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Modulation of influenza virus replication by alteration of sodium ion transport and protein kinase C activity

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ABSTRACT

In recent years, increasing levels of resistance to the four FDA-approved anti-influenza virus drugs have been described and vaccine manufacturers have experienced demands that exceed their capacity. This situation underlines the urgent need for novel antivirals as well as innovations in vaccine production in preparation for the next influenza epidemic. Here we report the development of a cell-based high-throughput screen which we have used for the identification of compounds that modulate influenza virus growth either negatively or positively. We screened a library of compounds with known biological activity and identified distinct groups of inhibitors and enhancers that target sodium channels or protein kinase C (PKC). We confirmed these results in viral growth assays and find that treatment with a sodium channel opener or PKC inhibitor significantly reduces viral replication. In contrast, inhibition of sodium channels or activation of PKC leads to enhanced virus production in tissue culture. These diametrically opposing effects strongly support a role for PKC activity and the regulation of Na^+ currents in influenza virus replication and both may serve as targets for antiviral drugs. Furthermore, we raise the possibility that compounds that result in increased viral titers may be beneficial for boosting the production of tissue culture-grown influenza vaccines.

1. Introduction

Influenza viruses are enveloped RNA viruses that belong to the family of *Orthomyxoviridae* (Palese and Shaw, 2007). Influenza A and B viruses are considered to be major human pathogens and in a normal season can cause between 3 and 5 million cases of severe illness and up to 500,000 deaths worldwide (WHO, 2003). Influenza A viruses can also cause pandemics such as those that occurred in 1918, 1957 and 1968. These outbreaks resulted in high mortality rates because of the lack of pre-existing immunity against the new virus strain. Since the emergence of the highly pathogenic avian H5N1 influenza virus in the late 1990s (Claas et al., 1998), there have been concerns that it may be the next pandemic virus, which has sparked renewed interest in the development of anti-influenza virus drugs. Currently we have only four U.S. Food and Drug Administration (FDA)-approved drugs available for the treatment and prevention of influenza. The adamantanes (amantadine and rimantadine) block the M2 ion channel of the virus and prevent the release of the viral genome into the host cell (Pinto and Lamb, 1995; Wharton et al., 1994). These drugs are effective if used prophylactically and if administered within 48 h of infection but are not effective against influenza B viruses. Unfortunately, the development of widespread resistance has precluded the use of adamantanes in recent influenza seasons (Bright et al., 2006) and isolates of the H5N1 influenza virus have been shown to be resistant to these drugs due to mutations in M2 (Cheung et al., 2006). The preferred treatment for influenza virus infection is now the use of the neuraminidase inhibitors, oseltamivir and zanamivir (Garman and Laver, 2004). By targeting the neuraminidase, these compounds prevent the release of the virus from the infected cell and halt the spread of the virus. As part of its pandemic preparedness plan, the World Health Organization (WHO) has advised that supplies of the neuraminidase inhibitors be stockpiled, but it is always advantageous to have at least two antiviral drugs (aimed at different targets) available due to the possible emergence of resistant virus strains. In fact the 2007–2008 influenza season in the Northern hemisphere has shown a marked increase in the number of H1N1 isolates that are resistant to oseltamivir (WHO, 2008) and concerns have also been raised regarding oseltamivir-resistant H5N1 influenza viruses isolated from patients in Southeast Asia (Le et al., 2005).

Abbreviations: PKC, protein kinase C; HTS, high-throughput screen.

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Vaccination is by far the best means we have of preventing infection or at least minimizing the severity of disease. Based on knowledge of the current circulating influenza virus strains, the WHO makes an annual decision as to which virus strains should be included in the influenza vaccine for the following season. Manufactures therefore have a relatively short time period in which to generate new vaccine stocks and this, combined with the increase in demand from the population, sometimes leads to shortages. Vaccine viruses are currently grown in embryonated chicken eggs which generally support high levels of virus growth; however the use of eggs has certain limitations. Vaccine production cannot easily be scaled up at short notice, as would be required during a pandemic, due to the reliance on a continuous supply of embryonated eggs. Furthermore if the pandemic virus was of avian origin it may be lethal in eggs, as occurred during the preparation of an H5N1 vaccine candidate (Takada et al., 1999). An avian virus would likely also affect the poultry industry and the egg supply may be greatly reduced. In an effort to avoid these problems, vaccine manufacturers are now establishing tissue culture systems for the growth of influenza virus vaccines (Oxford et al., 2005; Romanova et al., 2004; Tree et al., 2001). The major disadvantage being that wild-type human influenza virus strains often do not show optimal growth properties in this culture system, resulting in lower vaccine yields. In conclusion, there is an urgent need for the development of new antiviral drugs and also for the improvement of tissue culture-based vaccine production, in preparation for future influenza epidemics or pandemics.

With this in mind, we designed a cell-based, high-throughput assay that allowed us to screen for small molecular weight compounds that modulate influenza virus growth. In an initial screen of compounds with known biological activity, we identified compounds that negatively regulate influenza virus replication and may have potential as antiviral drugs. We also identified compounds that enhance influenza virus growth and we propose that these may be used as tools to boost the replication of influenza virus vaccine candidates in tissue culture.

2. Materials and methods

2.1. Cell lines, viruses and plasmids

Human alveolar basal epithelial (A549) cells, African green monkey kidney (Vero) cells, chicken embryo fibroblast (DF1) cells and Madin–Darby canine kidney (MDCK) cells, were obtained from the American Type Culture Collection (ATCC, Manassas, VA). A549 cells, Vero cells and DF1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Corp., Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (HyClone; South Logan, UT) and 100 U/mL penicillin G sodium and 100 μg/mL streptomycin sulfate (Invitrogen Corp., Carlsbad, CA). MDCK cells were cultured in Minimum Essential Medium (MEM) (Invitrogen Corp., Carlsbad, CA) supplemented with 10% FBS, 2 mM L-glutamine (Invitrogen Corp., Carlsbad, CA), 100 U/mL penicillin G sodium and 100 mg/L streptomycin sulfate and 0.15% $NaHCO_3$ (Invitrogen Corp., Carlsbad, CA).

Influenza A/WSN/33 virus and influenza B/Yamagata/88 virus were grown in MDCK cells in MEM-post-infection medium (MEM supplemented with 0.3% bovine serum albumin (BSA), 0.1% FBS, 2 mM L-glutamine, 100 U/mL penicillin G sodium and 100 μg/mL streptomycin sulfate and 0.15% $NaHCO_3$). Viruses were titered by standard plaque assay in MDCK cells. Vesicular stomatitis virus expressing the green fluorescence protein (VSV-GFP) was kindly provided by John Hiscott (McGill University, Montreal, Canada), and was grown and titered in Vero cells. Newcastle disease virus strain B1 (rNDV/B1) was grown in 10-day-old embryonated hens' eggs and titered in DF1 cells. The human isolates influenza A/Moscow/10/99 and A/Wyoming/03/2003 viruses were grown in 8-day-old embryonated hens' eggs and titered in MDCK cells. The recombinant 6:2 influenza A/PR/8/34 virus reassortant expressing a low virulence hemagglutinin (HA) and the neuraminidase (NA) of influenza A/VN/1203/04 virus (referred to as H5N1/PR8 in this study) was rescued using reverse genetics (Fodor et al., 1999). The HA of influenza A/VN/1203/04 virus was mutated by removing the multibasic cleavage site which is associated with high pathogenicity in chickens (Senne et al., 1996). Briefly, expression plasmids and pPol I plasmids for the HA and NA genes of influenza A/VN/1203/04 virus were co-transfected into 293T cells together with ambisense pDZ plasmids for the PB1, PB2, PA, NP, M and NS segments of influenza A/PR/8/34 virus. The supernatant was transferred 48 h later into 8-day-old embryonated hens' eggs to allow the recombinant virus to propagate. The egg-grown virus stock was sequenced and titered in MDCK cells.

