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A cell-based high-throughput approach to identify inhibitors of influenza A virus



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KEYWORDS

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Abstract Influenza is one of the most common infections threatening public health worldwide and is caused by the influenza virus. Rapid emergence of drug resistance has led to an urgent need to develop new anti-influenza inhibitors. In this study we established a 293T cell line that constitutively synthesizes a virus-based negative strand RNA, which expresses *Gussia* luciferase upon influenza A virus infection. Using this cell line, an assay was developed and optimized to search for inhibitors of influenza virus replication. Biochemical studies and statistical analyses presented herein demonstrate the sensitivity and reproducibility of the assay in a high-throughput format (Z' factor value > 0.8). A pilot screening provides further evidence for validation of the assay. Taken together, this work provides a simple, convenient, and reliable HTS assay to identify compounds with anti-influenza activity.

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1. Introduction

Influenza viruses are members of the family of Orthomyxoviridae and are classified into three types: influenza A, B and C viruses based on antigenic differences. Influenza A virus has caused significant morbidity in the last and in this century¹. To date, influenza-specific drugs include M2 ion channel blockers (amantadine and rimantadine) and neuraminidase inhibitors (oseltamivir and zanamivir), both of which were approved to prevent and treat influenza². However, rapid emergence of drug-resistant mutants restricts their utilization^{3,4}, and therefore leads to an urgent need to develop novel antivirals.

The use of high-throughput screening (HTS) technology for antiviral discovery is a fairly recent endeavor. Utilizing virus-inducible reporter genes to quantitate the infectivity has been achieved for several viruses^{5,6}. A reporter system mimicking the process of transcription/replication of influenza virus was constructed by Lutz et al.⁷. Cells were transfected with a reporter system that expressed *Firefly* luciferase in a response proportional to the infectivity of influenza virus. This luciferase assay is more rapid and simple than the standard plaque assays. Afterward de Vries et al.⁸ adapted this system by substituting *Gaussia* luciferase. *Gaussia* luciferase (hereafter referred to as Gluc), discovered from the marine copepod *Gaussia princeps*, can give high levels of light emission centered at 470 nm by catalyzing the oxidation of the substrate coelenterazine to coelenteramide⁹. This reaction requires no cofactors but O₂, and it generates over 100-fold higher bioluminescent signal intensity when compared with other frequently used luciferase reactions (e.g., *Renilla* or *Firefly* luciferase)^{10,11}. With the help of a secretory signal, Gluc was secreted into the culture medium. Lysis of the cells was not necessary, and the measurement of luciferase activity was simple and time-saving, making Gluc assays a promising technique. Nevertheless, transient transfection of the Gluc reporter system is likely to introduce variation between the experiments.

In this work, a stable cell line derived from 293T cell was established, named 293T-Gluc. It synthesizes the reporter protein Gluc when infected with influenza A virus. We then developed a reporter assay based on this engineered cell line. Antiviral activities of three anti-influenza compounds (ribavirin, amantadine hydrochloride and nucleozin) were assessed by this assay. Results demonstrated the ability of this assay to identify influenza inhibitors. Several parameters (*Z'* factor, CV, *S/B*, *S/N*) utilized to evaluate the quality of HTS (high-throughput screening) assays also validated our approach. These findings suggest that this cell-based assay is a promising tool to identify new anti-influenza drugs.

2. Materials and methods

2.1. Cells and viruses

MDCK and 293T cells were maintained in Dulbecco's modified Eagle's medium with 10% (*v/v*) fetal bovine serum (FBS; Invitrogen). The cells were cultured at 37 °C in a humidified atmosphere of 5% CO₂. Influenza A/WSN/33 (H1N1) was rescued from eight plasmids using cocultured MDCK and 293T cells¹². Influenza A/PR/8/1934 (H1N1), seasonal influenza B/Beijinghaidian/1386/2013 (Victoria) and influenza B/Massachusetts/02/2012 (Yamagata) were propagated in embryonated chicken eggs according to classical virological techniques. Virus titers were determined on

MDCK cells and represented as the median tissue culture infective dose (TCID₅₀), as previously described¹³. These viruses were provided kindly by Dr. Yuelong Shu at China CDC.

