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High-throughput screening of active compounds against human respiratory syncytial virus

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ABSTRACT

Human respiratory syncytial virus (RSV) is one of the predominant pathogens causing lower respiratory tract infection in infants and young children worldwide, whereas there is so far no vaccine or drug against RSV infection for clinical use. In this work, we developed and validated a fluorescence-based high-throughput screening (HTS) assay to identify compounds active against RSV, using RSV-mGFP, a recombinant RSV encoding enhanced green fluorescent protein (EGFP). Thereafter, among 54,800 compounds used for our screen, we obtained 62 compounds active against RSV. Among these hits, azathioprine (AZA) and 6-mercaptopurine (6-MP) were identified as RSV inhibitors with half maximal inhibitory concentration (IC_{50}) values of 6.69 ± 1.41 and 3.13 ± 0.98 μM, respectively. Further experiments revealed that they functioned by targeting virus transcription or/and genome replication. In conclusion, the established HTS assay is suitable to screen anti-RSV compounds, and the screened two hits of AZA and 6-MP, as potential anti-RSV agents targeting RSV genome replication/transcription, are worthy of further investigation on their anti-RSV activity *in vivo*.

1. Introduction

Human respiratory syncytial virus (RSV) is one of the predominant pathogens causing lower respiratory tract infection in infants and young children worldwide. Globally, there were around 1.4 million hospitalizations and about 27,300 deaths caused by RSV in children younger than 6 months in 2015 (Shi et al., 2017). In infants younger than one year, RSV can cause significantly higher mortality than influenza (Mackman et al., 2015), and is the second single pathogen causing the death of babies, next only to malaria (Drysdale et al., 2016; Ngwuta et al., 2015). Currently, despite decades of endeavor to develop vaccine and drug, there is no efficacious and safe vaccine or drug against RSV infection for clinical use. Ribavirin and palivizumab are the merely available drugs for the treatment of RSV infection. However, palivizumab is a costly humanized monoclonal antibody specific for RSV fusion glycoprotein (F), and only used as preventive treatment to pediatric patient population at high-risk (Bond et al., 2015; Noton et al., 2015). Besides, ribavirin has limited clinical use due to severe toxicity and uncertain efficacy (Bond et al., 2015; Perron et al., 2015; Xiong et al., 2013).

Therefore, the development of effective RSV antiviral drugs and the establishment of high-throughput screening (HTS) of anti-RSV treatments are urgently needed (Bonavia et al., 2011; Chung et al., 2013; Fu et al., 2017; Tiong-Yip et al., 2014). Plaque assay is of low cost and has been used to screen packs of compounds (Lundin et al., 2010), but it is time-consuming and relies heavily on the experience of the operator. RSV subgenomic replicon, deficient in the genes for entry and assembly for RSV, does not generate infectious virus particles and thus can meet the biosafety requirement (Tiong-Yip et al., 2014). However, the compounds screened by RSV subgenomic replicon assay can't cover the

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whole RSV replication cycle but only the RSV RNA polymerase activities. As for the minigenome assay, it requires that the cells were transfected by plasmids encoding cDNA copy of an RSV minigenome and then co-infected by RSV or co-transfected four helper plasmids encoding individual components of the viral ribonucleocapsid proteins (RNPs) to provide the viral RNP complex essential for minigenome replication (Fu et al., 2017). The problems related to the plasmid transfection limit its utility for high throughput anti-RSV compound screenings.

Recombinant RSV expressing an enhanced green fluorescent protein (EGFP) was generated more than ten years ago and has been used for some assays involving RSV infection *in vitro* (Hallak et al., 2000). The most important characteristic of such recombinant RSV is its observability by fluorescence microscopy due to the expression of the ectopic gene coding for EGFP in infected cells. Moreover, compared with recombinant RSV carrying luciferase-encoding reporter gene, the fluorescence signal from cells infected by the EGFP-encoding virus does not need any substrate. Therefore, the replication of RSV can be analyzed and quantified in a continuous and non-damage way by fluorescence microscopy. Furthermore, when combined with automated plate reader, it is a convenient tool for high throughput antiviral compound screenings (Kwanten et al., 2013; Xu et al., 2018; Rameix-Welti et al., 2014). However, the optimal parameters for HTS assay need to be explored and determined.

Here, we describe the development, optimization and application of an HTS assay for screening of compounds active against RSV using a recombinant virus expressing mGFP (RSV-mGFP). Following the validation of the established HTS assay by 2 known RSV replication inhibitors, P13 and ribavirin, we screened 2,000 known bioactive compounds and 52,800 synthesized compounds, and identified 62 active compounds that presented ≥50% RSV inhibition, two of which, azathioprine (AZA) and 6-mercaptopurine (6-MP), were identified as RSV inhibitors with half maximal inhibitory concentration (IC_{50}) values of 6.69 ± 1.41 and 3.13 ± 0.98 μM, respectively. It is likely that these compounds function through targeting virus transcription or/and genome replication. Therefore, the established HTS assay provided a practical and robust tool to develop novel RSV treatments.

2. Materials and methods

2.1. Cells and viruses

The recombinant RSV-mGFP was engineered by replacing the mCherry gene (Rameix-Welti et al., 2014) with the mGFP gene. RSV-mGFP and wild type subgroup A RSV Long strain (*wt*RSV, kindly provided by Prof. Y. Qian, Capital Institute of Pediatrics, Beijing, China) were propagated in HEp-2 cells (ATCC, Rockefeller, MD, USA) in DMEM (Gibco BRL, Gaithersburg, MD, USA) supplemented with 2% fetal bovine serum (FBS, Hyclone, Logan, UT, USA), L-glutamine (2 mM), penicillin G (40 U/ml), streptomycin (100 μg/ml) and 0.2% sodium bicarbonate. RSV-mGFP and *wt*RSV were purified by sucrose ultracentrifugation and titrated for infectivity by immunoplaque assay (Jiao et al., 2017), and expressed as plaque-forming units (PFU) per ml. The cell of BHK-T7 is a gift from Prof. W. Y. Zhu, National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing, China.