For the construction of the influenza mini-genome reporter (pPolI-358Luc) the firefly luciferase open reading frame from pGL3 (Promega Corp., Madison, WI) was amplified by PCR and the 5′ and 3′ ends of the cRNA promoter of the influenza A/WSN/33 virus NP segment were incorporated on either end (Neumann and Hobom, 1995). This product was then inserted into the pPolI vector (Pleschka et al., 1996) with the luciferase gene in the negative sense.

2.2. Small molecular weight compounds

The Prestwick Chemical Library (1120 compounds; Prestwick Chemical, Inc., Washington, DC), the NINDS custom collection 2 (1040 compounds; National Institute of Neurological Disorders and Stroke; Bethesda, MD) and BIOMOL Known Bioactives-2 library (480 compounds; BIOMOL, Plymouth Meeting, PA) were provided by The Institute of Chemistry and Cell Biology (ICCB), National Screening Laboratory for the Regional Centers of Excellence in Biodefense and Emerging Infectious Disease (NSRB) (Harvard University, Boston, MA). The compounds were dissolved in DMSO at 2 mg/mL for the Prestwick library, 5 mg/mL for the BIOMOL2 library and at 10 mM for the NINDS2 library.

For secondary analyses 2′,4′-dichlorobenzamil and SDZ-201106 were purchased from BIOMOL (Plymouth Meeting, PA) while 3′,4′-dichlorobenzamil, phenamil, phorbol 12-myristate 13-acetate (PMA), mezerein, rottlerin, staurosporin, ouabain and lanatoside C were purchased from Sigma–Aldrich (St. Louis, MO). All compounds were dissolved in either H_2O or dimethyl sulfoxide (DMSO) to a stock concentration of 10 mM. The final concentration of DMSO in the culture medium did not exceed 0.004%.

2.3. High-throughput screening

The assay was performed in duplicate using solid white 384-well tissue culture-treated plates (Corning Life Sciences; Lowell, MA). A549 cells were cultured to 90% confluency, trypsinized with 0.05% Trypsin–EDTA (Invitrogen Corp., Carlsbad, CA), and resuspended in phenol red-free DMEM growth medium supplemented with 10% FBS at 7.2×10^5 cells/mL. Transfections were done in bulk and for each well, 12.5 ng of the reporter pPolI-358Luc was diluted in 6.25 μL OptiMEM (Invitrogen Corp., Carlsbad, CA) and mixed with 6.25 μL OptiMEM containing 0.025 μL Lipofectamine2000 (Invitrogen Corp., Carlsbad, CA). The transfection mix was incubated for 20 min before adding 12.5 μL of resuspended A549 cells (approximately 9×10^3 cells). The medium also included 0.25 μg/mL Scriptaid (BIOMOL, Plymouth Meeting, PA) and 1.0 μg/mL 5-aza-2′-deoxycytidine (Sigma–Aldrich; St. Louis,

MO), which were added to enhance reproducibility of the assay (Hellebrekers and Griffioen, 2007). The mix of cells and reporter DNA was transferred into 384-well plates using the Matrix Wellmate plate filler. Loaded plates were subsequently centrifuged at 1000 rpm for 5 min to ensure an equal distribution of cells within each well. The cells were incubated for 18 h at 37 °C, 5% CO_2, 95% humidity before the addition of 100 nL of compounds by the Epson compound transfer robot (Epson America, Inc., Long Beach, CA). The cells were incubated for a further 6 h before infection with influenza A/WSN/33 virus directly into the medium at an MOI of 2.5. The virus was added automatically to the plates using the Matrix Wellmate plate filler and the plates were subsequently centrifuged at 1000 rpm for 2 min. Each plate also contained mock-infected cells that were used as a positive control and cells that were infected but untreated, which were used as a negative control. The two approved classes of influenza antiviral drugs could not be used as positive controls in this screen because (i) influenza A/WSN/33 virus is resistant to amantadine and (ii) the assay does not detect multicycle replication which is necessary to see the effects of oseltamivir as it targets virus release. Infection was allowed to proceed for 18–20 h at 37 °C, 5% CO_2, 95% humidity. At that time 50% of the medium in each well was removed and the plates were equilibrated to room temperature for at least 20 min. The Matrix Wellmate plate filler was used to add 16 μL of BrightGloLuciferase reagent (Promega Corp., Madison, WI) to each well automatically and following a 2-min centrifugation at 2000 rpm, luminescence was measured for 0.1 s/well with the EnVision2 plate reader (PerkinElmer Inc., Waltham, MA).

To eliminate cytotoxic compounds that appear as false positives, a counter screen was performed in parallel. This consisted of A549 cells transfected with pGL3 and seeded at a density of 2500 cells per well of a 384-well plate. The remainder of the assay was performed as described above except that the cells were not infected.

2.4. Data analysis

To evaluate the HTS assay robustness, 384-well plates containing no compounds were run separately on two different days. Statistical parameters were determined as follows: $Z' = 1-((3\sigma_i + 3\sigma_m)/|\mu_i - \mu_m|)$, where μ_i is the mean signal for the negative control (infected cells), σ_i the standard deviation for the negative control, μ_m the mean signal for the positive control (mock-infected cells), and σ_m is the standard deviation for the positive control. The percent coefficient of variation (CV) = $\sigma_i/\mu_i \times 100$, the signal-to-background ratio (S/B) = μ_i/μ_m and the signal-to-noise ratio (S/N) = $(\mu_i - \mu_m)/((\sigma_i)^2 + (\sigma_m)^2)^{1/2}$ (Ghosh et al., 2005; Zhang et al., 1999).

The data from the influenza HTS assay and counter screen were analyzed with Microsoft Office Excel. The average of the negative control of each plate was set at 100% luminescence and the percent luminescence of each compound-containing well was determined in relation to this internal control. The average percent luminescence for the duplicate screenings was calculated and the compounds were classified into strong or medium inhibitors based on a 90–100 or 70–89% reduction in luminescence, respectively. Compounds leading to an increase in luminescence were considered as enhancers with at least a twofold induction above the negative control. The HTS data were compared to the corresponding data from the counter screen. A reduction in luminescence greater than 20–30% in the counter screen was considered to be caused by cytotoxicity and therefore the compound was defined as a false positive and eliminated from further analysis. This threshold was decreased down to 50% in cases where the compound caused a >95% reduction of luminescent signal in the influenza HTS assay.

2.5. Cell viability assay

The CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay (referred to as the MTS assay in this study) (Promega Corp., Madison, WI) was used to detect cell viability according to the specifications of the manufacturer. Briefly, A549 cells were seeded into 96-well plates (Corning Life Sciences, Lowell, MA) at 5×10^3 cells per well and allowed to incubate for 24 h at 37 °C, 5% CO_2. After incubation, the medium was aspirated and replaced with 100 μL of fresh DMEM containing the compounds at various concentrations. Following a further 24 h incubation, the MTS solution was added to each well and left to incubate for 2 h before measuring absorbance at 450 nm using a Beckman Coulter DTX 880 plate reader (Beckman Coulter, Inc., Fullerton, CA).

