

# A Cell Based High-Throughput Screening Approach for the Discovery of New Inhibitors of the Influenza H5N1 virus

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## Abstract

Using a highly reproducible and robust cell-based HTS assay, we screened the NIH Molecular Libraries Screening Centers Network (MLSCN) 100,000 compound library at 50  $\mu$ M compound concentration against influenza strain AV/VN/1203/2004 (H5N1). The "hit" rate (>25% inhibition of the viral cytopathic effect) from the single dose screen was 0.32%. The hits were evaluated for their antiviral activity, cell toxicity and selectivity in dose response experiments. The screen yielded five active compounds (SI50 value > 3). One compound showed an SI50 value of greater than 3.45, three compounds had SI50 values ranging from greater than 13.84 to 34.29, while the most active compound displayed an SI50 value of 94.64. The active compounds represent two different classes of molecules, benzoquinazolinones and thiazoloimidazoles which have not been previously identified as having anti-viral/anti-influenza activity.

## Methods

**Cell Growth Conditions and Media.** Madin Darby Canine Kidney cells (ATCC CCL-34, American Tissue Culture Type) were maintained as adherent cell lines in Eagle minimum essential medium with 2 mM L-glutamine and 10% fetal bovine serum (FBS) at 37°C in a humidified 5% CO<sub>2</sub> atmosphere as were described previously (1). Cells were passaged as needed and harvested from flasks using 0.25% trypsin-EDTA. Cells used for the assay were not used past passage 70.

**The Compound Library and Controls.** The positive control drug for this assay, Ribavirin (#196066, MP Biomedicals, Solon, OH) was solubilized at 8 mg/ml in dimethyl sulfoxide (DMSO, Sigma). The stock solution was diluted to final concentration of 40 mg/ml in assay media (DMEM without phenol red, 1.0% BSA, 4 mM L-glutamine, 100 U/ml penicillin and 100 U/ml streptomycin; Gibco) immediately before use. Final DMSO concentration in each well was 0.5%.

The NIH Molecular Libraries Screening Centers Network (MLSCN) compound library was screened in this assay, which was solubilized at 10 mM in DMSO. Before each experiment, all compounds were diluted to 50  $\mu$ M in assay media for the screen.

**Influenza HTS CPE Assay.** The high-throughput cell-based assay for the identification of potential inhibitors of Influenza virus has been previously described (1). Briefly, MDCK cells (3 x 10<sup>5</sup> cells/ml) were dispensed into black, clear-bottom, 384-well plates at a density of 6,000 cells/well in 20  $\mu$ l assay medium, using a Matrix WellMate and incubated 24 hours at 37°C, 5% CO<sub>2</sub>, with high humidity. The next day, 5  $\mu$ l of compounds were added to cells using a Biomek FX liquid handler (Beckman Coulter, Fullerton, CA). This resulted in a final drug concentration of 50  $\mu$ M (<1% DMSO) for all samples. The plates were then immediately transferred from HTS facilities to a class II Biosafety Cabinet within the BSL-3 laboratory where the cells were infected with 5  $\mu$ l of diluted virus (influenza AV/VN/1203/2004) at a concentration of 100 TCID50 doses (diluted from amplified virus stock in egg allantoic fluid into assay media for a final virus stock dilution of 1:10,000) using a Matrix WellMate to the required plate wells, which corresponds to an MOI of 0.005 PFU/cell. Internal controls consisted of wells containing cells only, cells infected with virus, and virus infected cells treated with ribavirin. Plates were then allowed to incubate at 37°C, 5% CO<sub>2</sub>, for 72 hours within the BSL-3 laboratory. After incubation, 30  $\mu$ l of Promega Cell Titer Glo was added to each well using a Matrix WellMate and incubated at room temperature for 10-30 minutes. Luminescence was then measured using a Perkin Elmer Envision plate reader (Wellesley, MA). This step was also performed within the BSL-3 facility.

**Secondary Confirmation assays.** For dose response assays, compounds were serially diluted in serum-free media (maintaining a 0.5% DMSO final concentration in the wells) in a plate to plate matrix rather than in a well to well matrix. This allows 320 compounds in one plate to be diluted together resulting in a 10 point dose response dilution series. This method is referred to as "stacked plate". It can be visualized as a serial dilution series proceeding vertically through a stack of plates with the high dose plate on top and the low dose plate on the bottom (final plate well concentration ranging from 60  $\mu$ M to 0.117  $\mu$ M and a final DMSO concentration of  $\leq$ 1%).