2.2. Drugs and compounds

Azathioprine (AZA), 6-mercaptopurine (6-MP), ribavirin and P13 were purchased from Shanghai Topscience Company (Shanghai, China). P13, AZA and 6-MP was dissolved in dimethyl sulfoxide (DMSO), and ribavirin was dissolved in deionized water. The compounds from synthesized compound library or commercial compound library were dissolved in DMSO and kept in storage in Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences, Beijing, China.

2.3. Development and optimization of HTS assay

HEp-2 cells were plated in 96-well flat bottom black plates (Corning Incorporated, Corning, NY, USA). After 24 h, HEp-2 cells were infected with 3-fold serially diluted viral inocula starting from 3×10^4 PFU/well. Relative fluorescence units (RFU) were measured using multimode microplate reader (Molecular Devices, Silicon Valley, CA, USA) with excitation wavelength of 479 nm and emission wavelength of 517 nm at 24 h, 48 h and 72 h post infection. Non-infected HEp-2 cells served as a reference for fluorescence background level.

2.4. Statistical parameter analysis for the evaluation and validation of HTS assay

To determine whether this established antiviral screening assay is qualified for HTS assay, the Z′ factor and other statistical parameters (Zhang et al., 1999; Iversen et al., 2006) such as percentage of coefficient variation (% CV), signal-to-noise ratio (S/N) and signal-to-background ratio (S/B) were calculated using the following formula.

$$Z' = 1 - \frac{3 \times (SD_V + SD_C)}{\mu_V - \mu_C}$$

$$S/B = \mu_V/\mu_C \text{.....(Recommendation: } > 3)$$

$$S/N = \frac{\mu_V - \mu_C}{\left(SD_V{}^{\frac{1}{2}} + SD_C{}^{\frac{1}{2}}\right)^2} \text{.....(Recommendation: } > 10)$$

$$\%\ CV = SD_V/\mu_V \text{.....(Recommendation: } < 20)$$

SD represents the standard deviation; μ refers to the average value; v is the virus infection group; c is the control group.

2.5. Large scale screening assay

HEp-2 cells were plated in 96-well flat bottom black plates at a density of 2×10^4 cells/well. After 24 h, HEp-2 cells were infected with RSV-mGFP at 3×10^3 PFU/well, and the test compounds were added using the high-throughput semi-automatic pipetting workstation (D.C. Labware, Foshan, China). Plates were incubated at 37 °C for 48 h. Fluorescence was measured as described above. Cell control group received the same amount of DMSO only.

2.6. Cell viability assay

After plated in 96-well plates at 37 °C for 24 h, the HEp-2 cells were either infected with RSV-mGFP (mock-infected cells as negative control) and calculated with formula A, or treated with the selected compound (mock-treated cells as negative control) and calculated with formula B. Following incubation for another 48 h, 20 μL of MTS (Promega, Madison, WI, USA) at 2 mg/ml were added into the 96-well plates, and cells were further incubated for 3 h at 37 °C in a 5% CO_2 incubator. After shaking the plate for 10 s, the absorbance was measured with a microplate reader (Tecan, Mannedorf, Switzerland) at the wavelength of 490 nm. The mock-infected cells or the mock-treated control were used as a 100% reference standard for cell survival.

$$(A)\ \textit{Cell viability}\ (\%\ \textit{control}) = \left(\frac{\text{the absorbance } \textit{of virus infected cells}}{\text{the absorbance } \textit{of virus non} - \textit{infected cells}}\right) \times 100\%$$

$$(B)\ \textit{Cell viability}\ (\%\ \textit{control}) = \left(\frac{\text{the absorbance } \textit{of compound treated cells}}{\text{the absorbance } \textit{of mock} - \textit{treat cells}}\right) \times 100\%$$

The compound concentrations that reduced cell viability by 50% (CC_{50}) were calculated using the software Graphpad Prism 5 (La Jolla, CA, USA).

2.7. RSV inhibition assay

HEp-2 cells were plated in 96-well plates and infected with RSV-mGFP at 3×10^3 PFU/well or *wt*RSV (50 PFU/well) in the presence of the tested compounds or DMSO. After 1 h of incubation, the virus inoculum was replaced and covered by 1% methyl cellulose with the same concentrations of compounds or DMSO (Lundin et al., 2013) and the plates were incubated at 37 °C for another 3–4 days. Then, the virus amount or titers were expressed as RFU measured by multi-mode microplate reader or PFU per ml by immunoplaque assay.

The quantification of the anti-RSV activity of the tested compounds, expressed as inhibition ratio, were calculated according to the following formulas of A and B for data based on RFU and PFU, respectively.

(*A*) *Inhibition ratio* (%)

$$= \left(\frac{RFU\ of\ virus\ infected\ cells\ -\ RFU\ of\ virus\ infected\ and\ compound\ treated\ cells}{RFU\ of\ virus\ infected\ cells} \right) \times 100\%$$

(*B*) *Inhibition ratio* (%)

$$= \left(\frac{PFU\ of\ virus\ infected\ cells\ -\ PFU\ of\ virus\ infected\ and\ compound\ treated\ cells}{PFU\ of\ virus\ infectied\ cells} \right) \times 100\%$$

The concentration of compounds that reduced the PFU of *wt*RSV by 50% (IC_{50}) was calculated using the software of Graphpad Prism 5.

2.8. Time-of-addition assay

The time-of-addition assay was finished as the previous report (Dirk et al., 2011). The method was introduced briefly as follows. HEp-2 cells were plated into 96-well plates at a density of 1.5×10^4 cells/well. After 24 h, HEp-2 cells were infected with *wt*RSV at a multiplication of infection (MOI) of 5 for 2 h at 37 °C. The cells were washed with PBS and the medium containing AZA or 6-MP at 50 μM was added at −1, 0, 2, 4, 6, 8, 10, 12 and 14 h post infection. Then, the treated cells were further cultured and collected at 24 h post infection, and the total RNA was isolated from the HEp-2 cells using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) and reverse transcribed with GoScript Reverse Transcriptase kit (Promega). RT-qPCR was performed as described previously (Jiao et al., 2017).