2.6. Viral growth assays in the presence of inhibitors or enhancers

A549 cells were seeded into 6-well plates at 5×10^5 cells per well. After incubation for 24 h at 37 °C and 5% CO_2, the cells were washed with phosphate buffered saline (PBS) (Invitrogen Corp., Carlsbad, CA) and the medium was replaced with DMEM supplemented with 0.3% BSA, 0.1% FBS and 100 U/mL penicillin G sodium and 100 μg/mL streptomycin sulfate containing the compound of interest. Compounds that enhance viral replication were used at their most potent concentration (400 nM for 2′,4′-dichlorobenzamil and 3′,4′-dichlorobenzamil, 10 μM for phenamil, 250 nM for mezerein and 250 nM for PMA). Compounds that inhibit viral replication were used at their most potent, but non-toxic, concentration (12.5 μM for SDZ-201106 and 1.25 μM for rottlerin). Cardioactive glycosides were tested at concentrations that maintained at least an 80% cell viability (20 nM for ouabain and 78 nM for lanatoside C). The cells were incubated in the compound-containing media for 6 h prior to infection. When testing the response of influenza A/WSN/33 virus and of H5N1/PR8 virus to enhancers, infections were done at a multiplicity of 0.001, whereas a multiplicity of 1 was used when testing the response of influenza A/WSN/33 virus to inhibitors. For the human isolates, influenza viruses A/Moscow/10/99 and A/Wyoming/03/2003, infections were done at an MOI of 0.01 when testing enhancers. For influenza B/Yamagata/88 virus, infections were done at a multiplicity of 5 when testing inhibitors and a multiplicity of 0.1 when testing enhancers. Compounds were absent during the 1 h incubation with the virus but were present in the post-infection medium (DMEM supplemented with 0.3% BSA, 0.1% FBS and 100 U/mL penicillin G sodium and 100 μg/mL streptomycin sulfate). For infection with influenza B virus, the human influenza A virus isolates and the H5N1/PR8 virus this post-infection medium also contained 1 μg/ml TPCK-treated trypsin (Sigma–Aldrich; St. Louis, MO). The infected cells were incubated at 37 °C with the exception for influenza B virus infected cells, which were incubated at 33 °C. The viral titers for all viruses were determined at various times post-infection by standard plaque assay in MDCK cells. When testing the effects of the cardioactive glycosides on the growth of NDV and VSV-GFP, infections were performed at an MOI of 1. Viral titers were determined at 24 h post-infection by standard plaque assay in Vero cells for VSV-GFP and in DF1 cells for NDV/B1. The NDV plaques were visualized by immuno-staining with an anti-NP antibody (Matrosovich et al., 2006).

2.7. Apoptosis assay

The Caspase-3 Colorimetric Assay (R&D Systems, Inc., Minneapolis, MN) was used to detect whether the compounds have pro- or anti-apoptotic effects. A549 cells were seeded into 60 mm tissue culture-treated dishes (Corning Life Sciences, Lowell, MA) at

Table 1
Summary of statistical parameters to assess the robustness of the HTS assay in 384-well format

	Z'[a]	%CV[b]	S/B[c]	S/N[d]
Screen 1	0.56	14.7	14586	6.8
Screen 2	0.55	15.1	10734	6.6

[a] $Z' = 1 - ((3\sigma_i + 3\sigma_m)/|\mu_i - \mu_m|)$, where σ_i is the standard deviation for the negative control, σ_m is the standard deviation for the positive control, μ_i is the mean signal for the negative control (infected cells) and μ_m the mean signal for the positive control (mock-infected cells).

[b] %CV (coefficient of variation) = $\sigma_i/\mu_i \times 100$.

[c] S/B (signal-to-background ratio) = μ_i/μ_m.

[d] S/N (signal-to-noise ratio) = $(\mu_i - \mu_m)/((\sigma_i)^2 + (\sigma_m)^2)^{1/2}$.

1.5×10^6 cells per dish and allowed to incubate for 24 h at 37 °C, 5% CO_2. After incubation, the medium was aspirated and the cells were washed with PBS. Fresh DMEM post-infection medium was added, containing compounds at the same concentrations as were used for the viral infections. As a positive control for the induction of apoptosis, the cells were treated with staurosporin at a concentration of 5 μM. Cells were incubated for 6 h at 37 °C, 5% CO_2. Subsequently, they were harvested, washed twice with PBS, lysed and incubated with the DEVD-AFC substrate for an additional hour at 37 °C, 5% CO_2 before measuring fluorescence at 500 nm using a Versa Fluor Fluorometer (BioRad; Hercules, CA).

3. Results

3.1. Development of a novel HTS assay for influenza virus

We developed a cell-based HTS assay for the identification of small molecules that can negatively or positively affect influenza A virus replication. An influenza mini-genome was designed encoding firefly luciferase in the negative sense in between the cRNA promoter of the influenza A/WSN/33 virus NP segment. This construct was cloned into a plasmid flanked by a human RNA polymerase I promoter and the hepatitis D virus (HDV) ribozyme (Fig. 1A). Upon transfection of this reporter into human lung epithelial (A549) cells, RNA polymerase I transcription generates an RNA segment that mimics viral RNA. When these cells are subsequently infected with influenza virus, this segment is recognized by the viral polymerase resulting in the production of firefly luciferase mRNA. Luciferase activity therefore serves as a measurement of influenza virus replication and decreases or increases in this signal that are observed in the presence of specific compounds are indicative of inhibitory or enhancing activities, respectively. Due to the fact that a high multiplicity of infection provided the greatest reproducibility, the assay is more likely to detect compounds that act on steps up to and including translation. A multicycle format would be necessary to detect inhibitors of any of the later stages such as assembly, budding and release. In this respect our assay differs from other cell-based HTS assays for influenza virus which rely on virus-induced cytopathic effect (CPE) as a readout and which use a low multiplicity of infection (Noah et al., 2007). It should be noted that such an assay can only be performed in MDCK cells which display significant CPE in response to influenza virus infection. Our assay is performed in A549 cells which are a biologically relevant cell type for influenza virus infection. The assay was initially optimized in 96-well format and its validity for use in a high-throughput screen was confirmed by demonstrating a Z' factor (Zhang et al., 1999) of 0.74. For screening purposes the assay was further adapted to 384-well format and Table 1 shows the statistical parameters used to evaluate the robustness of the assay in this format. Here the Z' factor was determined as 0.55 and 0.56 in two separate runs. The discrepancy between the Z' factor values determined in 96-well plates and 384-well plates can be explained by the "edge effect" and higher variability in the miniaturized 384-well format. However, both formats meet the requirements for high-throughput screening. Assays with a Z' factor value of $1 > Z' \geq 0.5$ are considered to be excellent for use in HTS and the larger the value, the higher the data quality (Zhang et al., 1999). Additional parameters which verify that the assay is robust are the coefficient of variation, CV (14.9 ± 0.2%), the signal-to-background ratio, S/B (>10^4) and the signal-to-noise ratio, S/N (6.7 ± 0.1). The S/N ratio is slightly below the ratio of another reported HTS for influenza virus (S/N > 10) (Noah et al., 2007) and reflects a higher signal deviation in our assay which nonetheless is still better compared to a HTS assay for SARS coronavirus (S/N > 3) (Severson et al., 2007). Recently, a number of cell-based HTS assays were developed for screening compounds against different viruses. The S/B ratio of our assay of >10^4 is strongly above those of other HTS assays reported for influenza virus (S/B > 30) (Noah et al., 2007), for SARS coronavirus (S/B = ~10) (Severson et al., 2007), for human immunodeficiency virus (HIV-1) (S/B > 100) (Blair et al., 2005), for hepatitis C virus (HCV) (S/B > 13) (Zuck et al., 2004) and for dengue virus (S/B = 8) (Chu and Yang, 2007). This high S/B ratio in addition to a CV of ~15% proves the suitability of our assay for use in a high-throughput screen. Subsequently a pilot screen was conducted at the Harvard Institute for Cell and Chemical Biology (ICCB) where two library plates (704 compounds) were tested in duplicate. When screening a library plate containing uncharacterized compounds, we found one strong inhibitor (0.28%) while 18 strong inhibitors (~5%) were detected when screening compounds of known bioactivity. Overall, 19 strong inhibitors were identified, which equals a hit rate of 2.7%. This rate was relatively high due to the fact that cytotoxic compounds were included in these hits and in order to eliminate these false positives we ran a counter screen in parallel (as described in Section 2.3) for all future screens.

