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Identification of novel virus inhibitors by influenza A virus specific reporter cell based screening

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ABSTRACT

As influenza viruses have developed resistance towards current drugs, it is urgent to find potential novel antiviral inhibitors. Here we generated an influenza virus reporter cell line in which the luciferase gene was driven by the influenza virus promoter and screened a small compound library (NCI Diversity Set II). Ten compounds were identified to have inhibitory activity against influenza A virus H1N1. Among them, four compounds blocked influenza virus replication through inhibiting the activity of vRNP. The compound NSC 335506 inhibited HA-mediated membrane fusion. It showed the inhibitory activity against H1N1, H9N2 and H5N1 subtype but not H3N2. Our results demonstrated that influenza virus reporter cell is a very useful tool to identify novel inhibitors against influenza A virus.

1. Introduction

Influenza virus is a considerable threat to human health and the world economy. Seasonal influenza virus infection is always associated with significant morbidity and mortality, causing an estimated annual mortality of over 36,000 deaths in the United States alone (Thompson et al., 2003). Highly pathogenic avian H5N1 viruses, despite they can only replicate efficiently in the lower human respiratory tract (de Jong et al., 2006; van Riel et al., 2006) and have not acquired the ability to transmit from person to person, the mortality of humans infected by the viruses due to direct contact with birds has lead to 60% (Abdel-Ghafar et al., 2008; Gambotto et al., 2008). A novel swine-origin H1N1 influenza virus (S-OIV) which emerged in Mexico in April 2009 and spread efficiently among humans caused the first pandemic of the 21st centuries (Dawood et al., 2009). Although the S-OIV infection causes mild symptoms and does not lead to high mortality, new treatment and prevention approach are urgently needed due to the spread efficiency and the potential of reemergence of a more virulent strain.

Currently there are two kinds of drugs that have been used in influenza treatment, the M2 ion channel inhibitors (amantadine and rimantadine) and NA inhibitors (oseltamivir and zanamivir) (De Clercq, 2006). The current clinical use of M2 inhibitors is limited due to severe drug resistance (Bright et al., 2005, 2006; Deyde et al., 2007). Only NA inhibitors are widely used in clinical treatment of seasonal and pandemic influenza infection (Moscona, 2008). However, oseltamivir resistant viruses with NA H275Y mutation were widespread in seasonal H1N1 since 2007 and they were also isolated in the swine-origin pandemic H1N1 (Baz et al., 2009; Dharan et al., 2009; Hurt et al., 2009; Meijer et al., 2009; Moscona, 2009; Weinstock and Zuccotti, 2009). Due to continuing threat of the resistant stain towards public health, it is urgent to identify new anti-influenza drugs to treat both the seasonal and pandemic influenza.

Cytopathic effect assay is the conventional and efficient strategy to search for anti-influenza compounds (Kao et al., 2010; Severson et al., 2008; Su et al., 2010). Alternatively, influenza virus or the host cells can be genetically modified to readout the virus replication level. Modified influenza virus expressing luciferase reporter has been used to screen for host factors required for influenza virus replication (Hao et al., 2008; Konig et al., 2010). While the idea of generating an influenza specific reporter cell line has been carried out (Lutz et al., 2005) and used to screen for host factors involved in virus budding process (Karlas et al., 2010), this approach can be adapted to screen for anti-influenza compounds.

In this report, we generated an influenza virus specific reporter cell line and identified several novel anti-influenza compounds from the NCI compound library, which provide an efficient way to screen for anti-influenza drugs.

