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Development and Validation of a High-Throughput Screen for Inhibitors of SARS CoV and Its Application in Screening of a 100,000-Compound Library

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The authors have developed a high-throughput screen (HTS) that allows for the identification of potential inhibitors of the severe acute respiratory syndrome coronavirus (SARS CoV) from large compound libraries. The luminescent-based assay measures the inhibition of SARS CoV-induced cytopathic effect (CPE) in Vero E6 cells. The assay was validated in 96-well plates in a BSL3 containment facility. The assay is sensitive and robust, with Z values > 0.6, signal to background (S/B) > 16, and signal to noise (S/N) > 3. The assay was further validated with 2 different diversity sets of compounds against the SARS CoV. The “hit” rate for both libraries was approximately 0.01%. The validated HTS assay was then employed to screen a 100,000-compound library against SARS CoV. The hit rate for the library in a single-dose format was determined to be approximately 0.8%. Screening of the 3 libraries resulted in the identification of several novel compounds that effectively inhibited the CPE of SARS CoV in vitro—compounds which will serve as excellent lead candidates for further evaluation. At a 10- μ M concentration, 3 compounds with selective indexes (SI₅₀) of > 53 were discovered. (*Journal of Biomolecular Screening* 2007:33-40)

Key words: high-throughput screening, severe acute respiratory syndrome, coronavirus, cytopathic effect

INTRODUCTION

Severe acute respiratory syndrome (SARS), an emerging infectious disease with severe mortality (15%) and morbidity, is a viral respiratory illness that first appeared in southern China in 2002 and rapidly spread to several countries.¹ The epidemic resulted in 8096 reported probable SARS cases in 28 countries, with a total of 774 deaths reported by the World Health Organization (WHO). The causative agent of SARS was identified by several groups²⁻⁴ who each reported the isolation of novel coronaviruses from patients with SARS. The coronaviruses (order *Nidovirales*; family *Coronaviridae*; genus *Coronavirus*) are members of a family of positive-sense RNA viruses that replicate in the cytoplasm of animal host cells. They are a diverse group of large, enveloped viruses that infect many mammalian and avian species causing upper respiratory, gastrointestinal, hepatic, and

central nervous system diseases. It is generally accepted that SARS coronavirus (CoV) is transmitted by close person-to-person contact through coughs or sneezing through the respiratory droplets' deposit on the mucous membranes of the mouth, nose, or eyes of persons nearby. In addition, transmission can occur through contact with surfaces contaminated with the infectious droplets. Infections caused by SARS CoV produce high fever, headache, and a dry, nonproductive cough, which can lead to hypoxia, pneumonia, and the need for mechanical ventilation.

During the SARS outbreak in Asia and Canada, a number of SARS patients were treated with the combination of ribavirin and/or corticosteroids.⁵⁻⁸ This treatment strategy was not proven to be effective. Recently, Barnard et al.⁹ have reported the enhanced infectivity of SARS CoV with inosine monophosphate (IMP) dehydrogenase inhibitors, which underscores the need for new antiviral therapies for SARS. Several focused screening efforts have resulted in lead-candidate antiviral drugs against SARS CoV, including protease inhibitors,^{10,11} fusion inhibitors,¹² and interferon.¹³ As yet, a rapid, sensitive, and validated assay for high-throughput screening (HTS) of chemical libraries against SARS CoV has not been developed. Although the disease caused by SARS CoV is now considered a rare human disease, it is impossible, as with many other zoonotically transmitted viral diseases (e.g., Ebola virus, monkey pox, and hantavirus), to predict when and where this disease will emerge again. Hence, it is

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critical to identify new drugs to be prepared for the reemergence of these and other emerging pathogens. Toward this goal, a validated HTS approach can be used to identify lead candidates.