3.2. A high-throughput screen identifies compounds that modulate influenza virus growth

As part of an initial study, we screened three compound libraries consisting of collections from NINDS, Prestwick and BIOMOL, and totaling 2640 small molecules. All of these compounds have known biological activity (i.e. their cellular targets are known) which facilitates the downstream analyses. A549 cells transfected with the reporter plasmid in bulk were plated in solid white 384-well plates and incubated overnight at 37 °C. Six hours prior to infection, 100 nL of the test compounds in library-defined concentrations were added automatically to each well, in duplicate. Influenza A/WSN/33 virus was added to the media at a multiplicity of infection (MOI) of 2.5, and infection was allowed to proceed for 18–20 h at 37 °C. After adding the luciferase substrate, luminescence was measured and compared to control wells that received no compound as well as to the results of the counter screen for elimination of false positives. From the 2640 screened compounds, 59 (2.2%) were identified as strong inhibitors with the luciferase signal reduced by 90–100%. An additional 43 compounds (1.6%) were found to reduce the signal by 70–89% and 4 of the compounds (0.15%) increased luminescence by at least twofold (Fig. 1B). Some of the same compounds were present in either two or all of the libraries and were identified as hits independently two or three times. Therefore in total, we identified 47 (1.8%) unique strong inhibitors, 37 (1.4%) unique moderate inhibitors and 4 (0.15%) enhancers. Table 2 shows the functional classes of the hit compounds that were identified as inhibitors in the HTS. About 24% of the inhibitors are compounds that interfere with DNA. Another group of 21% consist of antibiotics, antifungals and antiparasitic drugs. Roughly 13% of the inhibitors target different cellular kinases like protein kinase A, protein kinase C and receptor tyrosine kinases and more than 8% of the inhibitory compounds are Na^+/K^+/ATPase pump inhibitors (ouabain, lanatoside C, digoxin,

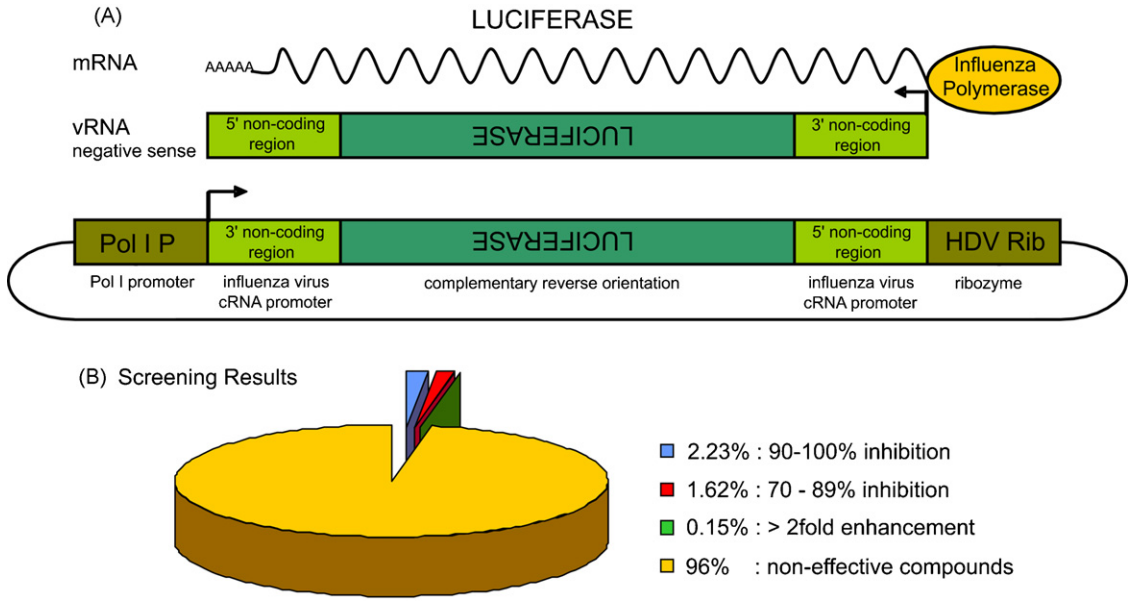

Fig. 1. Influenza virus mini-genome construct and high-throughput screening results. (A) Schematic of the influenza virus luciferase reporter construct. The firefly luciferase open reading frame was inserted in the reverse orientation and complementary sense between the influenza virus non-coding regions which serve as the viral promoter. This cassette is flanked by a human RNA polymerase I (Pol I) promoter and a hepatitis delta virus (HDV) ribozyme. The transcribed RNA (vRNA) has exact ends and mimics an influenza virus genome segment. Upon infection, the influenza virus polymerase recognizes the promoter and the reporter gene is transcribed and expressed. (B) Results of the HTS of known bioactive compounds. Three libraries (NINDS, Prestwick and BIOMOL) were screened containing 2640 known bioactive compounds. Strong inhibition was defined as a 90–100% reduction in luminescence, medium inhibition as a 70–89% reduction and an increase in luminescence of at least twofold was classified as an enhancement.

strophanthidin), known as cardioactive glycosides. The initial effect of these compounds and of SDZ-201106, a sodium channel opener which was also identified as an inhibitor, is to raise the intracellular Na^+ concentration. Interestingly, one of the compounds that resulted in increased luciferase signals (phenamil) is an amiloride analogue that acts as a sodium channel inhibitor. These opposing effects, by compounds that have contrasting effects on sodium channels, indicates that influenza virus is sensitive to changes in intracellular ion concentrations and that this may be a way of modulating influenza virus replication. In support of this, it has been reported that influenza virus can inhibit these amiloride-sensitive sodium channels in the respiratory epithelium (Chen et al., 2004; Kunzelmann et al., 2000). Amongst the group of inhibitory compounds we also found several protein kinase C (PKC) inhibitors, the strongest of which was rottlerin. PKC inhibitors have previously been shown to inhibit influenza virus (Kurokawa et al., 1990; Root et al., 2000; Sieczkarski et al., 2003) and furthermore, there is evidence that PKC activity is involved in the regulation of epithelial sodium channels (Booth and Stockand, 2003; Kunzelmann et al., 2000; Stockand et al., 2000; Yamagata et al., 2005). For further investigation into their effects on influenza virus growth, we therefore focused on those compounds that target either sodium ion transport or PKC.