2.2. Reagents and plasmids

Coelenterazine-h was purchased from Promega. Nucleozin and TPCK-treated trypsin were obtained from Sigma. The Microsource Library (2000 compounds) was obtained from commercial sources and compounds from this library were dissolved in DMSO at 2 mmol/L. Plasmid pHH-Gluc (a kind gift from Dr. Erik de Vries⁸) was used as template for Gluc reporter system (containing the RNA polymerase I promoter/terminator and influenza A/WSN/33 NP segment UTRs) amplification. The primers used are as follows: forward primer 5'-TATGAATTCGGAAAAACGCCAGC AAC-3' and reverse primer 5'-ATAAGGGCCCCAAAATCTTCTTT-CATCCGC-3'. PCR products were cloned into pLenti6/V5-DEST vector (Invitrogen) generating pLenti6-Gluc.

2.3. Establishing a stable reporter cell line

293T cells were transfected with pLenti6-Gluc using lipofectamine2000 in accordance with the manufacturer's protocol, and then were selected with 10 µg/mL Blastidicin 24 h post-transfection. Under antibiotic selective pressure several clonal colonies were obtained and tested for luciferase expression. One clonal cell line demonstrated high-level expression of luciferase; this cell line was named 293T-Gluc and was used for subsequent experiments.

2.4. Gluc reporter assay

293T-Gluc cells were cultured to 90% confluence, released with 0.25% trypsin-EDTA and resuspended in DMEM with 10% FBS at 7×10^5 cells/mL. Cells were then seeded in the wells of 96-well plate at 100 µL/well. After an overnight incubation at 37 °C and 5% CO₂, cells were infected with the indicated influenza viruses, which were contained in 10 µL serum-free DMEM culture medium. Post-infection medium also contained TPCK-treated trypsin with the final concentration of 1 µg/mL.

In specificity studies, infection was allowed to proceed for 24 h at 35 °C. For influenza A/PR/8/1934, influenza B/Beijinghaidian/1386/2013 (Victoria) and influenza B/Massachusetts/02/2012 (Yamagata), infections were carried out at a multiplicity of 0.1, 1 and 10, respectively, whereas a multiplicity of 0.1 and 1 was used when testing the response of influenza A/WSN/33 virus to Gluc reporter assay.

The dynamic signal range of the Gluc reporter assay was assessed by infecting 293T-Gluc cells with varying quantities of influenza A/WSN/33 virus (MOI of 0.0001, 0.001, 0.01, 0.1 and 1) and determining Gluc activity at various times post-infection (12, 24, 36 and 48 h post-infection).

For evaluation assay of antivirals and high-throughput screening, 1 µL of each tested compound was added to cells and incubated for 2 h prior to infection, after which cells were infected with influenza A/WSN/33 virus at an MOI of 0.05. After a further incubation for 24 h at 37 °C, the cell supernatant was collected and measured for Gluc activity. In each 96-well plate ribavirin and DMSO were used as positive and negative controls, respectively. The inhibition rate of the tested compounds was calculated with the following equation, where RLU indicates relative light unit:

$$\text{Inhibition rate} = \frac{(\text{RLU}_{\text{infected cells}} - \text{RLU}_{\text{tested compound}}) / (\text{RLU}_{\text{infected cells}} - \text{RLU}_{\text{mock-infected cells}})}{\times 100\%}$$

2.5. Luciferase activity assay

A stock of coelenterazine-h was prepared in ethanol to a concentration of 1.022 mmol/L and stored at -20°C . Before assay the stock was diluted into PBS by a factor of 60, which was used as working solution for luminescence. The working solution was held for 30 min at room temperature in the dark to stabilize it¹⁴. For luminescence analysis, 60 μL of working solution was added to 10 μL of cell-free conditioned medium and measured for 0.5 s using a 96-well microplate luminometer with automated substrate injection (Berthold Centro LB 960). The results were expressed in relative light units (RLUs).