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2. Materials and methods

2.1. Compounds, plasmids, cells and viruses

The compounds (total 1364) from the NCI Diversity Set II library (http://dtp.nci.nih.gov/branches/dscb/div2_explanation.html) were dissolved in DMSO at concentration of 10 mM. pREP4-FluA-Luc plasmid was kindly provided by Andrew Pekosz (Johns Hopkins University). Plasmids for expressing non-tagged PB1, PB2, PA and NP (A/WSN/33) were from influenza A virus reverse-genetics system. The cDNA of PB1, PB2, PA and NP of A/California/07/2009 (kindly provided by Jianfang Zhou, Chinese Center for Disease Control and Prevention) were subcloned into pcDNA 3.1. The polymerase I expressing plasmid carrying an influenza virus-like RNA encoding firefly luciferase (vNS-Luc) was kindly provided by Martin Schwemmle, University of Freiburg (Ghanem et al., 2007). HIV luciferase reporter vector pNLLucE-R – which contains the entire HIV gene except Env and Nef and pMT3-HXB2, was kindly provided by Po Tien, Institute of Microbiology, CAS (Su et al., 2008). pEWSN-HA and pCAGGS-NA (Neumann et al., 1999), which encode HA and NA of WSN strain, respectively, were from influenza A virus reverse-genetics system. Madin–Darby canine kidney (MDCK) cells, human embryo kidney 293T cells and HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS, PAA). GHOST-CXCR-4 cells were kindly provided by Prof. Po Tien and maintained in DMEM supplemented with 10% FBS and antibiotics (500 μg/ml G418, 100 μg/ml hygromycin, 1 μg/ml puromycin). Recombinant influenza virus A/WSN/33 was generated as previously described (Zhang et al., 2009). A/Chicken/Liaoning/1/00 (H9N2) was kindly provided by Wenjun Liu, Institute of Microbiology, CAS. A/Brisbane/10/07 (H3N2) was kindly provided by Jianfang Zhou.

2.2. Generation of HeLa-IAV-Luc cell

HeLa cells were transfected with pREP4-FluA-Luc plasmid for 30 h. Then the cells were selected with hygromycin (200 μg/ml) for 2 weeks and maintained in hygromycin (100 μg/ml), named as HeLa-IAV-Luc cell.

2.3. Virus infection and luciferase assay

To test the luciferase activity under different MOI, Hela-IAV-Luc cells in 96-well plate were infected at the indicated MOI for 14 h, the luciferase activity was measured with the Steady-Glo Luciferase substrate (Promega).

To measure the influenza viral polymerase activity, 293T cells in 12-well plate were transfected with plasmids for expressing influenza A virus (A/WSN/33 or A/California/07/2009) PB1, PB2, PA, NP and vNS-Luc (100 ng of each) with pCMV β-gal (50 ng) as an internal control for 24 h. The cell lysates were harvested and subjected to luciferase assay. The luciferase activity was normalized with β-gal activity.

2.4. Screening for anti-influenza compounds

To screening for anti-influenza virus compounds, Hela-IAV-Luc cells were inoculated to 96-well-plate (1.5×10^4 cells/well) for 12 h. Based on the screening optimization an MOI of 5 was chosen for the screening to ensure the screening efficiency. Then the cells were infected with WSN virus at an MOI of 5 and the compounds were added to a final concentration of 50 μM. DMSO was served as a negative control and ribavirin (10 μg/ml) was added to serve as a positive control. Fourteen hours post infection the compounds that showed obvious cell toxicity were excluded by checking under microscope. Then the luciferase activity was measured with Steady-Glo Luciferase substrate (Promega).

2.5. Immunobolttting

In order to observe viral protein expression level, MDCK or HeLa cells were infected with WSN virus at an MOI of 2 and treated with the indicated compounds (50 μM) at different time windows. Ten hours post infection, the cells were lysed with lysis buffer (1% Triton, 150 mM NaCl, 20 mM HEPES pH7.5, 10% Glycerol, 1 mM EDTA) with protease inhibitor cocktail (Roche). The lysates were then applied to immunoblotting with NP, M1and β-actin antibodies. The band intensity of proteins was quantified by using ImageJ (NIH) and normalized against β-actin.

2.6. Plaque assay

MDCK cell monolayers in 35 mm dishes were washed with PBS and 10-fold serial dilutions (10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}) of virus were adsorbed to the cells for 2 h. Unadsorbed virus was removed by washing with PBS, and the cell monolayers were then overlaid with DMEM supplemented with 3% low-melting-point agarose (Amresco) and 2 μg/ml TPCK-treated trypsin (Sigma). Visible plaques were counted and the titers were calculated after 3 days of incubation. All data were expressed as the mean of triplicate samples.

2.7. Pseudovirus inhibition assay

293T cells were transfected with pNLLucE-R-HIV-Luc together with pMT3-HXB2, pEWSN-HA and pCAGGS-NA, A/bar-headed goose/Qinghai/1/05 HA and pCAGGS-NA, or pCMV-VSVG for 36 h. The supernatants were collected, filtered through 0.45 μm filter, and stored at −80 °C as HIV, HA-HIVs, and VSVG-HIV pseudovirus stocks. GHOST cells were infected with HIV pseudovirus and 293T cells were infected with HA-HIVs and VSVG-HIV pseudovirus in the presence of the indicated compounds for 48 h. The cell lysates were harvested and subjected to luciferase assay.