The purpose of the present study was to develop, validate, and optimize an HTS assay to screen antiviral compound libraries rapidly using a high-throughput platform against the SARS CoV in vitro in the BSL3. Validation of this fluorescent-based assay included optimizing virus concentration and incubation time, determination of the highest concentration of DMSO that would not affect cell or virus viability, and determination of the IC_{50} of calpain inhibitor IV, a compound that exhibits antiviral activity against the SARS CoV. Once these conditions were optimized, the assay was further validated through variability testing. This included Z analyses, the signal-to-noise (S/N) ratio, and the signal-to-background (S/B) ratio to measure both the assay's signal dynamic range and the data variation associated with the sample measurement and the reference control measurement.¹⁴ To assess the performance of the cell-based assay, the Prestwick library, which consists of 880 drugs and bioactive natural compounds approved by the Food and Drug Administration (FDA), was screened in duplicate. To further determine assay reproducibility, the Microsource Spectrum library, which consists of 2000 FDA-approved drugs and natural compounds, was assayed in triplicate. The validated assay was used to screen a 100,000-compound library. The hit rate for the library was approximately 0.8% and resulted in several new lead candidates.

MATERIALS AND METHODS

Cell growth conditions and media

Vero E6 cells (ATCC# 3338237, American Tissue Culture Type) and Vero 76 were maintained as adherent cell lines in Dulbecco's modified eagle's medium (DMEM) with phenol red, supplemented with 10% fetal bovine serum (FBS), and 1% L-glutamine at 37 °C in a humidified 5% CO₂ atmosphere. Cells were passaged as needed and harvested from flasks using 0.25% trypsin-EDTA. Cells were counted and viability was determined by propidium iodide using the Coulter Epics XL-MLC.

Compound libraries and controls

The positive control drug for this assay, calpain inhibitor IV (Calbiochem, San Diego, CA), was solubilized at 1 µg/µL in DMSO (Sigma, St. Louis, MO) and stored at -80 °C. Aliquots were made from 1 common drug stock, and a previously unthawed aliquot was used for each experiment and discarded afterwards. The stock solution was diluted to 20 µg/mL in assay media (DMEM without phenol red, 5% FBS, 1% L-glutamine, and 1% penicillin/streptomycin; Gibco, Grand Island, NY) before each experiment.

Two small compound libraries were screened in this assay as part of the validation process: the Microsource Spectrum library,

which was solubilized at 1 mM in DMSO, and the Prestwick library, which was solubilized at 1 mg/mL in DMSO. Before each experiment, all compounds were diluted to 5 µg/mL (Prestwick) or 10 µM (Microsource) in assay media.

The validated assay was used to screen 100,000 compounds in duplicate from the National Institute of Neurological Disorders and Stroke (NINDS) compound library. The NINDS library was solubilized in 1 µg/µL DMSO, and all compounds were diluted to 10 µg/mL in assay media for the screen.

SARS CoV HTS cytopathic effect assay

Vero E6 cells were dispensed into black, clear-bottom, 96-well plates at a density of 10,000 cells/well in a 50-µL assay medium, using a Titertek Multidrop 384 (Titertek, Huntsville, AL), and incubated 24 h at 37 °C, 5% CO₂, with high humidity. The next day, 25 µL of compounds was added to cells using a Beckman Coulter Biomek[®] FX (Beckman Coulter, Fullerton, CA). The cells were transported to the BSL3, where they were infected with the Toronto-2 strain of SARS CoV (gift of Dr. Heinz Feldman) at a concentration of 100 TCID₅₀ using a Beckman Coulter Biomek[®] 2000. This resulted in a final drug concentration of 5 µg/mL or µM (0.5% DMSO) for all samples. Internal controls consisted of wells containing media only, cells only, cells infected with virus, and virus-infected cells treated with calpain inhibitor IV. Plates were then allowed to incubate at 37 °C, 5% CO₂, for 72 h. After incubation, 100 µL of Promega CellTiter-Glo[®] (Promega, Madison, WI) was added to each well using the Biomek[®] 2000. Plates were shaken for 2 min at speed 5 on a Labline Instruments (Kochi, India) plate shaker. Luminescence was then measured using a PerkinElmer Envision[™] plate reader (PerkinElmer, Wellesley, MA).