3. Results and discussions

3.1. Specificity for influenza A virus

In construction of pLenti6-Gluc, the open reading frame (ORF) of the influenza A/WSN/33 NP protein was replaced by *Gaussia* luciferase and this RNA segment was inserted in a human RNA polymerase I promoter/terminator cassette in the reverse orientation and complementary sense. As a stable cell line, 293T-Gluc cells that contain pLenti6-Gluc synthesize a viral negative strand RNA constitutively, which expresses Gluc under control of the untranslated regions (UTRs) of the influenza A/WSN/33 NP segment (Fig. 1). We assessed the response of this reporter cell line to different strains of influenza virus. As shown in Fig. 2, the infection with influenza A viruses induced much higher luciferase activity than did the two strains of influenza B virus. The observation that the 293T-Gluc cell line is more responsive to influenza A virus may reflect species specificity of UTR region for the corresponding RdRp, which is consistent with a previous observation⁷. Among all the virus strains tested, the A/WSN/33 strain exhibited the most potent ability to induce luciferase activity when the cells were infected at identical MOI. Therefore, influenza A/WSN/33 virus was used for all subsequent experiments.

3.2. Dynamic signal range of the 293T-Gluc reporter assay

To further optimize the assay a signal range was determined using serial 10-fold dilutions of influenza A/WSN/33 virus. As shown

in Fig. 3, infection at MOIs of 0.1 or higher induced a Gluc signal at 12 h post-infection, and signal intensity significantly increased thereafter and reached a peak at 36 h post-infection. When infected with virus at MOIs of 0.001 or lower, cells failed to generate a detectable Gluc signal. It should be noted that viral infection at an MOI of 1 induced significant CPE in 293T-Gluc cells like MDCK, while the cells kept a normal morphology during the 96 h time period of post-infection when the MOI was less than 0.1 (data not shown). This may explain the observation that the Gluc signal from the cells infected at an MOI of 1 was less than that of MOI of 0.1 (Fig. 3), most likely due to reduced cell viability. Based on this result, the amount of virus used and the measurement time point were set at an MOI of 0.05 and 24 h post-infection, respectively.

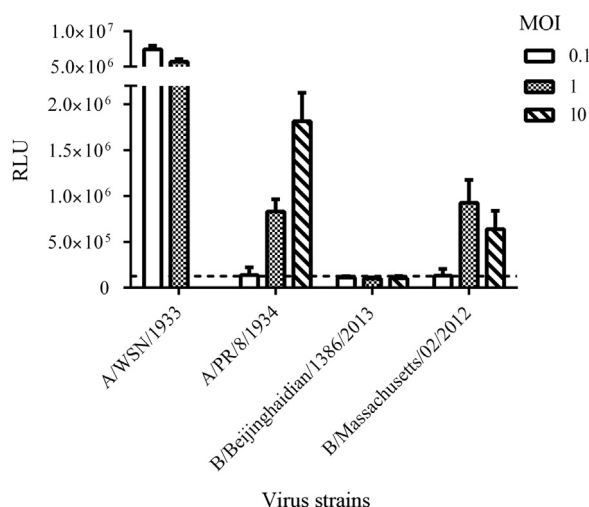


Figure 2 Specificity of Gluc reporter assay. Experiments were conducted as described in Materials and Methods. 293T-Gluc cells were seeded and infected with indicated viruses at various doses for 24 h. Infection with A/WSN/33 at an MOI of 10 was not tested. Gluc activity of mock-infected cells was regarded as background and indicated by a dashed line. Data were shown as average \pm standard deviation ($n=3$).