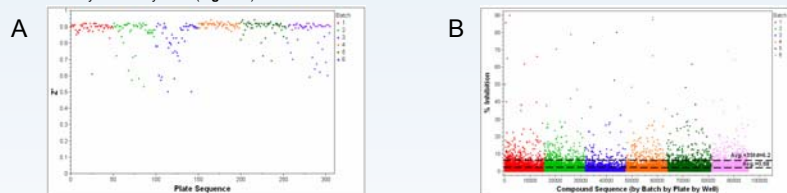
**Data Analysis.** Data was analyzed using ActivityBase software (IDBS, Inc, Guildford, UK). Percent cytopathic effect (CPE) inhibition was defined as  $100 - \frac{\text{Percent cell viability}}{\text{Percent CPE inhibition}} \times 100$ . An active compound, or "hit", was defined as a compound that exhibited a %CPE inhibition of >25% without compromising cell viability. Two dose response curves were calculated for each substance. One assessed cytopathic effect (% CPE Inhibition) at each dose; the other assessed cell viability at each dose. Percent CPE inhibition =  $100 \times (1 - \frac{\text{luminescence compound well} - \text{median luminescence virus control}}{\text{median luminescence cell control} - \text{median luminescence virus control}})$ . Percent viability =  $100 \times \frac{\text{luminescence compound well} - \text{median luminescence cell control}}{\text{median luminescence cell control} - \text{median luminescence virus control}}$ . An EC<sub>50</sub> (for % CPE inhibition) and IC<sub>50</sub> (for cell viability) were calculated for each substance using the 4 parameter Levenburg-Marquardt algorithm with parameter A locked at 0 and parameter B locked at 100. Standard deviation, normalized chi2 and hill slope were used to evaluate the curves. EC<sub>50</sub>/IC<sub>50</sub> values were also calculated to estimate the relative CPE inhibition and cell viability at which 50% inhibition and 50% cell viability would be achieved respectively. Values were not extrapolated beyond the tested range of concentrations.

## Introduction

Influenza A and B are negative strand RNA viruses (*Orthomyxoviridae*), that infect the upper and lower respiratory tracts causing substantial morbidity and mortality annually. In terms of epidemic and pandemic potential, Influenza A viruses, especially avian strains, pose a considerable threat. In the twentieth century three influenza pandemics have occurred making this illness the most devastating disease known to mankind. The catastrophic "Spanish" flu pandemic in 1918 infected a significant percentage (20% to 40%) of the world's population and resulted in the deaths of 675,000 people in the U.S. and an estimated 40 million people worldwide. Approximately 70,000 people in the United States and two to seven million people succumbed to the "Asian" flu pandemic of 1957 (2). The third and mildest pandemic in the 20th century, the "Hong Kong" flu pandemic of 1968 killed about 33,800 people in the U.S. The pandemics of 1957 and 1968 were quickly identified due to increased surveillance for flu outbreaks and technological advances in influenza biology. The survival rate for the 1968 pandemic was greatly increased due to improved medical care and the development of antimicrobials to protect against secondary bacterial infections. In 1997, the Special Administrative Region of China in Hong Kong garnered worldwide attention when an epidemic of highly pathogenic avian influenza (H5N1) virus was transmitted from poultry to humans resulting in 18 human cases, of which six were fatal (4). Clearly, the continued emergence of new influenza variants, drug-resistant mutants and potential pandemic strains demands our attention toward the discovery and development of more effective antiviral therapeutics. High-throughput screening (HTS) offers an important tool for drug discovery of new antiviral leads for new and emerging pathogens such as SARS CoV (3) and pandemic influenza. Toward this, we previously reported the development of a cell-based HTS that monitors virus-induced cytopathic effects (CPE) in MDCK cells (1). We have employed this assay to screen 100,000 compounds from the NIH Molecular Libraries Screening Centers Network (MLSCN) compound library at 50  $\mu$ M compound concentration to identify potential novel influenza inhibitors. We report the discovery of 5 hits with anti-influenza activity. The screening was performed with the support of the SRMLSC through the NIMH grant R03-MH081270-01.

## Compound Screening Results

A total of 95,512 compounds were initially screened at a final concentration of 50  $\mu$ M. Z' scores for this assay were consistently greater than 0.80 demonstrating that this is a very robust assay. The mean inhibition of the compound wells was 2.04% with a standard deviation of 5.36%. Inhibition values of greater than 18.12% were outside the calculated noise of the assay defined by + 3 $\sigma$  (Figure 1).



**Figure 1.** HT Screen of 95,512 compounds from the MLSCN library. (A) Z' sequence plot. (B) Influenza AV/VN/1203/2004 (H5N1) compound sequence plot. Reference lines were calculated from the percent (%) inhibition. Compounds from NIH MLSCN library, along with the Influenza AV/VN/1203/04 (H5N1), were added to 6 x 10<sup>5</sup> MDCK cells per well in 384-well plates. 72 hrs later, inhibitory effects were assessed using Cell Titer Glo (Promega). Control drug used was Ribavirin.