2.9. RSV minigenome assay

BHK-T7 cells were plated into 24-well plates at a density of 2×10^4 cells/well. After 24 h, the helper plasmids (pCITE-N、pCITE-P、pCITE-L and pCITE-M2-1) were mixed with the RSV minigenome plasmid encoding firefly luciferase (pBR322B-RSV-Gluc) in a microfuge tube containing 250 μL Opti-MEM. At the same time, the transfection reagent of Lipofectamine 2000 (Invitrogen, CA, USA) was diluted in 250 μL Opti-MEM in another tube. After 5 min, the plasmids were mixed with the transfection reagent and incubated at room temperature for 30 min. Then, the media in BHK-T7 culture were replaced with the transfection mixture and incubated at 37 °C, 5% CO_2. After 4–6 h, the mixture was replaced with media containing the compounds (AZA or 6-MP at 50 μM) and further incubated at 37 °C, 5% CO_2 for 24 h. Finally, the supernatant from the BHK-T7 cells was transferred into 96 well plate, and the Gluc activity was assayed by using LUMI star (BMG ABTECH, Ortenberg, Germany) according to the manufacturer's instructions after adding the chromogenic substrate of Gaussia Luciferase Flex Assay Kit (NEB, Ipswich, USA). The results were documented by photography and/or evaluated with OPTIMA software (BMG LABTECH). The resultant signal strength was expressed as relative light unit (RLU).

2.10. Statistical analysis

Statistical analyses of data were accomplished using GraphPad Prism 5 software. Differences were compared using an independent, two-sided Student's t-test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Development and optimization of an HTS assay for RSV inhibitors

To establish an HTS assay for RSV inhibitors using the recombinant RSV-mGFP, we first determined the RSV amount to be inoculated and the incubation time after RSV infection for optimal fluorescence readout. HEp-2 cells were infected with different titers of RSV-mGFP, followed by the measurement of RFU at 24, 48 and 72 h post infection. When the RSV-mGFP increased to about 10,000 PFU per well and the fluorescence signal was detected at 48 h post infection, the apex was reached (Fig. 1A), and turned down immediately with slightly increased infection dose. Given the 10,000 PFU per well is the point of transition downward, we think 3,000 PFU per well of RSV-mGFP was the reasonable virus titer for later assays, and the detection time was set as 48 h after RSV-mGFP infection, when the expression level of GFP was high and meanwhile a good dose-effect relationship between virus amount and fluorescence intensity was also established.

In addition, we analyzed the cytopathic effect of RSV-mGFP on HEp-2 cells and found that the cell viability remained over 90% even when the virus titer was increased to 3,000 PFU/well (Fig. 1A). Therefore, the cell viability was not either affected significantly at 48 h post infection when using 3,000 PFU/well. In conclusion, we chose to infect HEp-2 cells with 3,000 PFU/well of RSV-mGFP and a reading at 48 h post infection as standards for our further HTS assays.

To choose the optimal cell density for HTS assays, 96-well plates were seeded with 40,000 down to 5,000 HEp-2 cells per well, by using two-fold serial dilutions, and RFU were measured at 48 h post infection. As was shown in Fig. 1B, the maximum RFU were attained when seeding wells with 20,000 cells. This condition was used for further HTS assays.

3.2. Validation of the HTS assay with previously validated anti-RSV compounds

We next validated the assay using two known anti-RSV compounds including P13, a small-molecule with RSV membrane fusion inhibitor activity (Lundin et al., 2010), and ribavirin, a commercially available nucleoside analog capable of inhibiting RSV replication. Compared with the mock-treated control group, the inhibition of RSV-mGFP replication was evidenced in the presence of 0.1–100 μM of P13 (Fig. 2A), or 1–100 μM of ribavirin (Fig. 2B) in a dose-dependent manner. The calculated IC_{50} values of P13 and ribavirin were 0.57 ± 0.17 μM and 15.88 ± 3.38 μM respectively, which are close to the value previously reported (Lundin et al., 2010, 2013; Sudo et al., 2005; Liuzzi et al., 2005). Meanwhile, no significant cytotoxicity was observed in the concentration range. The CC_{50} values of P13 and ribavirin were 309.2 ± 45.94 μM and 273.80 ± 4.13 μM, respectively (Fig. 2A and B). The dose-response assessment provided proof-of-concept evidence to support the applicability of the assay to screen RSV inhibitors.