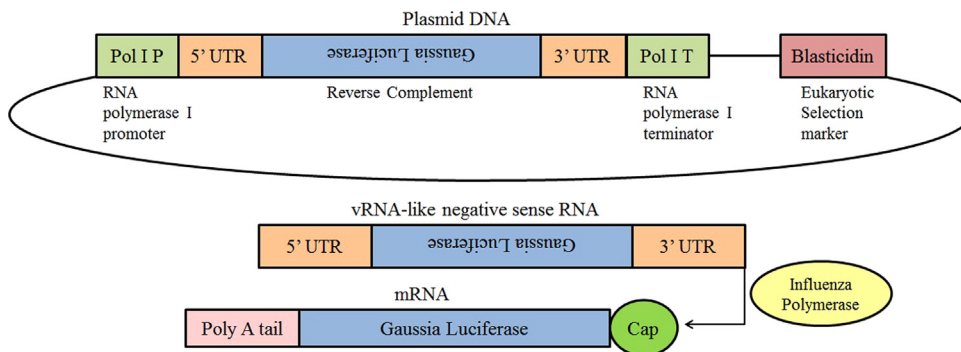


Figure 1 Schematic of the influenza virus Gluc reporter construct. The *Gaussia* luciferase open reading frame was inserted in the reverse orientation and complementary sense between the influenza virus untranslated regions (UTRs) which serve as the viral promoter. This cassette is flanked by a human RNA polymerase I (Pol I) promoter and terminator. The transcribed RNA is a vRNA-like negative sense RNA, which mimics an influenza virus genome segment. Upon infection, the influenza virus polymerase recognizes the UTRs and *Gaussia* luciferase is transcribed and expressed. A eukaryotic selection marker of Blasticidin was used to establish stable cell line 293T-Gluc.

3.3. Evaluation of anti-influenza virus agents

To ascertain assay sensitivity, three reference compounds, including ribavirin, amantadine hydrochloride and nucleozin, were examined in our assay. Ribavirin is used against several DNA and RNA viruses including influenza viruses^{15,16}. It is converted to ribavirin monophosphate, which disturbs GTP synthesis, leading to inhibition of RNA synthesis in cells¹⁷. Amantadine hydrochloride, approved for the treatment of influenza infections, acts in the early phase of the life cycle of influenza A virus to target the M2 ion channel^{18,19}. Nucleozin is a novel inhibitor with anti-influenza activity. It impedes influenza A virus replication by triggering the aggregation of NP and inhibits its nuclear accumulation²⁰. We used the Gluc reporter assay to evaluate these antivirals in dose-response experiments, measuring IC₅₀ (Fig. 4). All the compounds showed the ability to reduce the luciferase signal driven by influenza A/WSN/33 virus. IC₅₀ values determined from our experiments were comparable to data previously reported (Table 1), demonstrating the applicability of the Gluc reporter assay for screening influenza inhibitors. Furthermore, compared with the conventional plaque reduction assay (PRA)²⁴, the method herein is more rapid and simple for the determination of IC₅₀ values. At least 48 h were needed before the number of plaques could be calculated, whereas our assay can give results in 24 h after infection. Moreover, this unbiased and automated method of

infectivity quantitation avoids tedious plaque counting, making it an ideal approach.

3.4. High-throughput screening

The above results suggest the potential to use the Gluc reporter assay to screen compounds against influenza A virus. To ensure that the assay can be used in high-throughput screening, we assessed its accuracy by using several statistical parameters²⁵ (Table 2). *Z'* factor is a simple statistical characteristic for HTS assay. It can be used to evaluate the performance of an assay. A robust assay should have a *Z'* factor > 0.5 and the larger the value of *Z'* factor, the better the data quality of the assay²⁶. The *Z'* factor in our experiments was 0.84 (*n* = 48), which is considered to be excellent for use in HTS (Fig. 5). Normally, infected cells treated with a positive anti-influenza compound are used as positive control for calculating the *Z'* factor. Nevertheless, according to the assay validation guidance for agonist assays (<http://www.ncbi.nlm.nih.gov/books/NBK83783/>), the background signal is also suitable for use as a 'minimum signal'. Since viral infection acts as an agonist of luminescent protein, we used mock-infected cells as positive control in the validation analysis. Similarly, other work used mock-infected cells as positive control to calculate the *Z'* factor²⁷. In addition, as shown in Fig. 4, most of the anti-influenza compounds tested were able to achieve full inhibition in our assay. Moreover, the minimum signals derived from infected cells treated with one of the positive anti-influenza compounds were close to that of mock-infected cells.