Table 2
Functional catergories of the hit compounds with inhibitory activity

Functional category	Number of compounds	% of total
DNA interfering compounds	20	23.8
Antibiotics/antifungals/antiparasitics	18	21.4
Kinase inhibitors	11	13.1
Cardioactive glycosides	7	8.3
Cell redox metabolism interfering compounds	4	4.8
Other compounds	24	28.5
Total number of inhibitory compounds	84	100

3.3. Inhibition of influenza A and B virus replication by a sodium channel opener and a PKC inhibitor

The high-throughput assay revealed that the sodium channel opener, SDZ-201106 and the PKC inhibitor, rottlerin are potential influenza virus inhibitors. In order to confirm that these findings were specific, we first determined the cytotoxicity profiles of these compounds. A549 cells were seeded into 96-well plates and treated with increasing concentrations of SDZ-201106 or rottlerin for 24 h before performing an MTS assay to determine cell viability. The CC_{50} (concentration of 50% cytotoxicity) of SDZ-201106 was determined to be 29 μM and concentrations up to 12.5 μM were found to be non-toxic. The CC_{50} of rottlerin was determined to be 18.2 μM and concentrations up to 1.28 μM did not decrease cell viability. All further experiments with rottlerin were performed using a maximum concentration of 1.25 μM. To determine the IC_{50} (half maximal inhibitory concentration) for both inhibitors, A549 cells were infected for 24 h with influenza A/WSN/33 virus at an MOI of 1 in the presence of increasing compound concentrations. Viral titers were determined by plaque assay. The IC_{50} for SDZ-201106 was determined to be 4.1 μM. This results in a selective index ($SI = CC_{50}/IC_{50}$) of 7, which classifies this sodium channel opener as a weak inhibitor. The IC_{50} for rottlerin was determined to be 465 nM. With an SI of 39, this PKC inhibitor is considered to be a moderate inhibitor. Table 3 summarizes the CC_{50}, IC_{50} and SI values for both inhibitors. Subsequently the compounds were tested at their

Table 3
Potency of the inhibitors against influenza A virus in A549 cells

	CC_{50} (μM)[a]	IC_{50} (μM)[b]	SI (CC_{50}/IC_{50})[c]
SDZ-201106	29	4.1	7
Rottlerin	18.2	0.46	39

[a] CC_{50}—compound concentration of 50% cytotoxicity.
[b] IC_{50}—compound concentration of 50% inhibition of viral replication.
[c] SI—selective index.

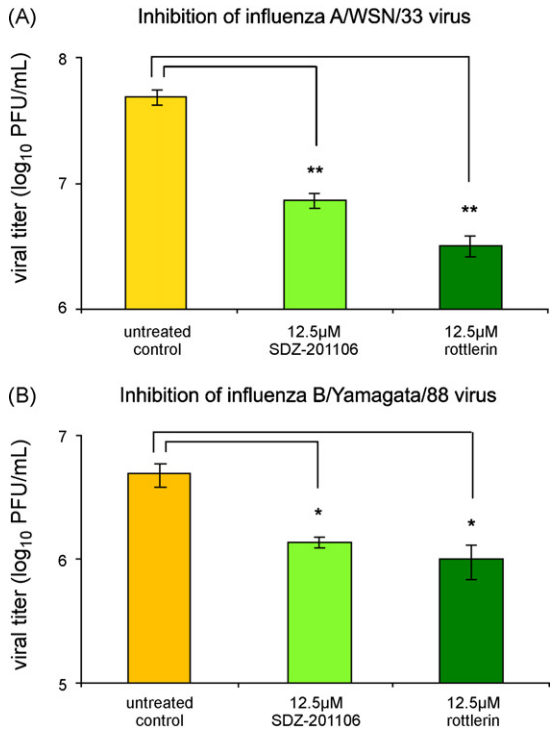

Fig. 2. Inhibition of influenza A and B viruses by a Na^+-channel opener and a PKC inhibitor. A549 cells were infected with either (A) A/WSN/33 (MOI = 1) or (B) B/Yamagata/88 (MOI = 5) in the presence of 12.5 μM SDZ-201106 (Na^+-channel opener) or 1.25 μM rottlerin (PKC inhibitor). Viral titers were determined 24 h post-infection by plaque assays in MDCK cells. The assay was performed in triplicate and is presented as the mean ± standard deviation. Student's *t*-test: $^*P \leq 0.05$; $^{**}P \leq 0.01$.

highest, non-toxic concentrations for their inhibitory activity against both influenza A/WSN/33 virus and influenza B/Yamagata/88 virus (Fig. 2). In the presence of 12.5 μM SDZ-201106 there is an 85% reduction in titers of influenza A/WSN/33 virus compared to the untreated control and the growth of influenza B/Yamagata/88 virus is reduced by 72%. Compared to the untreated controls, titers of influenza A/WSN/33 virus and influenza B/Yamagata/88 virus are significantly reduced by 93 and 80%, respectively, in the presence of 1.25 μM rottlerin.

3.4. Enhancement of influenza A and B virus replication by sodium channel inhibitors and PKC activators

The identification of enhancers is performed best under multicycle replication conditions. This is a limitation of our HTS assay which is performed with a high multiplicity infection and therefore probably only allows for the detection of strong enhancers. Furthermore compounds with enhancing activity may be marked as false negatives if the concentration at which the screen is performed is cytotoxic. Although we screened the PKC activator PMA we did not identify it as an enhancer, probably because the concentration of PMA used in the screen (~6 μM) was toxic. Nevertheless, we decided to investigate the effects of PMA and another PKC activator, mezerein, due to the link between PKC activity and sodium channel regulation and the fact that PKC inhibitors can down-regulate influenza virus growth. For the sodium channel inhibitors, in addition to phenamil, which was identified in the screen as a potential enhancer, we also examined the effects of a related sodium channel inhibitor, dichlorobenzamil. A549 cells were infected with influenza A/WSN/33 virus at a low multiplicity in the presence of increasing concentrations of each compound in order to find the most effective concentration (data not shown). Multicycle growth assays for influenza A/WSN/33 virus were then performed in the presence of 400 nM 2′,4′-dichlorobenzamil, 10 μM phenamil, 250 nM PMA or 250 nM mezerein (Fig. 3). The growth of influenza A/WSN/33 virus is significantly enhanced in the presence of the sodium channel inhibitors. Compared to the untreated control, the viral titer is increased 103-fold in the presence of 400 nM 2′,4′-dichlorobenzamil and 16-fold in the presence of 10 μM phenamil at 48 h post-infection (Fig. 3A). In the presence of the PKC activators, the titers of influenza A/WSN/33 virus increase 17-fold with 250 nM mezerein and 12-fold with 250 nM PMA, compared to untreated cells at 48 h post-infection (Fig. 3B). We also examined the enhancing effects of these compounds on the replication of influenza B/Yamagata/88 virus (Fig. 4). In the presence of 400 nM 2′,4′-dichlorobenzamil, the viral titer increased fourfold at 48 h post-infection compared to untreated cells. With mezerein, the viral growth enhancement is seen much earlier with an eightfold increase at 12 and 24 h post-infection. Therefore the growth of both influenza A and B viruses can be enhanced by the addition of sodium channel inhibitors and PKC activators.

We also examined whether the growth-enhancing effects of these compounds could be observed with human isolates of influenza virus that have not been adapted to cell culture. For this purpose we compared the growth of influenza A/Moscow/10/99 and A/Wyoming/03/2003 viruses in the absence and presence of mezerein and 2′,4′-dichlorobenzamil. Both of these viruses grow poorly in A549