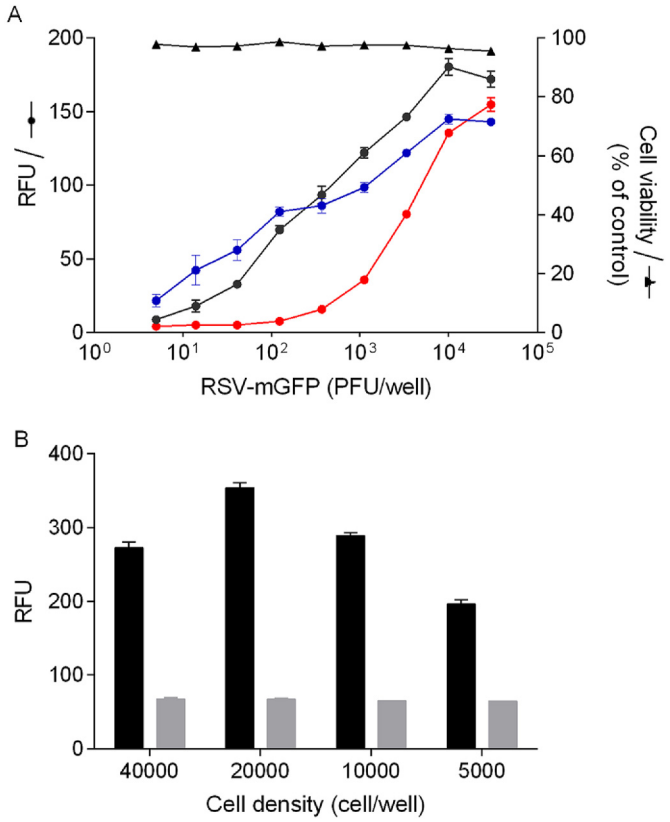

Fig. 1. Optimization of HTS assay. (A) Dose-effect relationships between the fluorescence signals and the inoculated virus titers were established following HEp-2 cells seeded in 2×10^4 cells/well in 96-well plates, infected by three-fold serially diluted RSV-mGFP starting from 8×10^4 PFU/well, and detected at 24 h (red solid round), 48 h (black solid round), and 72 h (blue solid round) post infection. Meantime, the cell viability was measured by MTS assay at 48 h (black solid triangle) post infection, in which the cell activity of mock-treated control wells left uninfected by RSV-mGFP was set as 100%. **(B)** The dose-effect relationships between the fluorescence signals and seeding density of HEp-2 cells (cells/well) were also established following the HEp-2 cells seeded in different densities, infected with RSV-mGFP of 3×10^3 PFU/well (black columns), or 0 PFU/well (grey columns), and detected at 48 h post infection. The results were shown as means ± standard deviations from triplicate of a representative experiment, expressed as relative fluorescence units (RFU) compared to the RFU in mock-treated control wells, or percent reduction in the cell viability relative to the mock-treated controls. PFU: plaque-forming units. In the case of cell viability, the bars for the standard deviation are obscured by the symbols because of the small margin of values.

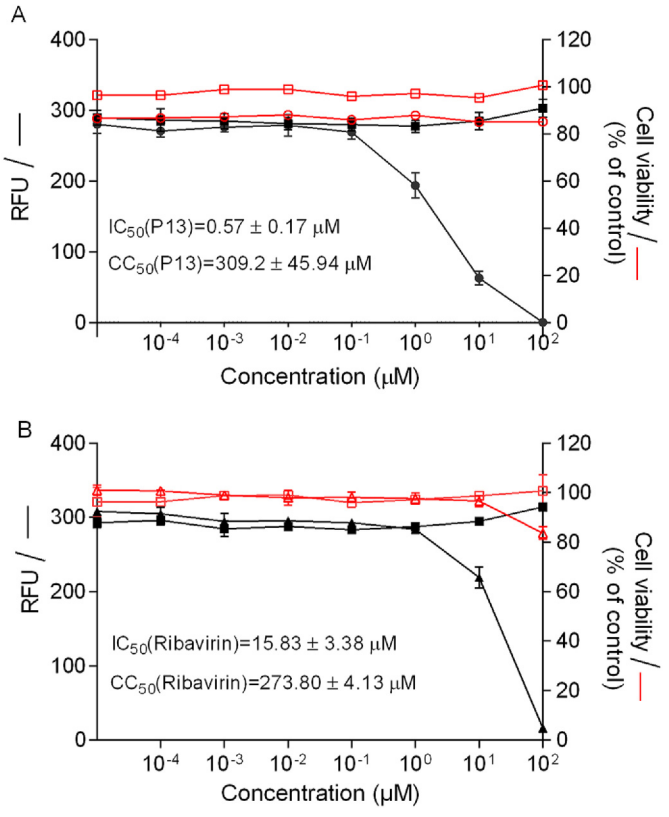

Fig. 2. Evaluation of the HTS assay with the known anti-RSV inhibitors. Ten-fold serially diluted samples of P13 **(A)** or ribavirin **(B)** were added to the HEp-2 cells, seeded in 2×10^4 cells/well in 96-well plates and infected by RSV-mGFP in 3×10^3 PFU/well. The antiviral activity, expressed as RFU, were measured at 48 h post treatment from P13 (black solid round), solvent of dimethyl sulfoxide (DMSO, black solid square), or ribavirin (black solid triangle). Simultaneously, the cell viabilities following the addition of P13 (red open round), DMSO (red open square), or ribavirin (red open triangle), were also analyzed by MTS assay at 48 h post treatment, in which the activity of the cells in the mock-treated control wells, infected but absent of the test compound, was set as 100%. CC_{50} and IC_{50} were also calculated as the described methods in sections of 2.6 and 2.7 and shown as means ± standard deviations for three independent experiments.

In order to evaluate the sensitivity, reliability and reproducibility of the established HTS assays, we tested and calculated the evaluation parameters of high throughput screening model such as Z′ factor, percentage of coefficient variation (CV %), signal-noise ratio (S/N) and signal-background ratio (S/B) (Zhang et al., 1999; Iversen et al., 2006; Ji et al., 2011). Z′ factor, reflecting datum variation associated with signal measurements and dynamic range of the test signal, is an important parameter for evaluating the quality of HTS model (Zhang et al., 1999). The results showed that the optimized HTS model for anti-RSV compounds had a Z' = 0.85 (n = 48), and met the criteria for HTS methods, and all the other HTS model evaluation parameters were also acceptable (Table 1). Taken together, all the values of the four kinds of HTS index factors indicate that the established HTS assay is suitable for the screening of compound candidates against RSV infection.

Table 1
Statistical parameters for the high-throughput screening (HTS) assay.

Statistical parameters	Recommended threshold	Value
Z′	> 0.5	0.85
CV[a] %	< 20	2.94
S/B[b]	> 3	3.53
S/N[c]	> 10	23.87

HEp-2 cells were plated in 96-well flat bottom black plates at a density of 2×10^4 cells/well. After 24 h, HEp-2 cells were infected with RSV-mGFP at 3×10^3 PFU and incubated for 48 h at 37 °C in a 5% CO_2 incubator. Finally, the expression of RSV-mGFP was detected by multi-mode microplate reader. Z′ factor, CV %, S/N and S/B were calculated according to the formulas in method section.

