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Development of a high-throughput replicon assay for the identification of respiratory syncytial virus inhibitors

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ABSTRACT

Respiratory syncytial virus (RSV) drug discovery has been hindered by the lack of good chemistry starting points and would benefit from robust and convenient assays for high-throughput screening (HTS). In this paper, we present the development and optimization of a 384-well RSV replicon assay that enabled HTS for RSV replication inhibitors with a low bio-containment requirement. The established replicon assay was successfully implemented for high-throughput screening. A validation screen was performed which demonstrated high assay performance and reproducibility. Assay quality was further confirmed via demonstration of appropriate pharmacology for different classes of RSV replication tool inhibitors. RSV replicon and cytotoxicity assays were further developed into a multiplexed format that measured both inhibition of viral replication and cytotoxicity from the same well. This provided a time and cost efficient approach to support lead optimization. In summary, we have developed a robust RSV replicon assay to help expedite the discovery of novel RSV therapeutics.

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1. Introduction

RSV is an enveloped, non-segmented negative-sense RNA virus in the *Paramyxoviridae* family. It is the leading cause of acute lower respiratory tract infections in young children and other high-risk populations (Collins and Melero, 2011). No efficacious RSV treatment or vaccine is available despite decades of research and drug discovery endeavor. Ribavirin, the only approved antiviral for RSV treatment, has limited clinical use due to toxicity and controversial efficacy. Immunoprohylaxis with RSV-neutralization antibodies is only effective as a preventive measure (Collins and Melero, 2011).

The urgent need to develop efficacious RSV treatments prompted several HTS campaigns for novel RSV inhibitors (Bonavia, 2011; Mason, 2004; Chung, 2013). These screens primarily used an RSV cytopathic effect (CPE) assay which evaluates compound protection against cell death induced by RSV infection (Bonavia, 2011). The CPE HTS protocol employs an extended assay time and requires biosafety level-2 containment which limits its robustness and practicality. Other approaches including cell-free RSV ribonucleoprotein complex (RNP) and minigenome assays have also been applied (Mason, 2004; Olivo, 1998). The RNP assay requires isolation of functional viral RNP from infected cells. The minigenome assay requires transient transfection of cells with five plasmids encoding each of the RNP components to reconstitute the viral RNP complex essential for viral RNA replication. The lack of robustness in both approaches has limited their utility as HTS screens.

A common alternative to screening for antivirals uses subgenomic replicon cell systems which have been proven to be robust and convenient tools for the discovery of viral replication inhibitors for many viruses. These autonomous replicons mimic the viral replication process in infected cells and do not release infectious virus particles, hence have the advantage of lowering the biosafety requirement (Rice, 2011; Yang, 2011; Masse, 2010). One prominent example is the HCV replicon system which has been the backbone of HCV drug discovery (Rice, 2011; Yang, 2011). The feasibility of achieving a stable and non-cytotoxic RSV replicon system was recently demonstrated, despite natural RSV infection being transient and cytopathic (Malykhina, 2011). This autonomous RSV replicon utilizes a modified viral RNA genome encoding the viral RNP proteins essential for RSV replication: The RNA-dependent-RNA polymerase (L), nucleoprotein (N), cofactor phosphoprotein (P), and second matrix protein (M2-1) (Fig. 1A). Viral genes involved in virus entry and assembly (G, F, SH) were deleted and replaced by the insertion of reporter and antibiotic-resistant genes. This allowed selection and enrichment of the stable replicon cells and generation of a robust assay readout. The cells can be passaged multiple times and still maintain the replicon RNA and protein expression, providing the foundation for a more efficient approach to large scale screening for RSV replication inhibitors.

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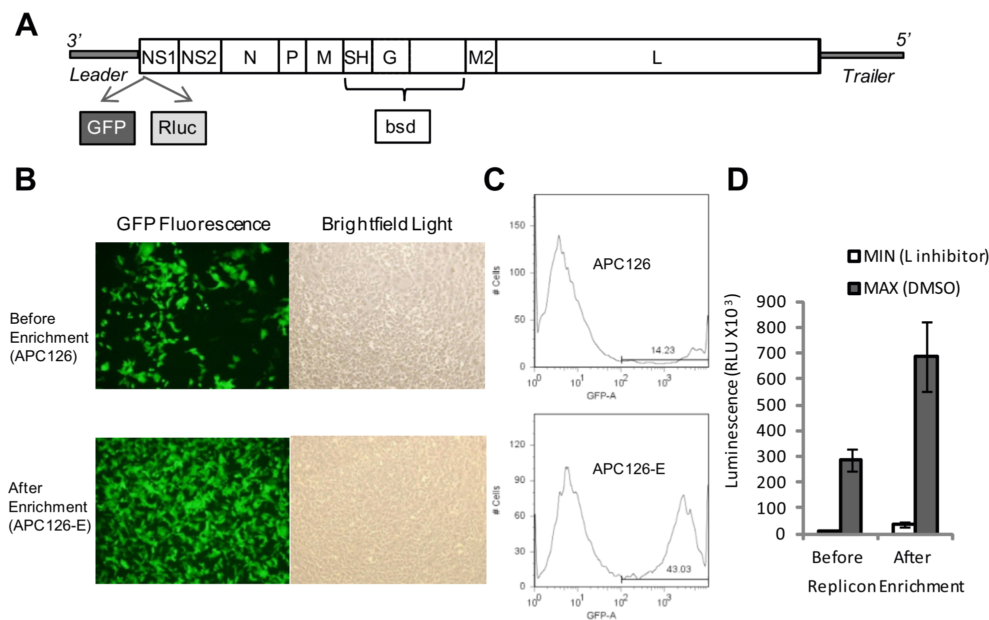