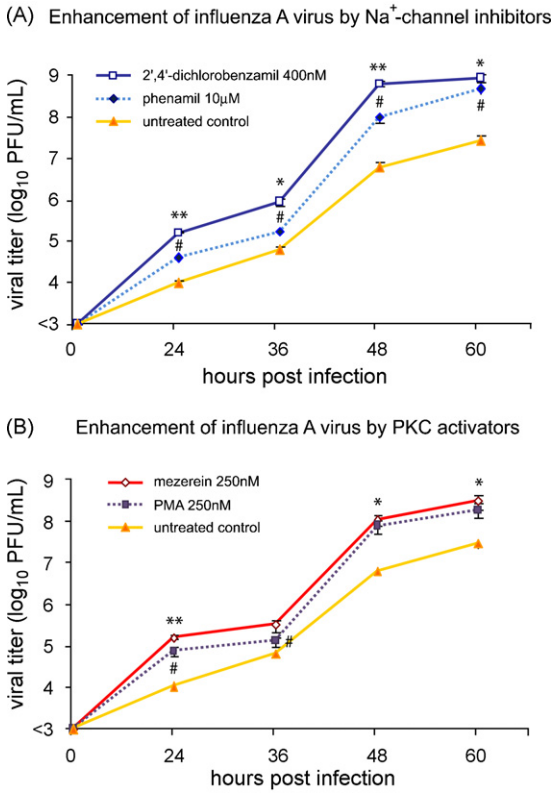

Fig. 3. Enhancement of influenza A virus replication by Na^+-channel inhibitors and PKC activators. A549 cells were infected with influenza A/WSN/33 virus (MOI = 0.001) in the presence of (A) Na^+-channel inhibitors (400 nM 2′,4′-dichlorobenzamil and 10 μM phenamil) and (B) PKC activators (250 nM mezerein and 250 nM PMA). Viral titers were measured at 24, 36, 48 and 60 h post-infection by plaque assay in MDCK cells. The assay was performed in triplicate and is presented as the mean ± standard deviation. Student's *t*-test: $^{*,\#}P \leq 0.05$; $^{**,\#\#}P \leq 0.01$ (*refers to data points for 2′,4′-dichlorobenzamil (A) and for mezerein (B), $^\#$refers to data points for phenamil (A) and PMA (B)).

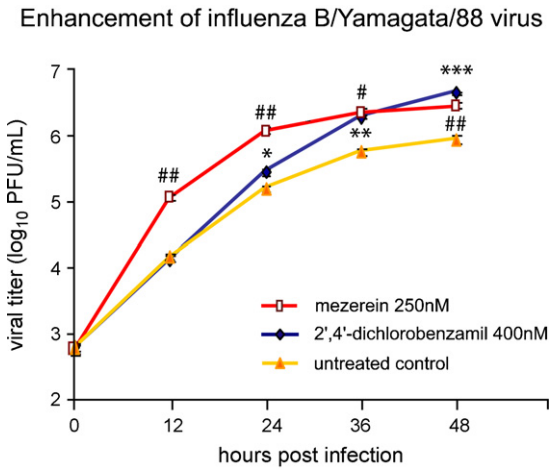

Fig. 4. Enhancement of influenza B virus replication by a Na^+-channel inhibitor and a PKC activator. A549 cells were infected with influenza B/Yamagata/88 virus (MOI = 0.1) in the presence of 400 nM 2′,4′-dichlorobenzamil (Na^+-channel inhibitor) and 250 nM mezerein (PKC activator). Viral titers were measured at 0, 12, 24, 36 and 48 h post-infection by plaque assay in MDCK cells. The assay was performed in triplicate and is presented as the mean ± standard deviation. Student's *t*-test: $^{*,\#}P \leq 0.05$; $^{**,\#\#}P \leq 0.01$; $^{***}P \leq 0.001$ (*refers to data points for 2′,4′-dichlorobenzamil and #refers to data points for mezerein).

cells but we observed a significant increase in the maximum viral titers in the presence of mezerein and 2′,4′-dichlorobenzamil compared to untreated cells (Fig. 5A and B). This suggests that the ability of these compounds to boost virus replication is a property that extends to many different influenza virus strains, which will be beneficial if used for production of influenza vaccines that change every year. For the current egg-grown vaccines, the seed strains for influenza A viruses are 6:2 reassortant viruses that contain the HA and NA genes of the vaccine virus in the background of influenza A/PR/8/34 virus. This is done to obtain high titers in eggs and to avoid the need to adapt each new virus strain. The same strategy is used for the H5N1 influenza vaccine that has been approved by the FDA, with the addition that the multibasic cleavage site present in the HA (which is associated with high pathogenicity in chickens) has been removed (Subbarao et al., 2003; Treanor et al., 2006). We examined the growth properties of this H5N1/PR8 vaccine virus in A549 cells that had been treated with 2′,4′-dichlorobenzamil and found that we could significantly increase the titers by ~20-fold compared to in untreated cells (Fig. 5C). We also observed a significant increase in viral titers in the presence of 2′,4′-dichlorobenzamil and mezerein (11-fold and 15-fold, respectively) when influenza A/WSN/33 virus was grown in Vero cells which is one of the approved cell lines for vaccine production (data not shown). As these compounds are acting on cellular pathways, differences between cell types will likely be observed and optimization or compound modification may be required to achieve the best possible effects.

To address the question of whether there is a correlation between the viral inhibitory or enhancing activities of these compounds and their ability to induce or inhibit apoptosis, we monitored the activity of caspase-3, an indicator of apoptosis induction. To mimic the condition of the cells at the time of infection, A549 cells were incubated with the compounds for 6 h and staurosporin (5 μM) was used as a positive control to induce apoptosis. Phenamil (which enhances influenza virus growth) showed a very slight induction (1.3-fold) of apoptosis during this time period, whereas all the other compounds did not display any significant increases or decreases in fluorescence compared to the untreated cells, indicating the absence of pro-apoptotic or anti-apoptotic activity (data not shown).

During the development stage, the activities of drug candidates can often be modulated by making small changes to the structure of the compound. We compared the effects of 2′,4′-dichlorobenzamil versus 3′,4′-dichlorobenzamil for their ability to enhance influenza virus growth (Fig. 6). Both compounds can boost influenza virus replication above that obtained with the untreated control, but the simple change of a chloride from position 2 in the benzyl group to position 3 makes it 10 times less efficient.

3.5. Inhibition of RNA viruses by sodium potassium ATPase pump inhibitors

The Na^+/K^+/ATPase pump inhibitors, ouabain, lanatoside C, strophanthidin and digoxin were all identified as potential influenza virus inhibitors in the HTS. These cardioactive glycosides, which are used in the treatment of congestive heart failure and cardiac arrhythmia, have also been shown to inhibit the replication of herpes simplex virus (HSV) (Dodson et al., 2007), vaccinia virus (Deng et al., 2007), murine leukemia virus (MuLV) (Tomita and Kuwata, 1978) and Sendai virus (Nagai et al., 1972). To confirm our initial findings, we examined the ability of ouabain and lanatoside C to inhibit influenza virus replication. The CC_{50} for ouabain and lanatoside C on A549 cells was determined to be 47 and 210 nM, respectively (data not shown) and for the viral replication assays

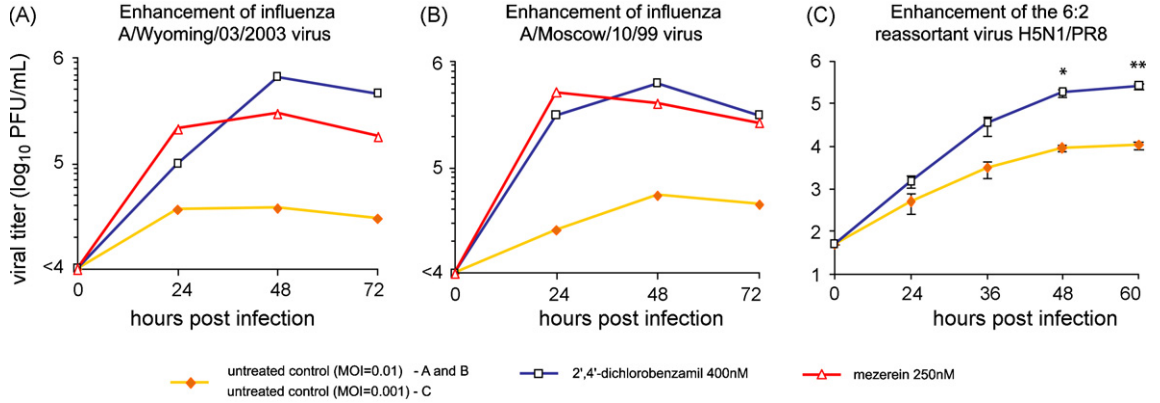

Fig. 5. Enhanced growth of human isolates of influenza virus and a reassortant H5N1 influenza vaccine strain. A549 cells were infected with (A) A/Wyoming/03/2003 (MOI = 0.01), (B) A/Moscow/10/99 (MOI = 0.01) and (C) 6:2 reassortant H5N1/PR8 (MOI = 0.001) in the presence of (A–C) 400 nM 2′,4′-dichlorobenzamil or (A and B) 250 nM mezerein. Viral titers were measured at the indicated time points post-infection by plaque assay in MDCK cells. (C) The assay was performed in triplicate and is presented as the mean ± standard deviation. Student's *t*-test: $^{*}P \leq 0.05$; $^{**}P \leq 0.01$.