Data analysis

Data were analyzed using Activity Base software (IDBS, Inc., Guildford, UK). Percent cytopathic effect (CPE) inhibition was defined as [(test compound - virus control)/(cell control - virus control)] * 100. Percent cell viability was defined as (test compound/cell control) * 100. An active compound, or "hit," was determined to be any compound that exhibited a %CPE inhibition of > 50% without compromising cell viability. Five concentrations of each drug were added to 96-well plates in triplicate to measure the effective concentration at which the drug inhibited viral CPE 50% (EC₅₀) or, in the absence of virus, the inhibitory concentration at which growth was inhibited by 50% (IC₅₀). The selective index (SI) was calculated as SI = IC₅₀/EC₅₀.

Calculations were as follows: the Z value was calculated as $1 - (3 * \text{standard deviation of cell control } [\sigma_c] + 3 * \text{standard deviation of the virus control } [\sigma_v]) / (\text{mean cell control signal } [\mu_c] - \text{mean virus control signal } [\mu_v])$. The S/B = mean cell control signal (μ_c)/mean virus control signal (μ_v). The S/N = mean cell control signal (μ_c) - mean virus control signal (μ_v)/(standard deviation

of the cell control signal $[\sigma_c]^2$ – standard deviation of the virus control signal $[\sigma_v]^2$.¹⁴

RESULTS AND DISCUSSION

Assay endpoint

The endpoint reagent is one of the most critical variables in assessment of an assay employed in an HTS. The endpoint markers typically used in automated assays measure a biochemical event that occurs in living cells but stops after cell death.¹⁵ The most suitable endpoint detection for an HTS assay gives the most accurate indication of the difference in cell numbers between virus and cell controls and the highest Z factor. In development of an assay in the BSL3, the endpoint reagent should also be easy to use and require minimal handling. Therefore, we compared 3 different kits and determined the S/N ratio, S/B ratio, and Z values for each to ascertain the optimal assay endpoint.

The first assay tested was the CellTiter 96[®] AQ_{ueous}. One Solution Cell Proliferation Assay, which is a homogeneous colorimetric method of determining the number of viable cells present. The reagent contains a tetrazolium compound (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium, inner salt; MTS) and an electron coupling reagent, which facilitates cellular reduction into a colored formazan product that is soluble in tissue culture medium. Assays were performed by adding 1/5 the assay volume MTS to culture wells, incubating for 1 to 4 h, and measuring absorbance at 490 nm. As shown in **Table 1**, there was a 2.6-fold difference in the S/N versus the S/B, with a Z value of 0.03. In addition, the MTS assay had several drawbacks. First, the assay did not provide an accurate indication of the difference in numbers between virus and cell controls. The assay also requires that a color control be used, increasing the amount of reagents and plates that are needed. The most important drawback is that a relatively long incubation period is required for viable cells to reduce the tetrazolium compound to generate a signal once the reagent is added. Clearly, these problems and the data indicate that the MTS assay was not a viable endpoint reagent for our cell-based HTS assay.

The second assay we examined was a Neutral Red–based cytotoxicity assay, which measures the ability of viable cells to incorporate and bind neutral red. Neutral Red is a weak, cationic dye that readily penetrates cell membranes by nonionic diffusion, accumulating intracellularly in lysosomes. Alterations of the cell surface or the lysosomal membrane lead to lysosomal frailty that becomes irreversible. This results in a decreased uptake and binding of Neutral Red, making this assay a highly sensitive indicator of cell viability.¹⁶ This assay also has several drawbacks. First, there are a number of steps involved in performing the actual assay. Media must first be removed and

Table 1. Comparison of Assay Endpoint Reagents

Assay Endpoint	Signal-to-Noise Ratio	Signal-to-Background Ratio	Z
MTS	1.5	3.9	0.03
Neutral Red	2.2	5.6	0.27
CellTiter-Glo [®]	2.9	16.0	0.68

replaced with an equal amount of media containing 0.066% Neutral Red. Plates are then incubated for 2 h and washed twice with phosphate-buffered saline (PBS). A buffer solution consisting of 50% ethanol and 1% acetic acid is added to plates, shaken for 2 min, and incubated for 10 min, and absorbance is measured at 540 nm. In addition, Neutral Red may have a relatively selective effect on the lysosomes of the cell, resulting in a deceptively low reflection of cell viability. Upon visual inspection of the cells under the microscope, there was an obvious difference in cell number between virus and cell controls that was not reflected accurately using Neutral Red. One last drawback of the assay is the precipitation of the Neutral Red dye into visible, fine, needle-like crystals. The S/B versus the S/N ratio was similar to the MTS assay, but the Z value increased but is significantly lower than the 0.5 cutoff Z value (0.27), making this endpoint reagent unacceptable for the HTS platform (**Table 1**).