CV is another parameter used for quality assessment. It reflects signal deviation within an assay and is recommended to be less than

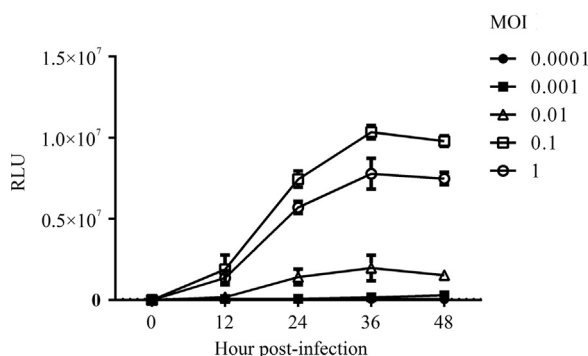


Figure 3 Signal range of the 293T-Gluc reporter assay. 293T-Gluc cells were infected with varying quantities of influenza A/WSN/33 virus. Infections were performed at MOI of 0.0001, 0.001, 0.01, 0.1 and 1. Gluc activities were measured at 4 times points with equal intervals of 12 h and that of mock-infected cells was regarded as background (indicated by a dashed line, which is very approachable to X-axis). Data are shown as average \pm standard deviation (*n* = 3).

Table 1 Inhibitory activity of the compounds against influenza A replication^a.

Compound	IC ₅₀		
	Tested (μmol/L) ^b	Reported (μmol/L)	Ref.
Ribavirin	26.62 \pm 7.15	11.06	21
Amantadine hydrochloride	24.94 \pm 6.10	78.32	22
Nucleozin	0.23 \pm 0.04	0.32	23

^aActivity against influenza A/WSN/33 virus.

^bValues are mean \pm standard deviation from three independent experiments.

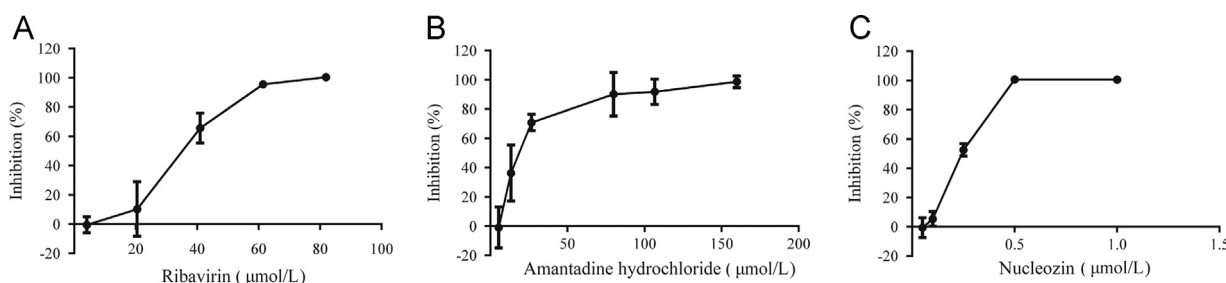


Figure 4 Dose-response relationships of three anti-influenza inhibitors. (A) Ribavirin; (B) amantadine hydrochloride; (C) nucleozin. Tested compounds were dissolved in DMSO at various concentrations. Data from the assay were analyzed and calculated by GraphPad Prism software. Three independent experiments were done, and representative data are shown as average \pm standard deviation (*n* = 3).

Table 2 Summary of statistical parameters to assess the robustness of the HTS assay.

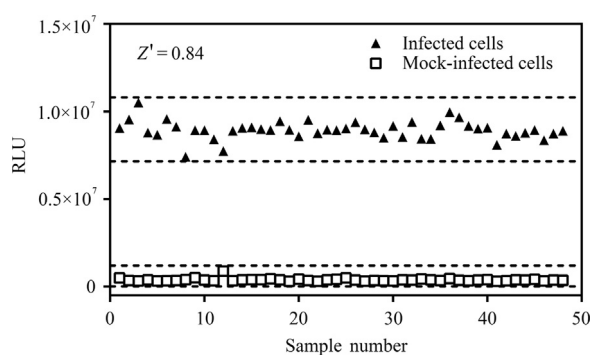
Statistical parameter	Value
Z' ^a	0.84
%CV ^b	5.79
S/B ^c	23.24
S/N ^d	16.29

^a $Z' = 1 - 3(\text{STD}_{\text{infected cells}} + \text{STD}_{\text{mock-infected cells}}) / (\text{MEAN}_{\text{infected cells}} - \text{MEAN}_{\text{mock-infected cells}})$.