2.8. HA assay

The HA assay was carried out in V-bottom 96-well plates. Serial 2-fold dilutions of virus samples were mixed with an equal volume of 1% (v/v) suspension of chicken erythrocytes with the compound (50 μM) or DMSO and incubated for 30 min. Wells containing an adherent, homogeneous layer of erythrocytes were scored as positive.

2.9. Polykaryon assay

Sub-confluent HeLa cells plated in 6-well-plate were transfected with 1 μg of pEWSN-HA for 30 h. The cells were treated with the indicated compound for 15 min and then incubated with fusion buffer (PBS, pH 5.0) in the presence of the compound at 37 °C for 3 min. Then the cells were cultured in the fresh medium for 4 h and fixed with 4% paraformaldehyde followed by staining with crystal violet (Sigma). The formation of polykaryon was observed under microscope.

2.10. Statistical analysis

All the experiments were repeated at least three times and the raw data was taken to do statistical analysis with Microsoft Excel. The statistical analysis was done using two-tailed Student's *t*-test.

Statistics with p value < 0.05 were considered significant and p value < 0.01 was considered very significant.

3. Results

3.1. Generation of influenza virus reporter cell line

In order to generate the influenza virus reporter cell line, HeLa cells were transfected with pREP4-FluA-Luc plasmid and selected with hygromycin. The HeLa cells carrying pREP4-FluA-Luc were named as HeLa-IAV-Luc as described in material and methods. pREP4-FluA-Luc contains firefly luciferase flanked by A/WSN/33 NP segment UTR which was driven by RNA polymerase I promoter. The luciferase will be transcribed and expressed in the presence of viral polymerase. Therefore the activity of vRNP could be monitored by measuring luciferase activity. As shown in Fig. 1A, when HeLa-IAV-Luc cells were infected with WSN virus, luciferase activity reached a high value while the activity could not be detected without virus infection. As the cells were treated with an anti-viral drug ribavirin, the luciferase activity was nearly reduced to basal level. These results demonstrated that the luciferase activity could represent influenza virus replication level in HeLa-IAV-Luc cells. In order to optimize the MOI for screening, HeLa-IAV-Luc cells were seeded in 96-well-plate (1.5×10^4 cells/well) for 12 h and then infected with virus at different MOI for 14 h. The cell lysates were harvested for luciferase assay. As shown in Fig. 1B, the luciferase activity is increased with the amount of virus when the MOI is under 40. It was almost linear as the MOI is below 5 (Fig. 1C).

3.2. Identification of novel compounds inhibiting influenza virus replication

To screen for compounds inhibiting influenza virus from the NCI Diversity Set II library, Hela-IAV-Luc cells were infected with WSN virus in the presence of the compounds with DMSO as negative control and ribavirin as a positive control. The cell lysates were collected for luciferase assay. Ten of the compounds (Fig. 2) caused the reduction of luciferase activity at least 2 log, indicating that they inhibited influenza virus replication efficiently. To examine