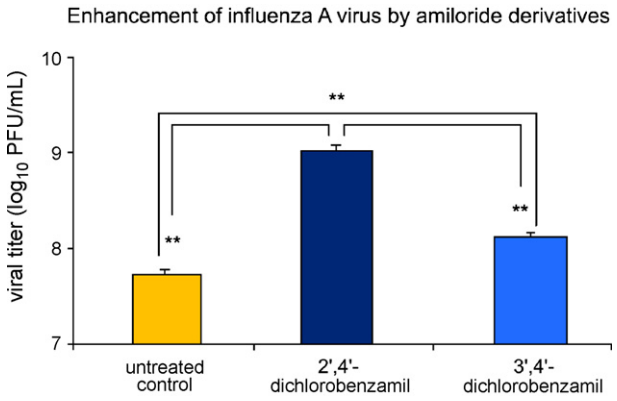

Fig. 6. Influenza A virus growth in response to 2′,4′-dichlorobenzamil versus 3′,4′-dichlorobenzamil. A549 cells were infected with influenza A/WSN/33 virus (MOI = 0.001) in the presence 400 nM of 2′,4′- or 3′,4′-dichlorobenzamil. Viral titers were determined at 48 h post-infection by plaque assay in MDCK cells. The assay was performed in triplicate and is presented as the mean ± standard deviation. Student's *t*-test: $^{**}P \leq 0.01$.

a CC_{20} was used for each compound. A549 cells were infected at an MOI of 1 with influenza A/WSN/33 virus in the presence of 20 nM ouabain or 78 nM lanatoside C and at 24 h post-infection the viral titers were found to be decreased by 99.1% with ouabain treatment and by 95.9% with lanatoside C treatment, compared to the untreated control (Fig. 7A). As these compounds have also been shown to inhibit the replication of other viruses, we investigated whether this effect on influenza viruses extends to other RNA viruses. We found that both ouabain and lanatoside C can significantly inhibit the replication of Newcastle disease virus (NDV) (Fig. 7B) and vesicular stomatitis virus (VSV) (Fig. 7C and D). This result indicates that these Na^+/K^+/ATPase pump inhibitors can inhibit multiple members of both RNA and DNA virus families. To address the possibility that their broad antiviral activity may be related to the induction of interferon, we examined the effects of the compounds on influenza virus replication in Vero cells, which do not produce interferon. We observed a similar level of inhibition as in A549 cells (data not shown), thereby excluding this possibility.

4. Discussion

An influenza pandemic caused by a virus of avian origin could not only have the potential to cause millions of deaths worldwide but could also affect the poultry industry which is crucial for the production of egg-derived vaccines. The primary concerns that need to be addressed in preparation for the next influenza epidemic or pandemic are the abilities of the manufacturers to produce enough doses of vaccine for the susceptible population and the availability of more antiviral compounds that are effective at preventing influenza virus infection. In our luciferase-based, high-throughput screen of 2640 compounds with known biological activity, we identified 84 unique compounds with at least 70% reduction in luciferase activity and 4 compounds that increased luciferase activity at least twofold. We reasoned that compounds found to suppress influenza virus replication are potential anti-influenza virus drugs, whereas those that enhance influenza replication could be used as a tool to boost the growth of vaccine viruses in tissue culture. Moreover, because the biological targets of these compounds are known, it also provides clues as to which cellular pathways and components are crucial for influenza virus replication. The use of antiviral drugs that target cellular proteins is also an advantage over the current drugs that target viral proteins and against which resistance is far more likely to develop. Of particular interest was the finding that sodium channel inhibitors and a sodium channel opener had opposing effects on viral replication. By adding 2′,4′-dichlorobenzamil, an amiloride-analogue that inhibits epithelial Na^+ channels (ENaC) and Na^+/Ca^{2+} exchange channels (Kleyman and Cragoe, 1988), we could enhance viral replication of influenza A/WSN/33 virus 103-fold. In direct contrast,

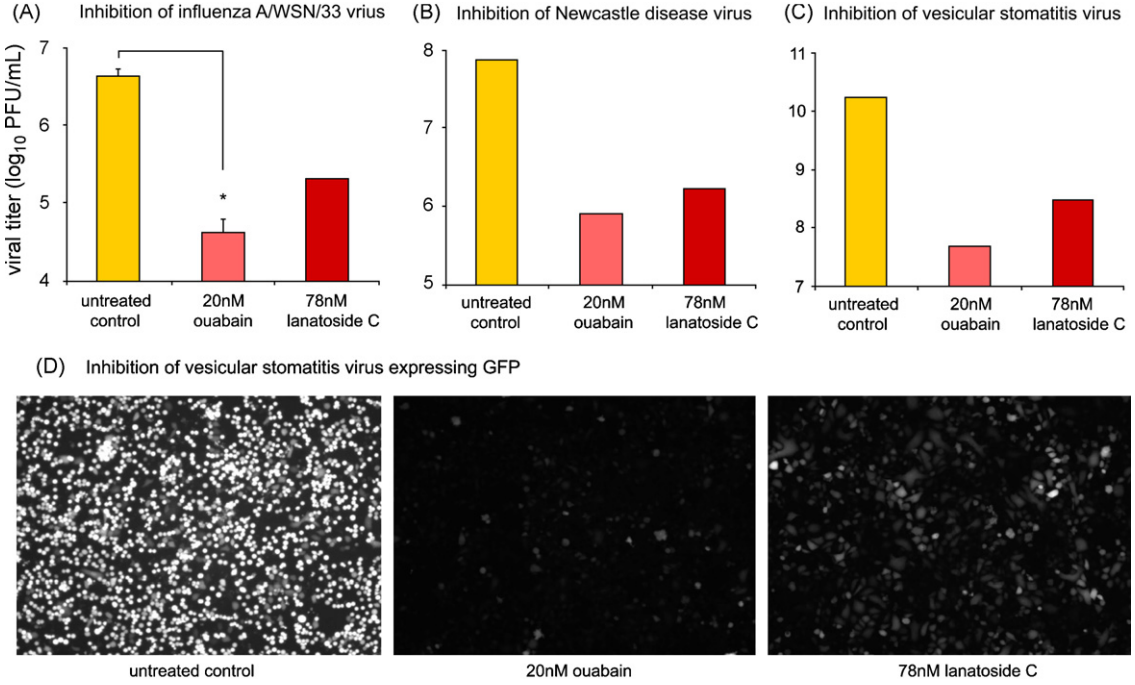

Fig. 7. Inhibition of negative sense RNA viruses by Na^+/K^+/ATPase pump inhibitors. A549 cells were infected with (A) A/WSN/33 (MOI = 1), (B) NDV/B1 (MOI = 1) or (C and D) VSV-GFP (MOI = 1) in the presence of 20 nM ouabain or 78 nM lanatoside C. Viral titers were determined 24 h post-infection by plaque assays (A–C) and the growth of VSV-GFP was in addition visualized by fluorescence microscopy (D). The assay was performed in triplicate for (A) testing ouabain and is presented as the mean ± standard deviation. Student's *t*-test: $^{*}P \leq 0.05$.

using the sodium channel opener SDZ-201106 at non-toxic concentrations we could decrease the titers of influenza A/WSN/33 virus by 85%. The effects of these compounds on influenza B virus replication were more modest (fourfold increase with 2′,4′-dichlorobenzamil and 72% decrease with SDZ-201106) but the overall trend was similar, suggesting that the cellular pathways affected by these compounds are important for both influenza A and B viruses. The fact that influenza virus has been shown to inhibit amiloride-sensitive sodium channels upon infection (Chen et al., 2004; Kunzelmann et al., 2000), suggests that this creates a cellular environment that is conducive to viral replication and one can speculate that the addition of chemical Na^+ channel inhibitors (such as phenamil or 2′,4′-dichlorobenzamil) prior to infection creates pre-optimized conditions and thereby boosts virus replication.