The final endpoint assay we examined was the CellTiter-Glo[®] Luminescent Cell Viability Assay. CellTiter-Glo[®] is a reagent that generates a luminescent signal directly proportional to the amount of adenosine triphosphate (ATP) present, which is proportional to the number of metabolically active cells. The assay has many advantages over the previous assays we examined. The assay is simple to use and requires no washing, removal of media, or multiple pipetting steps, which inherently introduce more error. Importantly, it requires only a 10- to 15-min period of time once the reagent is added to the cells for the mixture to equilibrate to ambient temperature, which reduces time in a BSL3 environment. The luminescent signal produced by the luciferase reaction has a half-life of greater than 5 h, which allows for continuous processing of multiple plates. To determine the number of cells required to optimize S/B and S/N ratios, cell density experiments were performed. To validate that the assay is linear with cell number, we plated a known number of cells in 96-well plates, incubated for 72 h, and measured luminescence using CellTiter-Glo[®]. As shown in **Figure 1**, there was a linear relationship ($r^2 = 0.9052$) between relative luminescence and cell number, with a range from 5000 to 12,500 cell/well, which corresponded to 2 doublings (data not shown). Subsequently, we chose an optimal cell density of 10,000 cells/well, which is within the linear range of detection.

To assess the quality of the assay, Z analyses were performed by running 3 separate plates on 3 days for a total of 9 plates. The

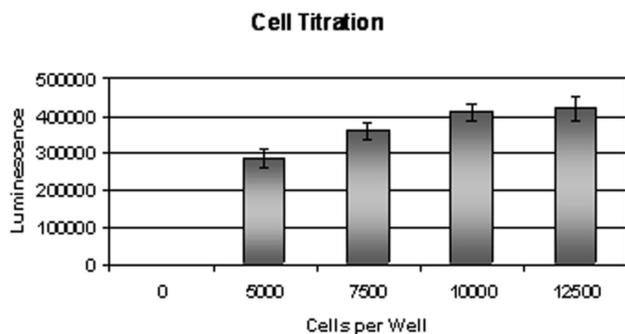


FIG. 1. Correlation of cell density and relative luminescence. Vero E6 cells were plated into 96-well plates at the indicated cell number per well and analyzed by CellTiter-Glo[®]. Then, 72 h later, the luminescence was measured using CellTiter-Glo[®] reagent and a PerkinElmer Envision[™] plate reader.

Z analysis is a statistical test designed to evaluate if an assay is robust enough for screening on an HTS platform. With a cutoff Z value of ≥ 0.5 and S/B ratio greater than 5, the more suitable the assay is for the HTS platform. Z analyses showed that this is an acceptable assay for the HTS platform, with consistent Z values of > 0.6 (data not shown). The assay was also reproducible and consistently exhibited a higher S/N ratio (2.9) and S/B ratio (16.0) (Table 1). In conclusion, CellTiter-Glo[®] was superior to Neutral Red or the MTS assay based on S/N, S/B, and Z.

DMSO effect on Vero E6 cells and SARS CoV

DMSO tolerance on assay performance is a critical parameter given its use as a compound solvent. To determine the highest DMSO concentration that the Vero cells and virus-infected cells could tolerate, we monitored the effect of serial 2-fold dilutions of DMSO (10% to 0.02%). Inhibition of cell growth was evaluated relative to cells not treated with DMSO. The data show that 0.5% DMSO was the highest concentration that would not affect cell viability (Fig. 2A). DMSO was included in all controls at a concentration of 0.5%.

To establish the signal dynamic range of the assay, we ascertained the optimal virus concentration that would give the largest differentiation between cells alone (the cell control) and cells infected with virus (the virus control). Dilutions of the SARS CoV were added to Vero E6 cells, and absorbance was measured using CellTiter-Glo[®]. Figure 2B shows the TCID₅₀ plot of cell viability versus decreasing concentration of added viral stock determined by the CellTiter-Glo[®] assay. We determined that the optimal concentration of the virus relative to the cell control was 6.85 TCID₅₀/mL 72 h postinfection for a multiplicity of infection (MOI) of 0.1 (Fig. 2B).