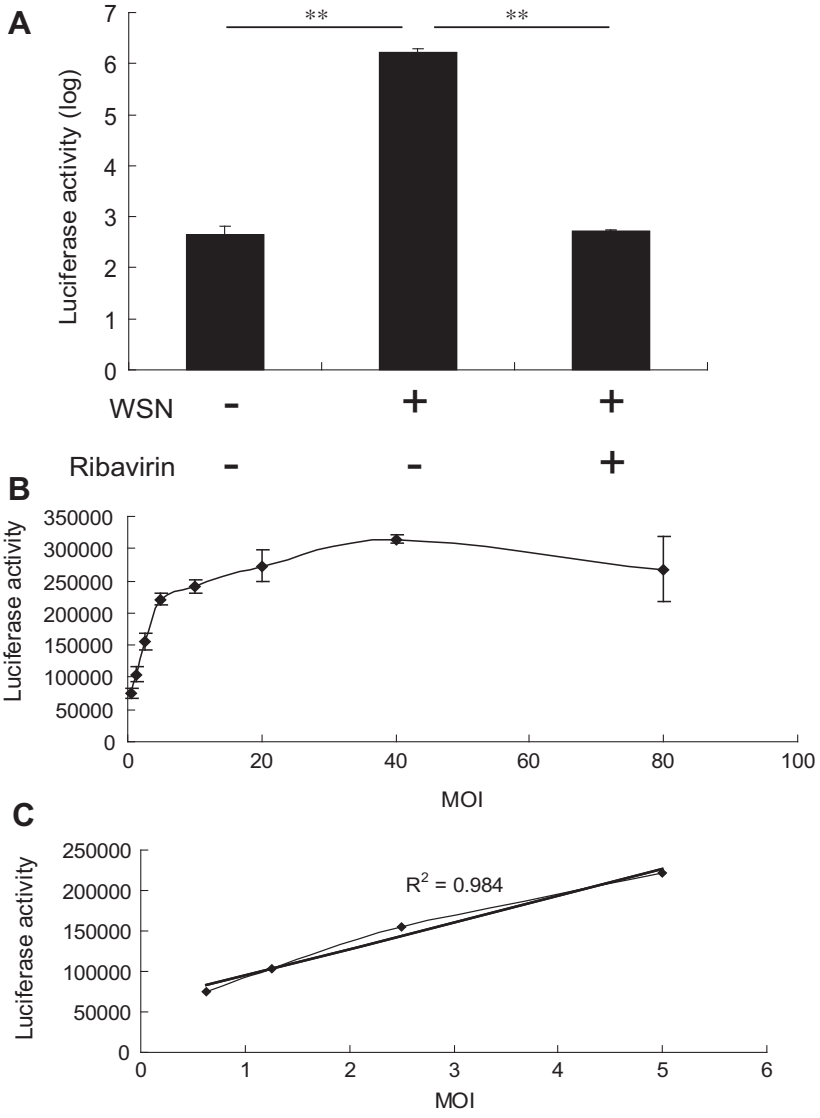

Fig. 1. (A) HeLa-IAV-Luc were infected with influenza virus A/WSN/33 at an MOI of 2, and treated with or without 10 μg/ml of ribavirin for 14 h. The luciferase activity was measured and the results represent the mean ± SD of three independent experiments performed in triplicate. (**$p < 0.01$). (B) HeLa-IAV-Luc cells in 96-well plate were infected with A/WSN/33 at different MOI and the luciferase activity was measured 14 h post infection. (C) The correlation of luciferase activity and the amount of virus (as MOI is under 5) was analyzed.

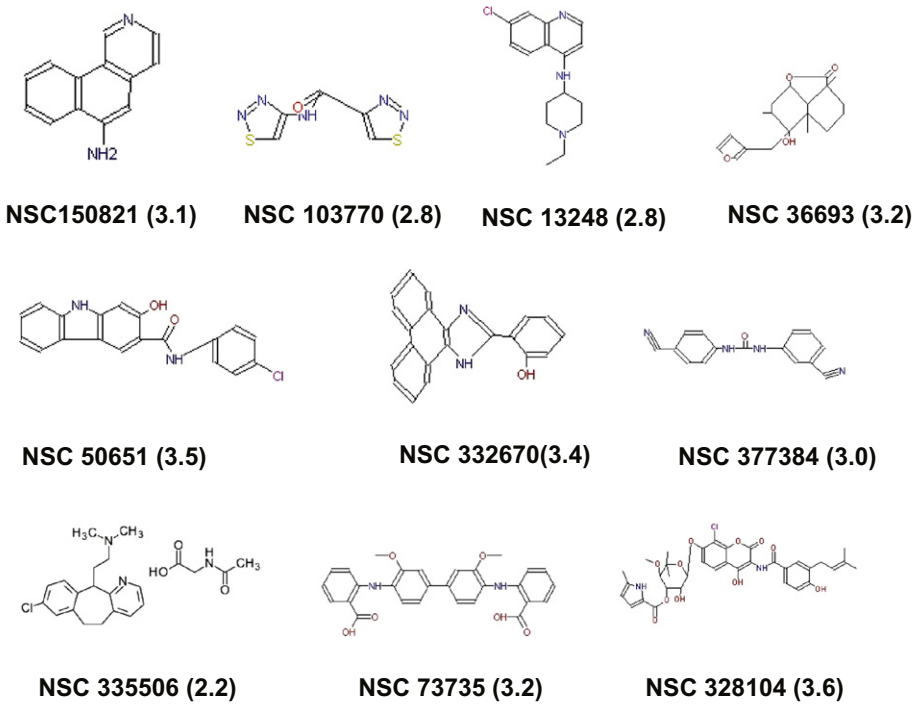

Fig. 2. Chemical structures of compounds with inhibitory activity on influenza virus. The fold of inhibition referred as log according to luciferase assay was shown in the parentheses.