[a] Coefficient variation.
[b] Signal-background ratio.
[c] Signal-noise ratio.

3.3. Screening of compounds active against RSV replication

Using the HTS assay, we first performed a pilot screening of a commercial library (sp100107), which includes 2000 compounds already used clinically. At a final concentration of 10 μM, one hundred and thirty-five compounds showed over 50% reduction in RSV replication and no visible cytotoxicity (Fig. S1). Next, the cytocidal activity of these positive compounds were tested using a MTS method, to exclude the effects of their cytotoxicity on the antiviral activity.

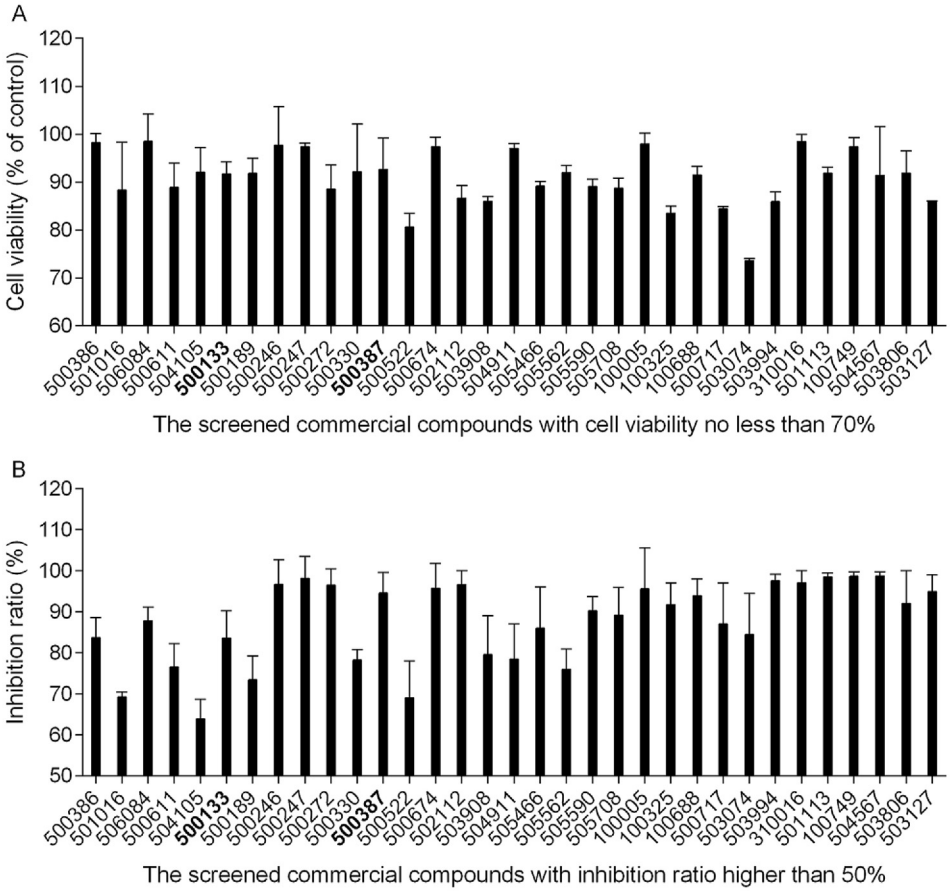

Fig. 3. Cytotoxicity and inhibition activity of the screened hit compounds from the commercial compound library further analyzed by MTS and immunoplaque sequentially. (A) Cell viability assay. The screened commercial compounds each at the concentration of 10 μM were added to HEp-2 cells for the cytotoxicity assay by MTS method. The hit compounds with cell viability no less than 70% of control were shown, in which the activity of the cells in the mock-treated control wells was set as 100%. **(B)** Inhibition activity of the nontoxic compounds. The selected nontoxic compounds each at the concentration of 10 μM were added to HEp-2 cells infected with *wt*RSV in 50 PFU/well and assayed by immunoplaque. Those compounds with inhibition ratio higher than 50% were further defined as active compounds. The results were shown as means ± standard and expressed as cell viability (% control) or inhibition ratio (%) = (virus infection control group-experimental group)/(virus infection control group-cell negative control group) × 100%. To be reminded: the compounds of 500133 and 500387 printed in bold represent AZA and 6-MP, respectively.

Moreover, the anti-RSV activity of the positive compounds was confirmed in a secondary screening using a *wt*RSV-based immunoplaque assay. As was shown in Fig. 3A and B, thirty-three compounds exhibited over 50% inhibitory effect in immunoplaque assay and no significant cytotoxicity (cell viability ≥ 70%) at a final concentration of 10 μM, in general, which are qualified to be regarded as the candidates of RSV inhibitor.

The potential application of the HTS assay for RSV inhibitor was further validated by a large scale screening of 52800 compounds in the chemical library at the Institute of Medicinal Biotechnology at a concentration of 10 μM. Eighty-six compounds were found to reduce more than 50% of RFU without visible cytotoxicity in the primary screening. Among them, forty-eight compounds displayed no or low cytotoxicity with cell viability ≥70% in a MTS-based cell viability assay. The immunoplaque assay showed that 29 of 48 compounds inhibited over 50% RSV replication (Fig. 4A and B). It should be noted that, although some compounds didn't display evident inhibitory activity based on the reduced plaque number, the size of the resulting plaques was strongly reduced when compared to the mock-treated control group. Taken together, these results demonstrate it is feasible that the established HTS assay serves as tool to screen compound candidates against RSV infection.