Fig. 1. Enrichment of reporter signal in RSV Replicon cells. (A) RSV replicon genome structure. Viral glycoprotein genes SH, G, F were replaced by selection marker bsd gene to acquire resistance to blasticidin. Green fluorescent protein (GFP) and renilla luciferase (Rluc) genes were inserted as reporters (Malykhina, 2011). (B, C) RSV replicon was enriched under the selection of blasticidin. Enrichment of replicon content in APC126-E cells was confirmed by increased GFP expression detected by fluorescence microscopy (B) and flow cytometry (C). (D) Enriched RSV replicon cells showed enhanced luciferase reporter signal and remained sensitive to RSV L inhibitor. APC126 and APC126-E replicon cells were treated with either DMSO (maximum signal) or a RSV L-inhibitor (minimum signal) for 48-h followed by luciferase activity detection. The data shown are mean values of the replicates from a representative experiment with standard deviation error bars.

Here we report the development and optimization of a robust HTS assay using this RSV replicon system. The fully optimized assay was further validated using known RSV replication inhibitors in a pilot screen. We also developed a multiplexed assay format which delivered both replicon and cytotoxicity readouts. These two robust versions of the RSV replicon assay provided new tools to advance HTS and chemistry campaigns towards the development of effective RSV treatments.

2. Materials and methods

2.1. Cells and media

2.1.1. RSV replicon cell lines

APC126 originating from Dr. Mark Peeples laboratory was licensed from Apath LLC. The cells were cultured in complete growth media consisting of Minimum Essential Medium (MEM) (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma), 2 mM L-glutamine (Sigma), 1 U/ml penicillin (Invitrogen), 1 μg/ml streptomycin (Invitrogen) and 10 μg/ml blasticidin-HCl (Invitrogen) at 37 °C in 5% CO_2. The enriched RSV Replicon Cell Line APC126-E was generated from APC126 cells following 4 weeks of culture and passaged in the presence of 40 μg/ml blasticidin-HCl.

2.1.2. Assay media

Assay optimization and validation work was performed using phenol red-free MEM supplemented with 10% heat-inactivated FBS and 2 mM L-glutamine. HTS validation was carried out using the same assay media with the addition of 1 U/ml penicillin and 1 μg/ml streptomycin.

2.2. Compounds

Ribavirin and oseltamivir were purchased from ACME Bioscience and EMD Chemicals. RSV inhibitors: YM53403 (Sudo, 2005), an analog of YM53403 with equivalent anti-RSV potency (Compound 1 in Xiong, 2013), and BI cpd D (Liuzzi, 2005) were synthesized in-house. Compounds were solubilized in 100% v/v DMSO at 10 mM and further diluted in assay media to a final DMSO concentration of 0.1% v/v for screening. The RSV L inhibitor used in HTS assay development refers to YM53403 analog and was used at 10 μM to generate the minimum assay signal.

2.3. Large scale APC126-E cell cryobank for HTS

APC126-E cells were propagated in complete growth media containing 40 μg/ml blasticidin-HCl and cultured at scale in twenty 10 chamber Cell STACKS (Corning Life Sciences) to generate an assay ready cryobank of 1.2×10^{10} cells. Cells were detached using accutase, centrifuged, and re-suspended in complete growth media at a density of 6×10^7 cells/ml. An equal volume of cryopreservation media (80% FCS/20% DMSO) was added and the cells were aliquoted for cryopreservation using a controlled rate freezer (Planer Kryo).

2.4. RSV replicon assay optimization and characterization

Cryopreserved APC126-E cells were plated into 384-well white assay plates (Falcon) at a density of 3000 cells/well in a 54 μl volume and incubated at 37 °C with 5% CO_2 for 4 h before compound treatment. Compounds were diluted as described in the Compounds section and transferred to assay plates at 6 μl/well followed by 48-h incubation at 37 °C in 5% CO_2. For replicon reporter signal detection, the assay media was removed from the assay wells and replaced with 20 μl of 50 μM EnduRen™ Live Cell Substrate (Promega). Cells were incubated for 1.5 h at 37 °C prior to luminescence detection using a Synergy 2 plate reader (Biotek).

2.5. Validation of the HTS RSV replicon assay

150 nl each of 7000 compounds at 4 mM in DMSO was dispensed into white 384-well tissue-culture treated microplates

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