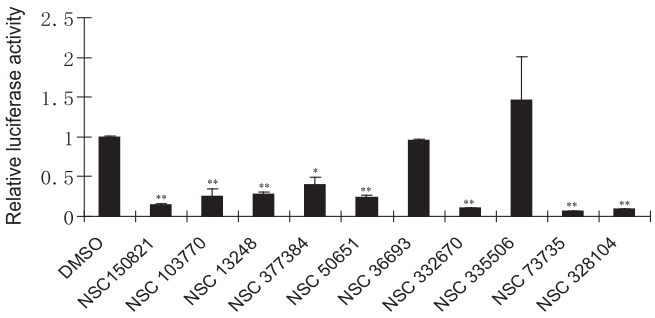

Fig. 3. GHOST cells were infected with HIV pseudovirus and treated with 50 μM of indicated compounds or DMSO as a negative control for 48 h. The cell lysates were harvested for luciferase assay. The relative luciferase activity was calculated against DMSO control (the average absolute value of DMSO control was 48,600). ($^{*}p < 0.05$, $^{**}p < 0.01$).

whether they inhibited influenza virus specifically, their inhibitory effect on HIV pseudovirus were assessed. As shown in Fig. 3, NSC 36693 and NSC 335506 showed no restriction on HIV while the other eight compounds inhibited HIV replication significantly. These data suggested that NSC 335506 and NSC 36693 may specifically inhibit influenza virus.

3.3. NSC 335506 inhibited HA-mediated membrane fusion

A time-of-addition experiment was conducted to analyze the effects of NSC 335506 on the replication of influenza virus. Both the virus particles released into the medium and the viral protein level in the infected cells were examined (Fig. 4A and B). The results indicated that NSC 335506 inhibited virus replication efficiently as it was added within 2 h of infection. It showed no inhibitory activity when it was added 2 h post infection. These results strongly suggested that NSC 335506 interfered with the very early stage of virus infection, probably before viral transcription and replication. Consistently, influenza viral polymerase promoter reporter assay indicated that NSC 335506 didn't inhibit the influenza viral polymerase activity (data not shown).

Then the pseudovirus assay was conducted to address whether NSC 335506 interfered with the HA function. The results showed that NSC 335506 significantly inhibited the infection of HA-HIV but not VSVG-HIV pseudovirus (Fig. 4C), which indicated that it may specifically target HA. To analyze whether NSC 335506 interfered with the binding between HA and receptor, hemagglutination assay was conducted and NSC 335506 did not influence the binding of influenza virus to chicken erythrocytes (data not shown), which excluded its inhibitory effect on the binding between HA and the receptor. To examine whether NSC 335506 inhibited HA mediated membrane fusion activity, we performed the polykaryon assay. The data showed that NSC 335506 greatly reduced the HA mediated HeLa cell fusion (Fig. 4D). Taken together, these results suggested that NSC 335506 may restrict influenza virus replication by preventing pH dependent HA conformational change. In addition, we tested inhibitory effect of NSC 335506 against other subtypes of influenza A virus. The data showed that NSC 335506 dramatically inhibited the expression of NP and M1 proteins in cells infected with A/Chicken/Liaoning/1/00 (H9N2) virus (Fig. 4E) while it showed no inhibitory effect against A/Brisbane/10/07 (H3N2), which was also resistant to ribavirin (Fig. 4F). For safety reasons, we analyzed whether NSC 335506 can inhibit the replication of high pathogenic H5N1 by pseudovirus assay. As shown in Fig. 4G, NSC 335506 could efficiently inhibit the replication of A/bar-headed goose/Qinghai/1/05 HA pseudovirus.

Like NSC 335506, NSC 36693 was effective only when added within 2 h of infection. But NSC 36693 didn't inhibit HA-HIV pseudovirus significantly (data not shown). The target of 36693 needs to be further investigated.