Control drug concentration

Of the several compounds that were tested for efficacy in inhibiting SARS CoV replication in vitro, the calpain inhibitor

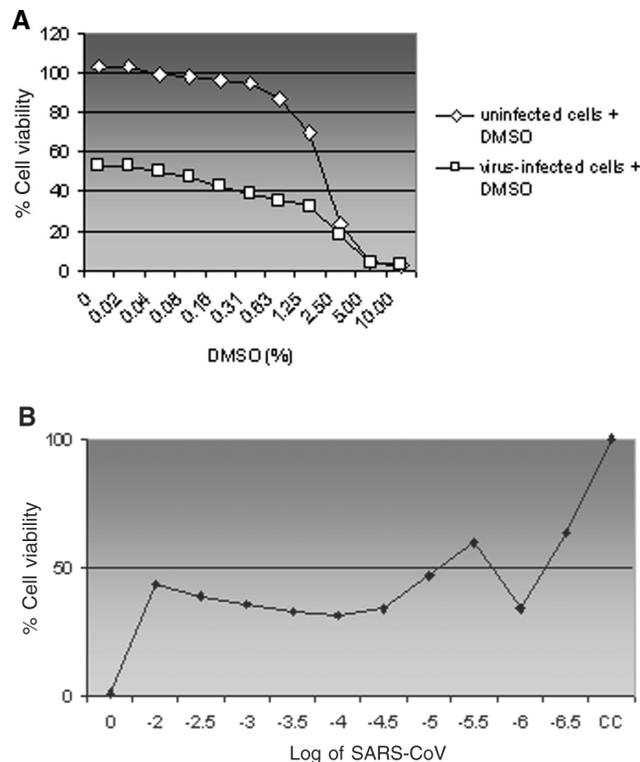


FIG. 2. Effects of DMSO on the assay performance. (A) Serial 2-fold dilutions of DMSO in assay media, along with the severe acute respiratory syndrome coronavirus (SARS CoV), were added to 1×10^4 Vero 76 cells in 96-well plates. Then, 72 h later, the cytopathic effect (CPE) of DMSO was assessed using CellTiter-Glo[®]. (B) TCID₅₀ plot of cell viability versus decreasing concentration of added viral stock determined by the CellTiter-Glo[®] assay. Intersection of the curve and 50% cell viability point shows that a $\sim 10^5$ -fold dilution of the SARS CoV caused CPE in 50% of cells when determined 72 h postinfection by CellTiter-Glo[®]. The corresponding virus titer of the stock was 10^6 plaque-forming units (PFU)/mL, determined by plaque assays of the cultured virus stock. CC indicates the uninfected cell control. TCID₅₀ is the tissue culture infectious dose at which the virus infects 50% of the cells.

IV gave the most consistent results. Therefore, this drug was used as the positive control in this assay. The positive control refers to the average value of the cells treated with the positive control drug and infected with the virus. This value was the same as the average value of cells alone, indicating that no CPE occurred in virus-infected cells that were treated with calpain inhibitor IV. Inhibition of SARS CoV was determined relative to virus not treated with calpain inhibitor IV.

To access the IC₅₀ value of calpain inhibitor IV, serial half-log dilutions of the drug were made in assay media, ranging from 100 to 0.107 $\mu\text{g}/\mu\text{L}$. The IC₅₀ of calpain inhibitor IV was 1 $\mu\text{g}/\mu\text{L}$ (Fig. 3). However, for this assay, we wanted to choose a concentration that would exhibit 100% inhibition of the SARS CoV without compromising cell viability. A dose response was run on the drug in duplicate on 3 different days to determine if the drug would