3.4. Azathioprine (AZA) and 6-mercaptopurine (6-MP) inhibit RSV replication

We next further explored the potential of two hits azathioprine (AZA) and 6-mercaptopurine (6-MP) identified herein as RSV inhibitor using the immunoplaque assay. AZA is a nucleoside analog prodrug and a thiopurine immunosuppressant for clinical use, and 6-MP is the first metabolite of AZA via a noneneymatic reaction, and subsequently metabolized to 3 kinds of metabolites of 6-MP-ribosidre/6-thioinosine (6-TI), or 6-methylmercaptopurine (6-MMMP), and 6-methylmercaptopurine riboside (6-MMPr). AZA and the resulting metabolites have been reported capable of inhibiting replication of hepatitis C virus (HCV), bovine viral diarrhea virus (BVDV), canine distemper virus (CDV), West Nile virus (MNV) and human cytomegalovirus (HCMV) *in vitro* but not herpes simplex virus (HSV) or varicella-zoster virus (VZV) (Shiraki et al., 1991; Spencer and Rob, 2008; de Carvalho et al., 2017; Carvalho et al., 2017; Lim et al., 2011).

Here, we would like to further explore the potential role of these two hits in inhibiting RNA virus replication using *wt*RSV and immunoplaque assay. As was shown in Fig. 5A, the IC50 values of AZA and 6-MP were 6.69 ± 1.41 and 3.13 ± 0.98 μM, respectively. In parallel, the cell viability was also tested, and the CC_{50} values of AZA and 6-MP were determined as 297.77 ± 23.93 and 183.73 ± 30.17 μM, respectively (Fig. 5B). The corresponding SI values were all above 50.

3.5. Investigation on the mechanism underlying AZA and 6-MP inhibiting wtRSV replication

To investigate the mechanism of RSV inhibition by AZA and 6-MP, a time-of-addition assay was carried out to identify the step(s) at which AZA and 6-MP inhibit RSV infection. AZA and 6-MP inhibited virus replication completely when added up to 6 h and 8 h post infection, respectively, similarly to both N protein inhibitor RSV604 (Challa et al., 2015), a less inhibition activity observed when added after 4 h (Fig. 6A), and genome replication stage-targeted inhibitor ribavirin (Lundin et al., 2013; Crotty et al., 2000), a marked inhibition on RSV replication when added up to 6 h after infection (Fig. 6A), which contrasted sharply with fusion inhibitor GS-5806, having inhibition activity only when added up to 0 h (Fig. 6A). Similarly, cyclopiazonic acid (CPA), a previously reported inhibitor, functions at the steps of virus