3.4. NSC 73735 inhibited viral polymerase activity

The other 8 compounds with inhibitory activity towards both influenza virus and HIV pseudovirus suggested that they may have

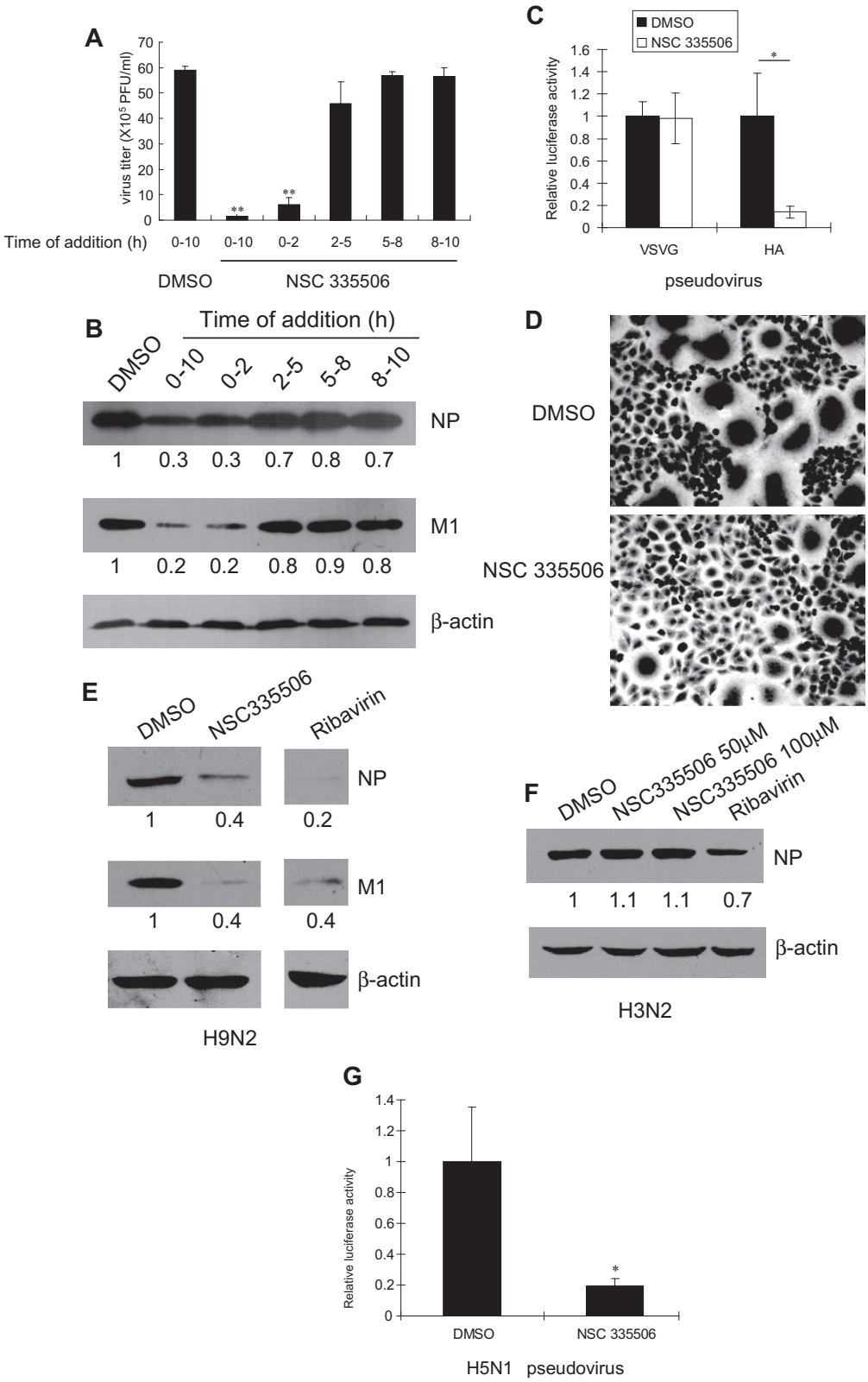

Fig. 4. (A) MDCK cells were infected with WSN virus at an MOI of 2. NSC 335506 was added at 0–10, 0–2, 2–5, 5–8, or 8–10 h to a final concentration of 50 μM. The supernatants were collected for plaque assay. (**$p < 0.01$) (B) The cells lysates were harvested and applied to immunoblot analysis. (C) 293T cells were infected with VSVG-HIV or HA-HIV pseudovirus for 40 h. The cell lysates were harvested for luciferase assay. The relative luciferase activity was calculated against DMSO control. The average absolute value of DMSO control (VSVG and HA) was 13,300 and 14,800, respectively. (*$p < 0.05$) (D) HeLa cells were tranfected with pEWSN-HA for 30 h. Then the cells were incubated with fusion buffer for 3 min at 37 °C with indicating compounds, and then covered with complete medium for 4 h to form polykaryon. Cells were fixed with 4% paraformaldehyde for 10 min and stained with crystal violet. (E, F) HeLa cells were infected with A/Chicken/Liaoning/1/00 (H9N2) (E) or A/Brisbane/10/07 (H3N2) (F) at MOI = 2 for 10 h with the indicated compounds or DMSO as control and cell lysates were applied to immunoblot analysis. In B, E and F, the relative amount of M1 or NP proteins were calculated and shown in their correspond figures. (G) 293T cells were infected with HA (H5N1)-HIV pseudovirus for 48 h and then luciferase activity was measured. The relative luciferase activity was calculated against DMSO control. The average absolute value of DMSO control was 13,900. (*$p < 0.05$).

broad antiviral effect like ribavirin. We took the influenza viral promoter reporter assay to examine whether these compounds could inhibit influenza viral polymerase activity. It turned out that NSC 377384, NSC 50651, NSC 73735 and NSC 328104 inhibited influenza viral polymerase activity by at least 70% (data not shown). NSC 73735 was most effective and the polymerase activity reduction was more than 2 log so it was chosen as an example of the viral polymerase antagonist for further analysis. The data from viral polymerase reporter assay showed that it strongly inhibited the viral polymerase activity of both A/WSN/33 (Fig. 5A) and swine origin H1N1 influenza virus A/California/07/2009 (Fig. 5B). It inhibited viral replication more effectively when added at the time of 0–2 h or 2–5 h, though it can inhibit viral replication as it was added at different infection time points (Fig. 5C). These results indicated that NSC 73735 was a potential candidate for anti-influenza virus drug which targeted influenza viral polymerase to restrict virus propagation.

4. Discussion

