenza RNA-dependent RNA polymerase activity. *Proceedings of the National Academy of Sciences of the United States of America*. 2010; 107(45):19151–19156. [PubMed: 20974907]
- Talon J, Salvatore M, O'Neill RE, Nakaya Y, Zheng H, Muster T, Garcia-Sastre A, Palese P. Influenza A and B viruses expressing altered NS1 proteins: A vaccine approach. *Proc Natl Acad Sci U S A*. 2000; 97(8):4309–4314. [PubMed: 10725408]

- Tannous BA, Kim DE, Fernandez JL, Weissleder R, Breakefield XO. Codon-optimized Gaussia luciferase cDNA for mammalian gene expression in culture and in vivo. *Mol Ther*. 2005; 11(3): 435–443. [PubMed: 15727940]
- Ujike M, Ejima M, Anraku A, Shimabukuro K, Obuchi M, Kishida N, Hong X, Takashita E, Fujisaki S, Yamashita K, Horikawa H, Kato Y, Oguchi A, Fujita N, Tashiro M, Odagiri T. Monitoring and characterization of oseltamivir-resistant pandemic (H1N1) 2009 virus, Japan, 2009–2010. *Emerging infectious diseases*. 2011; 17(3):470–479. [PubMed: 21392439]
- Wagaman PC, Leong MA, Simmen KA. Development of a novel influenza A antiviral assay. *J Virol Methods*. 2002; 105(1):105–114. [PubMed: 12176147]
- Ye J, Sorrell EM, Cai Y, Shao H, Xu K, Pena L, Hickman D, Song H, Angel M, Medina RA, Manicassamy B, Garcia-Sastre A, Perez DR. Variations in the hemagglutinin of the 2009 H1N1 pandemic virus: potential for strains with altered virulence phenotype? *PLoS pathogens*. 2010; 6(10):e1001145. [PubMed: 20976194]
- Zhan P, Wang L, Liu H, Chen X, Li X, Jiang X, Zhang Q, Liu X, Pannecouque C, Naesens L, De Clercq E, Liu A, Du G. Arylazolyl(azinyl)thioacetanilide. Part 9: Synthesis and biological investigation of thiazolylthioacetamides derivatives as a novel class of potential antiviral agents. *Arch Pharm Res*. 2012; 35(6):975–986. [PubMed: 22870806]
- Zhang J, Liu T, Tong X, Li G, Yan J, Ye X. Identification of novel virus inhibitors by influenza A virus specific reporter cell based screening. *Antiviral Res*. 2011
- Zhu W, Zhou J, Qin K, Du N, Liu L, Yu Z, Zhu Y, Tian W, Wu X, Shu Y. A reporter system for assaying influenza virus RNP functionality based on secreted Gaussia luciferase activity. *Virology journal*. 2011; 8:29. [PubMed: 21251302]

Highlights from “Genome Rearrangement of Pandemic H1N1 Influenza for Anti-Viral Drug Screening”

- The viral genome of A/California/04/2009 (H1N1) can be rearranged to express GFP or *Gaussia* luciferase (GLuc).
- Amantadine sensitive and resistant GLuc expressing variants were evaluated for anti-viral screening in the presence of amantadine.
- Viral expressed luciferase was shown to be suitable for *in vitro* and *in vivo* anti-viral screening.

Segment 2

Segment 8

Fig. 1. Schematic representation of segment 2 and 8 constructs for the rearranged virus strategy

NCR denotes non-coding region, ORF denotes open-reading frame, and ψ denotes packaging signal (consisting of both the UTR and a region of the ORF). GLuc and GFP denote *Gaussia* luciferase and green-fluorescent protein, respectively. NS1 splice constitutes NS1 with the donor and acceptor splicing sites knocked-out, a stop-codon inserted early in the NS2 ORF, and a truncation to encompass only NS1. Segments are drawn in the positive sense orientation and are not drawn to scale.

Fig. 2. Foreign gene expression from rearranged A/California/04/2009 (H1N1) and viral growth curves of amantadine sensitive and resistant GLucCa04

Panel (A) shows cells infected with GFPCa04 (MOI 0.01) at 16, and 24 hpi. The upper and lower panels are from 16 and 24 hpi. Left panels are phase contrast images, the middle panels are fluorescent light images and right panels are merged images, respectively. All images are at 100X magnification. Panel (B) shows viral titers of amantadine Sens and Res/ Ca04 and amantadine Sens and Res/GlucCa04 viruses determined by TCID₅₀. Panel (C) shows GLuc expression in MDCK cells infected with Sens and Res/GlucCa04 at MOI 0.01 over 72 h Sens and Res/GlucCa04 grew to significantly lower titers than their corresponding

parental viruses. * denotes significantly different from parental strains and negative control ($p < 0.05$).