^b%CV (coefficient of variation) = $\text{STD}_{\text{infected cells}} / \text{MEAN}_{\text{infected cells}} \times 100\%$.

^c S/B (signal-to-background ratio) = $\text{Mean}_{\text{infected cells}} / \text{Mean}_{\text{mock-infected cells}}$.

^d S/N (signal-to-noise ratio) = $(\text{Mean}_{\text{infected cells}} - \text{Mean}_{\text{mock-infected cells}}) / ((\text{STD}_{\text{infected cells}})^2 + (\text{STD}_{\text{mock-infected cells}})^2)^{1/2}$.

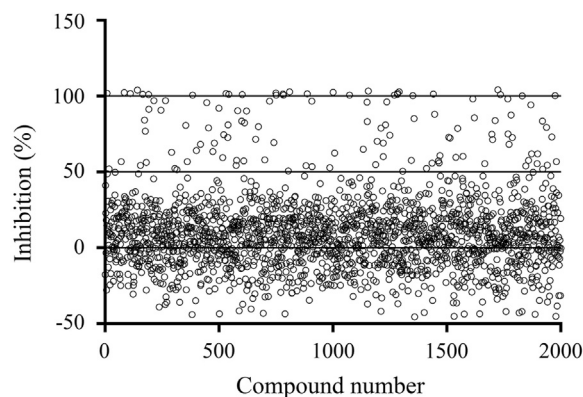
**Figure 5** Determination of Z' factor of the 293T-Gluc report assay. Approximately 7×10^4 293T-Gluc cells were seeded per well of a 96-well tissue culture plate and half were infected with influenza A/WSN/33 virus at an MOI of 0.05. At 24 h post-infection, Gluc activity was measured and Z' factor value was calculated as described.

or equal to 20% (<http://www.ncbi.nlm.nih.gov/books/NBK83783/>). The CV of our assay was 5.79%, which meets the requirements for HTS. In addition, the S/B ratio (23.24) and the S/N ratio (16.29) were comparable to those of other HTS assays reported^{28,29}, proving our assay was suitable for use in a high-throughput screen.

As a proof of concept, we carried out a pilot screening of the Microsource Library as described above and the results are plotted in Fig. 6. In each 96-well plate, ribavirin and DMSO were used as a positive and negative control, respectively. Majority of the 2000 screened compounds did not decrease the luminescence signal by more than 50%. Seventeen showed strong luminescence reduction by 90%–100%, which translates to a hit rate of 0.85%. Compared with assays exploiting the principle of cytopathic effects (CPE) protection³⁰, the approach established here is rapid and simple for anti-influenza inhibitor screening. We anticipate that this approach will facilitate the discovery of compounds with anti-influenza activity and lead to drug development.

4. Conclusions

Influenza virus remains a health threat, and much more work needs to be done to discover new antivirals. In this work we reported a cell-based high-throughput assay to identify inhibitors for influenza virus. The assay used *Gaussia* luciferase as a readout. Several

**Figure 6** Results of the pilot high-throughput screening. Two thousand compounds from Microsource library were automatically added to cells by machine arm and final concentrations were 20 $\mu\text{mol/L}$. Each circle represents the inhibition rate of each compound. Seventeen compounds showed 90%–100% inhibition of luminescence signal with a hit rate of 0.85%.

criteria for HTS were evaluated, including Z' factor and CV. Results obtained validated the robustness of our assay. Furthermore, we used this high-throughput assay to screen 2000 small molecules at 20 $\mu\text{mol/L}$. Seventeen compounds showed 90%–100% inhibition of luminescence signal for a rate of 0.85%. Utilization of this cell-based high-throughput assay will benefit identification of new anti-influenza lead compounds in future work.

Acknowledgments

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