The aim of this study was to generate influenza A virus specific reporter cell and test its efficiency in identifying novel influenza virus inhibitors. We manipulated HeLa cells to generate a specific reporter cells to monitor influenza A virus infection. By using the screening system several compounds were hit and they all showed strong inhibitory activity against influenza A virus in vitro, which demonstrated that this method was efficient for anti-influenza compounds screening.

The screening method was based on the hypothesis that viral polymerase activity could reflect virus replication level and our data indicated that the polymerase activity was nearly linear with the original virus amount (Fig. 1B). Therefore the screening system provided an effective influenza A virus titration method *per se*. The whole process could be completed within less than 10 hours instead of waiting 2–3 days for plaque formation. More importantly, our screening method was easily adapted to high throughput screening. It is also noteworthy to mention that the method could cover the whole process of influenza virus infection. In our screening we just screened the antiviral activity prior to virus transcription and replication. In order to screening for compounds disrupting virus budding process, virus supernatants from the compound treated infected cells should be transferred to the reporter cells, then the compounds targeting budding process could be covered. The same strategy has been used in a genome-wide RNAi screening to find human host factors crucial for influenza virus assembly and budding (Karlas et al., 2010).

Our reporter cell based screening have some advantages compared to reporter virus based screening and viral protein staining based screening. The reporter cell based screening is more stable than the reporter virus based screening, since the reporter cells can be propagated easily while reporter virus has to be generated by reverse genetics every time. Our screening method costs less and is easier to process than viral protein staining based screening since the later has to be performed with a large amount of specific antibodies and more steps. However our reporter cell based screening, together with other's reporter virus based screening, and viral protein staining based screening methods all suffer from the side effect of compound toxicity to the cells, which could lead to false positive effect. An additional step was needed to exclude those compounds that were toxic to the cells. In our small-scale screening, we just excluded the toxic compounds by microscope checking. Alternatively some high-throughput screening used cytopathic effect (CPE) assay to screen for anti-influenza compounds and the CPE assay was low cost and efficient for large amount of screening especially primary screening.