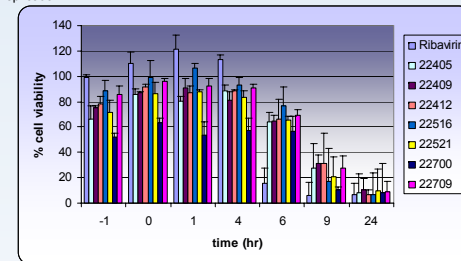
Sixty-four compounds that showed protective effect of > 25% CPE inhibition were identified as hits. Because of the low number of hits identified in the primary single dose screen, structure activity analysis was performed and analogs to these sixty-four compounds were identified within the compound library. A total of three hundred five compounds were ordered for follow up screening. These compounds were then tested in dose response at ten concentrations, ranging from 60  $\mu$ M to 0.117  $\mu$ M for both antiviral and cytotoxicity activity. Of the compounds screened in dose response, five were confirmed as active. The EC<sub>50</sub> (CPE inhibition) values ranged from 0.634 - >100  $\mu$ M. The IC<sub>50</sub> (cytotoxicity) values ranged from 3.6 - >100  $\mu$ M. The selectivity index (SI = IC<sub>50</sub>/EC<sub>50</sub>) values ranged from 3.45 - 94.64 and compounds with SI values >2 were considered active. The antiviral activities of selected compounds are shown in Table 1.

**Table 1. Inhibitory Activities of Selected Influenza AV/VN/1203/04 (H5N1) Antiviral Compounds ( $\mu$ M)**

PubChem ID	Structure	% INH CPE	EC50 $\mu$ M	EC90 $\mu$ M	IC50 $\mu$ M	IC90 $\mu$ M	SI50	SI90
7975011		48	0.634	>60	>60	>60	>94.64	0
862635		86	1.75	>60	>60	>60	>34.29	0
847638		66	3.596	>60	>60	>60	>16.69	0
14743025		70	4.336	27.206	>60	>60	>13.84	<2.205
14738273		27	17.383	>60	>60	>60	>3.45	0

## Time of Addition Assay Results

Determination of compound antiviral activity, cell toxicity and selectivity enabled us to group the active compounds into two different classes of molecules, benzoquinazolinones and thiazoloimidazoles. From structure activity relationships (SAR) several compounds were synthesized from novel scaffolds that exhibited sub-micromolar activity (EC<sub>50</sub> < 1  $\mu$ M). Therefore, we employed an assay to determine the point in the influenza virus life cycle that seven of these compounds inhibited. This screen allowed us to ascertain if the inhibition activity of the compound was early (entry) or late (replication) in the virus life cycle. In this screen, compounds were added in triplicate to plates at time points -1, 0, 1, 4, 6, 9 and 24 hours p.i. All seven compounds exerted antiviral activity when they were added one hour before infection or 1 to 6h after infection (Figure 2). These results suggest these compounds may impede an early to middle stage of influenza replication.



**Figure 2.** Time of addition compound screen against influenza AV/VN/1203/04 (H5N1). MDCK cells were plated in 96 well black tissue plates at 15,000 cells per well and incubated 24 h at 37°C, 5% CO<sub>2</sub>. Test compounds were diluted in media to give a final concentration of 20  $\mu$ g/ml, and added to plates at time points -1, 0, 1, 4, 6, 9 and 24 h post infection. Cells were infected with influenza AV/VN/1203/04 at an MOI of 0.005 and incubated 72 h at 37°C, 5% CO<sub>2</sub>. CTG was added and luminescence was read at 560/590nm on an Envision plate reader. Ribavirin was used as a control compound.

## Conclusions

The aim of this study was to gain access to the Molecular Libraries Screening Center Network (MLSCN) high throughput screening resources to facilitate the discovery of new molecular probes for the inhibition of avian influenza strain H5N1 virus. Using our high-throughput cell-based assay a total of 95,512 compounds were initially screened in single dose. Three hundred five compounds were evaluated for their antiviral activity, cell toxicity and selectivity in dose response experiments. Of these compounds, only 5 compounds met the MLSCN criteria of active compounds: the selectivity index (SI = IC<sub>50</sub>/EC<sub>50</sub>) values were greater than 2. Activity data from the confirmatory and secondary assays were analyzed in-depth to identify key scaffolds of interest, further investigate SAR, and contribute to probe optimization efforts. Subsequently, we synthesized several compounds for secondary testing. Preliminary time of addition assays suggest these compounds are most efficacious from 1 hour before infection to 6 hours after infection, similar to the control drug Ribavirin. In addition, dose response and toxicity assays indicate that these compounds are efficacious against influenza A/Hong Kong/156/97 (H5N1) and A/Udm/72 (H3N2). In view of these data, we believe that evaluation of these targeted analogues of these various scaffolds in an iterative fashion should lead us toward the identification of compounds that can be developed into clinically useful therapeutic agents.

## References

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