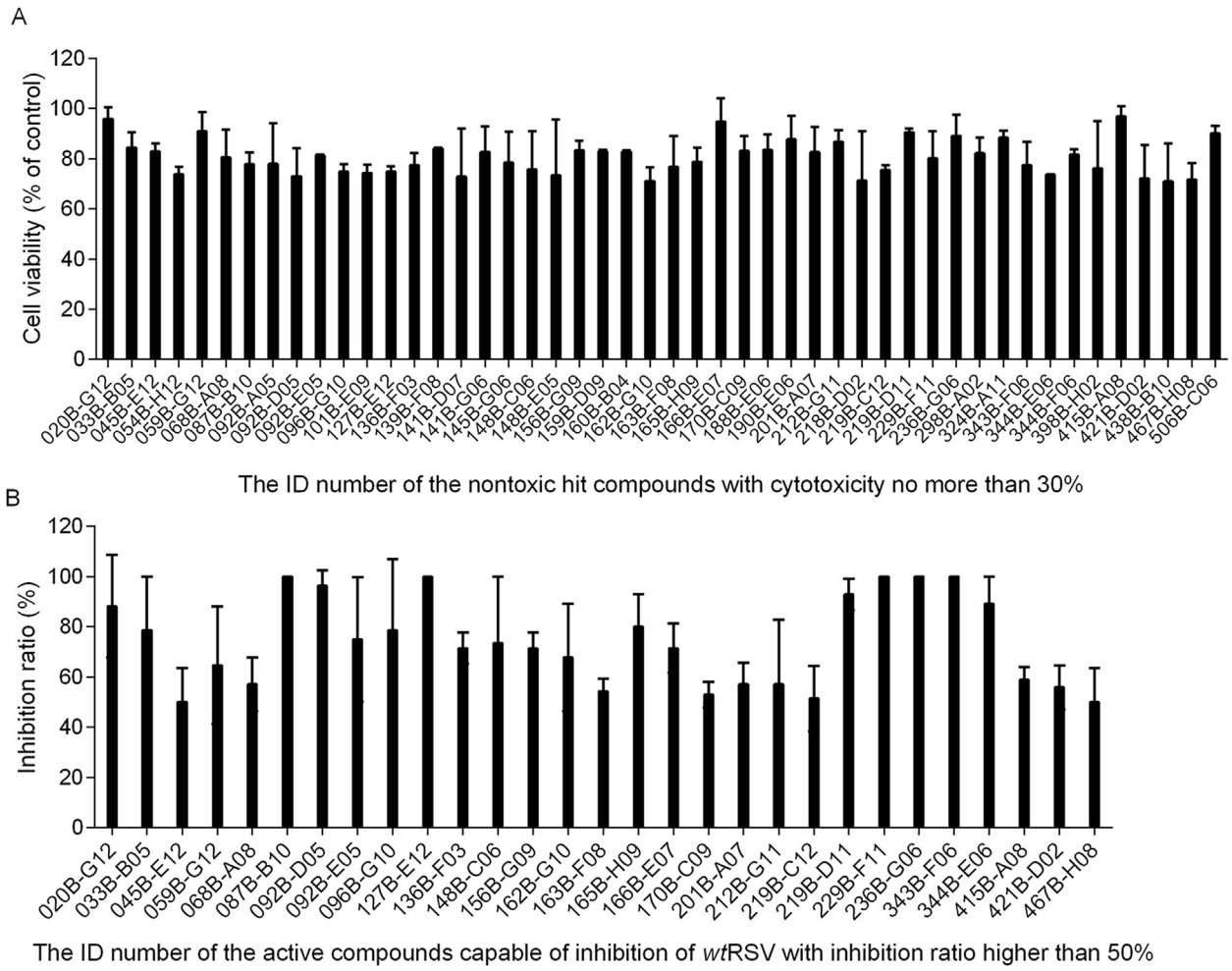

Fig. 4. Cytotoxicity and inhibition activity of the screened hit compounds from the synthesized compound library further analyzed by MTS and immunoplaque sequentially. (A) Cell viability assay. Each hit compounds, at the concentration of 10 μg/ml, was added to HEp-2 cells infected with *wt*RSV in 3×10^3 PFU/well in 96-well plates, and tested at 48 h post treatment, in which the cell activity in the mock-treated control wells was set as 100%. Nontoxic compounds, defined as the remaining cell viability no less than 70% or the resulting cytocidal activity no more than 30%, were selected and used for the further inhibition activity assay. **(B)** Inhibition activity of the nontoxic compounds. The selected nontoxic compounds at the concentration of 10 μg/ml each were added to HEp-2 cells infected with *wt*RSV in 50 PFU/well and assayed by immunoplaque. Those compounds with inhibition ratio higher than 50% were further defined as active compounds. The results were shown as means ± standard and expressed as cell viability (% control) or inhibition ratio (%).

genome replication and/or transcription and inhibits RSV replication when added up to 8 h post infection (Cui et al., 2016).

Further, HEp-2 cells were incubated with the mixture of *wt*RSV (MOI 5) and 100 μM AZA or 6-MP for 2 h at 37 °C. Then, the cells were extensively washed with PBS and continually incubated with 100 μM AZA or 6-MP for 0, 10, 30, 60, 90 min and until 9 h with 1-h interval, respectively. After extensive washing of the treated cells with PBS at the indicated incubation time above (also defined as time-of-removal experiment (Loregian et al., 2010)), total RNA was isolated and tested using RT-qPCR. As was shown in Fig. S2, both of the two test compounds had inhibitive effect on RSV transcription or/and genome replication, which is consistent with the conclution obtained from the time-of-addition assay.

In the subsequent RSV minigenome assay, we co-transfected RSV minigenome plasmid of pBR322B-RSV-Gluc with four auxiliary plasmids into BHK/T7 cells. After 24 h, we detected the expression of Gluc in the supernatant of BHK/T7 cells. The results were shown as Fig. 6B. Compared with the negative control group (mock), AZA and 6-MP, similar to ribavirin, could inhibit the expression of Gluc, suggesting that AZA and 6-MP act in the genome replication/transcription phase.

Altogether, these data suggest that AZA and 6-MP exert their inhibitory action at the post-entry stage of RSV replication, possibly by targeting RSV transcription or/and genome replication, which is consistent with previous report on their antiviral effects likely occurring on the stages of RNA replication rather than on viral entry, packaging or release (Spencer and Rob, 2008).

4. Discussion

In this study, we established a fluorescence-based HTS assay aiming to screen novel anti-RSV compounds from both the commercial and the synthesized libraries. Our results showed that the HTS assay was time-saving and convenient for this purpose. By screening compounds active against RSV from the commercial library containing 2000 compounds applicable to clinical use, we identified 33 compounds with ≥50% RSV inhibition activity. Subsequently, the library containing 52,800 synthesized compounds was screened, and we identified 48 hit compounds with ≥50% RSV inhibition activity, among which 29 active compounds were finally confirmed as active by immunoplaque assays performed with *wt*RSV. The total screening rates were 1.65% and 0.05% from these two libraries, respectively. This different hit rates may result from the differential capacity and constituent of these two libraries. For the two commercial compounds AZA and 6-MP, nucleotide analog (Ruel et al., 2019; Dogan et al., 2015), they are found capable of inhibiting

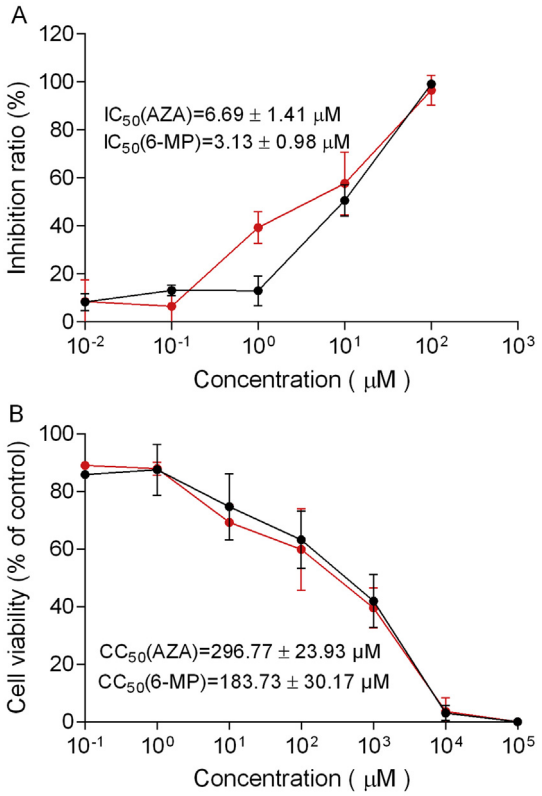

Fig. 5. Inhibitory and cytotoxic effects of two hit compounds of azathioprine (AZA) and 6-mercaptopurine (6-MP) on RSV infection and HEp-2 cells, respectively. (A) Inhibition activity assay. Two-fold serially diluted samples of AZA (red solid round) or 6-MP (black solid round) were added to HEp-2 cells, seeded in 2×10^4 cells/well in 96-well plates and infected with *wt*RSV at 50 PFU/well. Immunoplaque analyses were performed at 3–4 d post treatment. **(B)** Cell viability assay. The HEp-2 cells, infected with *wt*RSV at 50 PFU/well and added ten-fold serially diluted samples of AZA (red solid round) or 6-MP (black solid round), were analyzed by MTS assay at 48 h post treatment. The results of inhibition ratio and cell viability were shown as means ± standard deviations from three independent experiments and expressed as inhibition ratio (%) or cell viability (% control). CC_{50} and IC_{50} were also calculated as the described method above and shown as means ± standard deviations for three independent experiments.