Our results demonstrated that HA and viral polymerase were promising targets for antiviral compounds. HA plays a critical role in virus binding to host cells and internalization and it can arise efficient response during infection and vaccination. Other reports also found compounds that target HA, and those compounds also inhibited pH dependent conformation change of HA (Vanderlinden et al., 2010). It could be postulated that antigenetic drift will soon lead to resistant virus. And it was reasonable that HA specific inhibitors could not restrict all influenza virus strain. For example, NSC 335506 could not inhibit seasonal H3N2 virus strain while it

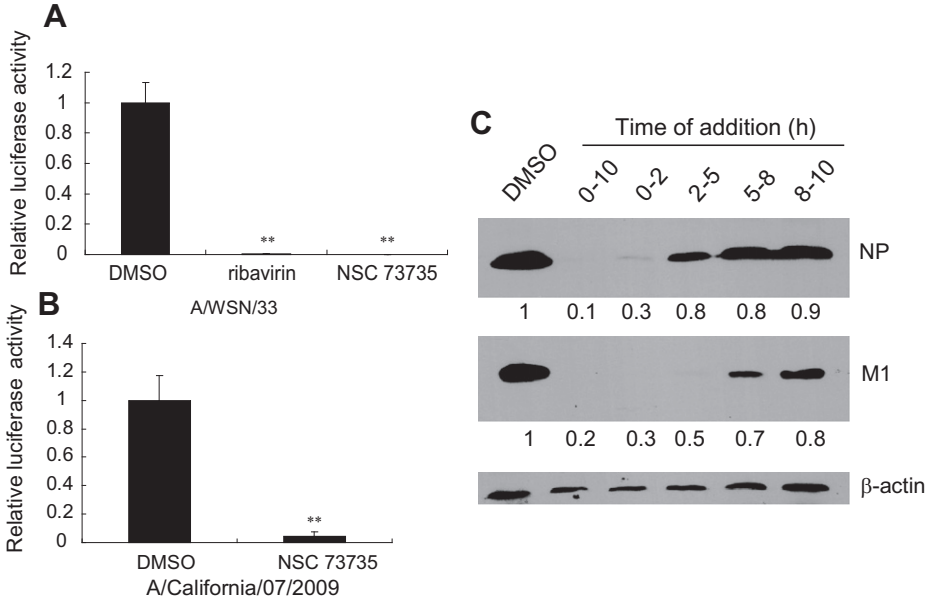

Fig. 5. (A) (B) 293T cells were transfected with PB1, PB2, PA, NP from influenza A virus A/WSN/33 (A) or A/California/07/2009 (B) with vNS-Luc and pCMV β-gal for 24 h, then the compounds were added to a final concentration of 50 μM for 12 h. The cell lysates were harvested for luciferase assay and normalized with β-gal activity. The relative luciferase activity was calculated against DMSO control. The average absolute value of DMSO control was 1,863,900 (A) and 22,971,500 (B). (**$p < 0.01$) (C) HeLa cells were infected with WSN virus at an MOI of 2. NSC 73735 was added at 0–10, 0–2, 2–5, 5–8, 8–10 h to a final concentration of 50 μM. The cell lysates were harvested 10 h post infection and subjected to immunoblot with NP and M1 antibodies. The relative amount of M1 or NP proteins were calculated and shown in the figure.

showed good activity against H1N1 virus. Among the 10 hit compounds, four of which were effective in restricting influenza virus transcription activity, which demonstrated that vRNP was a promising target. Other reports also indicated that NP and polymerase were targeted by antiviral compounds. Nucleozin was found in two independent screening and it triggered the aggregation of NP and inhibited its nuclear accumulation (Kao et al., 2010; Su et al., 2010). PB1 was also a promising target in the same report (Su et al., 2010). PA subunit was targeted by L-742,001 (Nakazawa et al., 2008). T705 was an effective polymerase inhibitor both in vitro and in vivo (Furuta et al., 2002, 2005; Kiso et al., 2010). The reason that polymerase was attracting as new drug target was based on the conception that polymerase was more conserved among different virus strains than membrane protein such as HA and NA. NSC 73735 showed significantly restrictive effect against the new S-OIV, which also strengthened the conserved characterization of influenza virus polymerase.

In summary, we generated an influenza A virus specific reporter cell line based on HeLa cells. Several antiviral compounds were found with this screening system which demonstrated that the method was effective in finding new anti-influenza compounds.

Acknowledgements

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