Fig. 3. Growth kinetics and luciferase expression of Sens/GlucCa04 in the presence of amantadine or oseltamivir

Growth kinetics experiments were performed with Sens/GlucCa04 in the presence of 0, 0.1, 1, or 10 $\mu\text{g/mL}$ amantadine hydrochloride (AMT) or oseltamivir carboxylate (OST). Panel (A) and (B) represent GLuc activity, and panels (C) and (D) show viral titers in the presence of amantadine and oseltamivir, respectively. * denotes significantly different from all other conditions ($p < 0.05$). † denotes significantly different from 0.1 $\mu\text{g/mL}$ amantadine ($p < 0.05$). δ denotes significantly different from 0.1 $\mu\text{g/mL}$ oseltamivir ($p < 0.05$).

Fig. 4. Amantadine anti-viral screen using rearranged Sens and Res/GlucCa04

(A), (B), (C), and (D) represent 16, 24, 48 and 72 hpi, respectively. Significant differences can be observed between Sens and Res/GlucCa04 with increasing amantadine concentration at all time points. Data is expressed as a percentage of the non-drug, 0 $\mu\text{g/ml}$ Amantadine condition. MOI include 0, 0.01, and 0.001. * denotes significantly different from Res/GlucCa04 MOI 0.01. † denotes significantly different from Res/GlucCa04 MOI 0.001.

Fig. 5. Luciferase expression and viral titers over serial passage of Res/GlucCa04
 Res/GlucCa04 was serially passaged as three separate lineages, designated lineage 1–3. (A) and (B) show GLuc expression and viral titer, respectively, for each lineage. * denotes significantly different from all other lineages ($p < 0.05$).

Fig. 6. Microneutralization titers determined by hemagglutination and GLuc expression
 MN were performed with Res/Ca04 and Res/GLucCa04. At 24, 48, and 72 h, separate plates for each virus were assayed for hemagglutination, and the Res/GLucCa04 infected plates were assayed for GLuc expression. Panel (A), (B), and (C) denote micro neutralization titers on three separate serum samples, respectively. For hemagglutination, the titer recorded is the highest serum dilution at which there is no hemagglutination activity, while for GLuc, the titer recorded is the highest serum dilution at which there is less than 10-fold luciferase expression above background (uninfected cell culture supernatant).

Fig. 7. *In vivo* anti-viral screen using Sens and Res/GlucCa04 combined with amantadine treatment

Three groups of DBA/2 mice were used for this experiment and included: 1) PBS, 2) Sens/GlucCa04, and 3) Res/GlucCa04. In each virus-infected group, half of the mice were treated with amantadine (AMT). Shown are results from days 3 and 5 post infection. Panel (A) displays relative luciferase expression from lung homogenates of mice, and (B) shows levels of replicating virus titrated from the lungs. Displayed as a proportion is the number of mice that had detectable virus. Panel (C) displays weight loss of inoculated mice. * denotes significantly different from corresponding untreated group ($p < 0.05$).

Fig. 8. *In vivo* anti-viral screening using a mixture of Sens and Res/GlucCa04 and Ca04 viruses
 Four groups of DBA mice were used and consisted of 1) PBS, 2) Sens/GlucCa04 + Sens/Ca04, 3) Res/GlucCa04 + Res/Ca04, and 4) Sens/GlucCa04 + Res/Ca04. In each virus-infected group, half of the mice were treated with amantadine (AMT). Panel (A) shows relative luciferase expression from lung homogenates, (B) displays pulmonary viral titers by TCID₅₀. All mice except for those in the PBS group had virus isolated from the lungs. Shown in panel (C) is weight loss, and (D) displays survival curves for all groups of mice. * denotes significantly different from corresponding untreated group ($p < 0.05$). ** denotes significantly different from untreated groups and groups receiving Res/GlucCa04. Only the Sens/GlucCa04 + Sens/Ca04 group that received amantadine treatment had reduced GLuc expression and viral lung titers. This group of mice showed minimal body weight loss and had 100% survival. Shown in panels C and D is an additional control group consisting of 4 mice inoculated with PBS and treated with amantadine. Amantadine treatment did not result in weight loss or decreased survival.

Table 1

Inhibitory Concentration –50 (IC₅₀) of amantadine determined by GLuc expression using Sens and Res/
GlucCa04.

Time (hrs)	Sens/GlucCa04 MOI 0.01 (µg/mL)	Res/GlucCa04 MOI 0.01 (µg/mL)
16	0.007762	10.04
24	0.01021	5.179
48	0.005058	48.89
72	0.02883	>400

IC₅₀ values were calculated for MOI 0.01 and are expressed in µg/mL.