In terms of mechanism of action, these data indicate that an increase in the intracellular Na^+ concentration caused by opening the sodium channels leads to a decline in influenza virus titers, whereas a decreased intracellular Na^+ concentration caused by the inhibition of sodium channels can boost viral replication. However, because the different intracellular ion currents are tightly linked to one another, further investigation is required to evaluate the contribution of Ca^{2+} due to regulation of the Na^+/Ca^{2+} exchange channel. As many of these compounds can target more than one type of channel, it is difficult to attribute their effects on virus replication to a specific channel. That said, several of the amiloride analogues have more potent activity against a specific channel. In our study, 2′,4′-dichlorobenzamil shows slightly stronger pro-viral activity than phenamil. A comparison of their reported potencies against ENaC or the Na^+/Ca^{2+} exchange channel indicates that phenamil is more specific for ENaC while 2′,4′-dichlorobenzamil is more specific for the Na^+/Ca^{2+} exchange channel (Kleyman and Cragoe, 1988). We also tested another amiloride analogue, 3′,4′-dichlorobenzamil, which compared with 2′,4′-dichlorobenzamil, is less effective at enhancing virus replication and has less potent activity against the Na^+/Ca^{2+} exchange channel than 2′,4′-dichlorobenzamil (Kleyman and Cragoe, 1988). Therefore it appears that the ability to enhance the growth of influenza virus correlates with the strength of inhibition of the Na^+/Ca^{2+} exchange channel. It should be noted that in contrast to our findings, amiloride derivatives have been reported to inhibit the replication of several RNA viruses such as human immunodeficiency virus (HIV-1) (Ewart et al., 2004), human rhinovirus (Gazina et al., 2005), coxsackievirus (Harrison et al., 2008) and coronaviruses (Wilson et al., 2006). For HIV-1 (Ewart et al., 2002), coronaviruses, hepatitis C virus (Premkumar et al., 2004) and dengue virus (Premkumar et al., 2005), these compounds have been shown to act by inhibiting the formation of the viral ion channel. There is no evidence that the influenza virus M2 ion channel activity is adversely affected by amilorides and our results rather show that for influenza virus, these compounds have a pro-viral effect.

It is possible that the effects of these compounds on virus growth are not due to changes in ion transport directly but rather due to changes in other cellular activities that are influenced by these compounds. For example pre-treatment of the cells with the compounds prior to infection could be inducing either a pro- or anti-apoptotic state which then influences virus replication. However, as judged by caspase 3 activity, neither the inhibitory nor the enhancing compounds were found to exhibit any anti- or pro-apoptotic effects in the cells at the time of infection. Therefore this is not likely to be an explanation for the observed effects of these compounds on influenza virus replication. Interestingly, it has been shown that influenza virus-mediated inhibition of Na^+ channels requires PKC activity (Chen et al., 2004; Kunzelmann et al., 2000) and also, that there is a stimulation or inhibition of Na^+ transport in the presence of PKC inhibitors or activators, respectively (Kunzelmann et al., 2000). These data correlate with our findings that influenza replication is inhibited in the presence of a PKC inhibitor (rottlerin) but conversely, enhanced in the presence of a PKC activator (PMA or mezerein). Influenza virus entry requires PKC activity and this is believed to be stimulated upon the binding of virus to cellular receptors (Arora and Gasse, 1998; Root et al., 2000; Rott et al., 1995; Sieczkarski et al., 2003). However, to our knowledge this is the first demonstration that a PKC activator can enhance influenza virus growth. The activation of PKC has also been found to down regulate the surface expression of the beta and gamma subunits of ENaC, resulting in a decrease in epithelial Na^+ re-absorption (Booth and Stockand, 2003; Stockand et al., 2000). Therefore it appears that there is a connection between the activation status of PKC and the transport of Na^+ and that influenza virus replication favors the presence of activated PKC and a low intracellular Na^+ concentration.

We also demonstrated that the Na^+/K^+/ATPase pump inhibitors, ouabain and lanatoside C, can inhibit the replication of influenza virus, NDV and VSV, representatives of three different RNA virus families. These data, combined with the results of previous studies that show inhibition of HSV-1, vaccinia virus, MuLV and Sendai virus (Deng et al., 2007; Dodson et al., 2007; Nagai et al., 1972; Tomita and Kuwata, 1978), indicate that these cardioactive glycosides have broad antiviral activity. Through the use of Vero cells, we determined that these compounds do not act by inducing interferon, as the same degree of virus inhibition was observed in these cells as seen in A549 cells. One other common feature shared by these viruses is that they all possess a lipid envelope, however data on ouabain-mediated inhibition of HSV-1 indicates that it acts at a post-entry stage of the viral life cycle (Dodson et al., 2007). The main function of the Na^+/K^+/ATPase pump is to pump Na^+ out of the cell and K^+ into the cell to maintain the cell potential as a driving force for several membrane transport proteins (e.g. the Na^+-glucose symporter, the Na^+-amino acid symporter or the Na^+-hydrogen antiporter). This gradient is also important for the removal of Ca^{2+} by the Na^+/Ca^{2+} exchange channel. Thus in the presence of an inhibitor such as ouabain, there is an increase in the intracellular Na^+ concentration as well as the Ca^{2+} concentration and this is probably similar to the effects of a sodium channel opener such as SDZ-201106, which also inhibits influenza virus replication. Whether or not these compounds share the same mechanism of action for their antiviral activity will be the subject of future research, but this study has shown that influenza viruses are sensitive to changes in intracellular ion concentrations and that this may be a suitable target for novel antiviral drugs. Similarly, knowledge of these crucial factors that are required for optimal virus growth may also be used to boost virus production, as we have demonstrated through the use of PKC activators and Na^+ channel inhibitors. This technology could be used for the production of influenza virus vaccines which will most likely make the transition to in vitro culture systems in the near future. As a demonstration of this potential application, which would require activity for a wide range of influenza viruses, we used the enhancing compounds identified in the study to boost the replication of different influenza A viruses, influenza B virus and the FDA-approved H5N1 vaccine virus strain.

5. Conclusion

The identification of viral inhibitors and enhancers validates the functionality of our high-throughput screen assay and raises the possibility of finding novel antiviral or pro-viral drugs by screening libraries of uncharacterized small molecular weight molecules. In summary, through the use of a high-throughput screen of

biologically active compounds, we have shown that influenza virus growth can be modulated both positively and negatively by chemical manipulation of Na^+ transport or PKC activity. These findings provide insight into the cellular pathways involved in influenza virus replication and for the first time demonstrate the feasibility of using pro-viral drugs to enhance the growth of influenza virus vaccines in tissue culture.

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