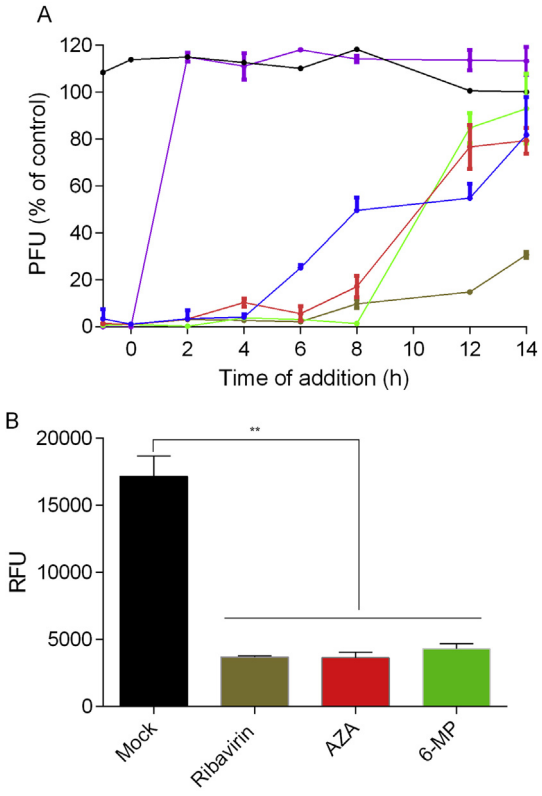

Fig. 6. Investigation on the mechanism underlying AZA and 6-MP inhibiting *wt*RSV replication (A) Time-of-addition analyses of AZA and 6-MP on RSV replication. The HEp-2 cells were infected with 5 MOI *wt*RSV at the time point of 0 h and incubated up to 2 h at 37 °C, the cells were washed by PBS and further cultured with test medium. The test medium containing AZA (red solid round), 6-MP (green solid round), DMSO (negative control, black solid round), 10 μM RSV-604 (blue solid round), 100 μM ribavirin (brown solid round) or 1 μM GS-5806 (purple solid round) were added into HEp-2 cells at −1, 0, 2, 4, 6, 8, 10, 12 and 14 h separately and incubated till 24 h post infection. Then, the cells were harvested and lysed for RNA extraction and RT-qPCR assay. The results were a representative of three independent experiments, and shown as means ± standard and expressed as % negative control, although in many cases the bars for the standard deviation were obscured by the symbols given the small margin of values. **(B)** RSV minigenome assay. After 24 h, we co-transfected RSV minigenome plasmid of pBR322B-RSV-Gluc with four auxiliary plasmids into BHK/T7 cells, and detected the expression of Gluc in the supernatant of BHK/T7 cells. The results were shown as means ± standard deviations for three independent experiments, $^{**}P < 0.01$.

RSV by a post-entry mechanism, suggesting that they should interfere with viral transcription or/and replication. To our knowledge, this is the first report on their inhibition activity against RSV replication. Therefore, our HTS assay represents an invaluable tool to develop anti-RSV compounds effectively and rapidly.

Previously, an HTS method using recombinant RSV expressing EGFP was developed by Kwanten et al. (2013). However, neither the optimal parameters critical as the guideline for the application of HTS, nor the characteristic HTS index factor, including Z′, CV%, S/N and S/B, were available. Here, we optimized the three parameters, validated HTS assay by index factor, and performed high-throughput RSV inhibitor screenings by using two compound libraries. Furthermore, based on the experience to establish and apply this HTS assay, we find many advantages of the RSV-mGFP-based HTS over other screening methods. First, it is suitable for robotized screenings, making it more efficient and convenient for large scale screenings. Second, this established HTS assay provides digital readout, avoiding subjective interference by the operator. Third, in contrast to minigenome or minireplicon assays, it is suitable for screening compounds targeting any step of the infection cycle of RSV. Fourth, the fluorescence readout of EGFP does not require any substrate or other auxiliary factor, and is absent of cytotoxicity (Cao et al., 2014). Fifth, not only is it sensitive enough to screen compounds active against RSV infection, but it also displays pretty good hit rate. In addition, most of the antiviral compounds identified by these screenings are effective when subjected to the traditional immunoplaque method. Concerning the gap existing between these two screening methods in the hit rate, we think it could be attributed to the higher sensitivity of RSV-mGFP-based HTS. So, compounds only reducing plaque size and classified as non-inhibitors of RSV by immunoplaque assays might be ones active against RSV infection by using RSV-mGFP-based HTS assay.

As we know, AZA and 6-MP have been used as immunosuppressive/anti-inflammatory, antiviral or anticancer agents. Besides, AZA, a precursor of 6-MP, can be quickly converted to 6-MP *in vivo* (Ruel et al., 2019; Aldinucci et al., 2010; Prufer et al., 2014). From 6-MP, there are several active metabolites further transformed. Among them, 6-MMPr is known to inhibit purine synthesis and shows the greatest antiviral potential (Spencer and Rob, 2008). This work herein provides new data supporting that AZA and 6-MP are effective inhibitors of RSV replication *in vitro*, and target a post-entry stage of viral life cycle as the likely mechanism.

In conclusion, we established and validated a robust HTS assay for screening RSV inhibitor, and identified 62 compounds including AZA and 6-MP that possess anti-RSV activity with low cytotoxicity *in vitro*. Moreover, the anti-RSV mechanism of AZA and 6-MP, targeting on RSV genome replication/transcription, was also explored, which paves the way for the further investigation on of their *in vivo* inhibitory activity on RSV.

Conflicts of interest

We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virol.2019.07.002.

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