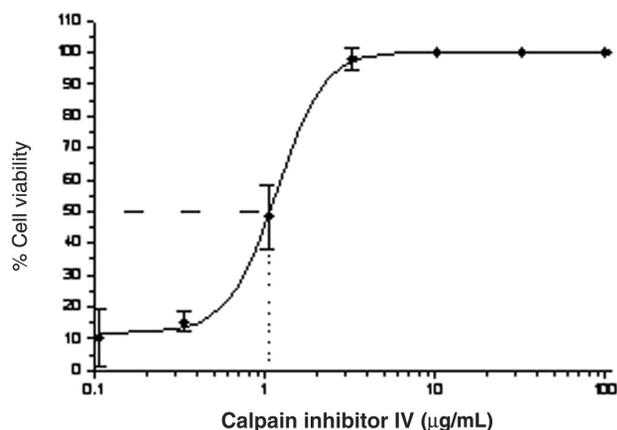


FIG. 3. IC_{50} curve of calpain inhibitor IV using a CellTiter-Glo[®] endpoint. Serial half-log dilutions of calpain inhibitor IV in media, along with the severe acute respiratory syndrome coronavirus (SARS CoV), were added to 1×10^4 Vero 76 cells in 96-well plates. Then, 72 h later, the inhibitory effects of the drug were assessed using CellTiter-Glo[®].

give reproducible results. It was determined that a 5- μ g/mL dose of the drug consistently showed 100% inhibition of the virus-induced CPE (**Fig. 3**).

An assessment of the screening data variability showed that this assay is able to identify “hits” with confidence. This was done by measuring the coefficient of variation, or CV, which is calculated as (standard deviation of the sample/mean of the sample). The lower the value of the CV, the less variation is associated with the assay. For this experiment, cells were infected with virus in all wells of a 96-well plate, luminescence was measured, and the CV was calculated. These experiments were performed repeatedly on different days, all with CVs of ~5% (data not shown).

Compound screen results

As part of the validation for this assay, 2 small compound libraries were screened to determine the reproducibility of the hits and the hit rate. A hit is identified as an active compound that either activates or inhibits the assay signal above a defined threshold value from the sample mean signal (1). A hit for this assay was considered any compound exhibiting a %CPE inhibition of > 50%. The Microsource library was screened in triplicate, and the average at 10 μ M is shown (**Fig. 4B**). The Prestwick library was screened in duplicate, and the average at 5 μ g/mL is shown (**Fig. 4A**). The hit rate for both libraries was determined to be approximately 0.01%.

Following these initial studies, we screened 100,000 compounds in duplicate from the NINDS compound library against SARS CoV using the validated HTS assay. The hit rate for the library (compounds that inhibited CPE by > 50%) in a single-dose (SD) format was determined to be approximately 0.8%. To

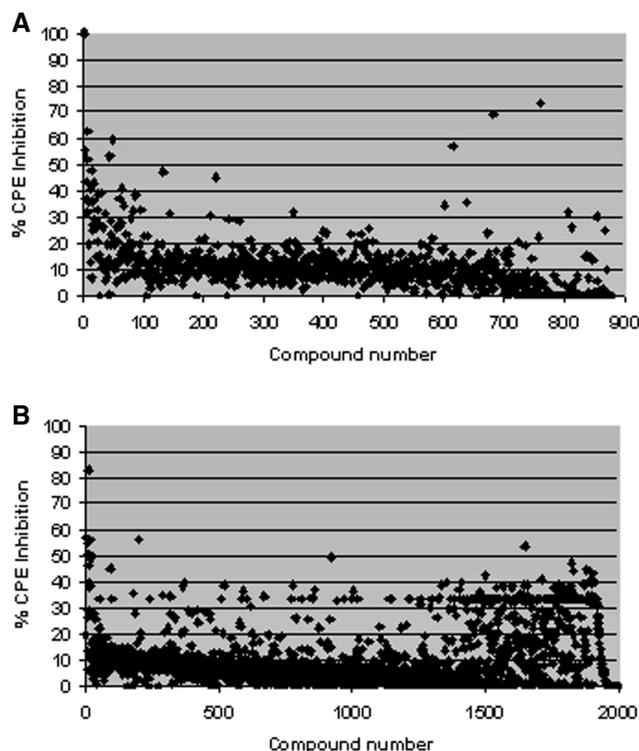
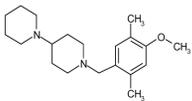
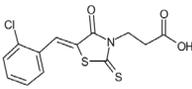
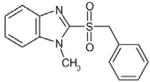
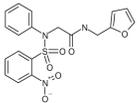
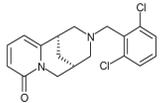
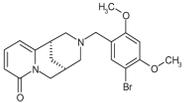
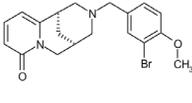
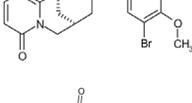
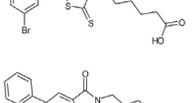
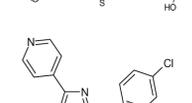
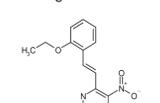
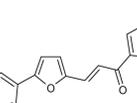
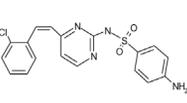
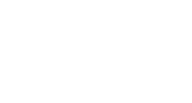


FIG. 4. Prestwick and Microsource library screen. (A) Average of duplicate Prestwick screen at 5 μ g/mL. Compounds from Prestwick library, along with the severe acute respiratory syndrome coronavirus (SARS CoV), were added to 1×10^4 Vero 76 cells in 96-well plates. Then, 72 h later, inhibitory effects were assessed using CellTiter-Glo[®]. (B) Average of triplicate Microsource screen at 10 μ M. Compounds from Microsource library, along with the SARS CoV, were added to 1×10^4 Vero 76 cells in 96-well plates. Then, 72 h later, inhibitory effects were assessed using CellTiter-Glo[®]. Control drug used was β -interferon. CPE = cytopathic effect.

screen selected hits from the SD assay, compounds were confirmed in a dose-response (DR) format. Furthermore, an assay was employed to determine the point in the SARS CoV life cycle that the DR hits inhibited. In effect, this simple screen allowed us to ascertain if the inhibition of the compound was early (entry) versus late (replication). In this screen, compounds were added to cells 6 h after infection with SARS CoV. The assay was executed for 77 of the DR hits from the NINDS library. The criteria for determining compound activity are based on a compound’s selective index (SI_{50}). We classify the compounds with an SI_{50} value of < 4 as not active, SI_{50} = 4 to 9 as slightly active, SI_{50} = 10 to 49 as moderately active, and an SI_{50} value > 50 as highly active. Forty-eight of the 77 compounds were slightly active, 25 were moderately active, and 3 were highly active. Representative compounds are shown in **Table 2**. The EC_{50} , IC_{50} , and calculated SI values for the 3 compounds that displayed high activity in reducing SARS CoV-induced CPE in Vero E6 cells

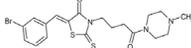
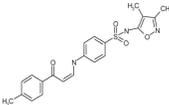
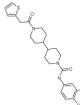
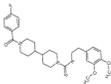
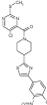
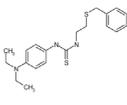
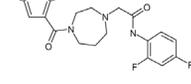
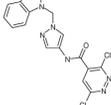
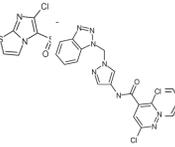
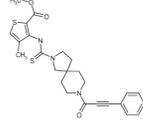
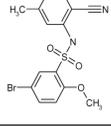
Table 2. Structures and Inhibitory Activities of Selected Compounds ($\mu\text{g/mL}$)

ARB #	Structure	%CPE	EC_{50}	EC_{90}	IC_{50}	IC_{90}	SI_{50}	SI_{90}
05-010887		73.1	8.8	> 100	> 100	> 100	> 11.4	0
05-014335		65.2	4.7	18.2	52.2	> 100	11	> 5.5
05-014927		50	2.8	13.3	> 100	> 100	> 35.9	> 7.6
05-016757		58.1	6.7	> 100	> 100	> 100	> 14.9	0
05-018137		93.5	0.4	1.4	27.7	77.5	76.9	56.1
05-018139		57.4	3.8	> 100	> 100	> 100	> 26.2	0
05-018147		66.9	0.7	2.7	19.9	> 100	28.9	> 36.8
05-021554		80.4	4.4	8.5	80.6	98.2	18.5	11.6
05-022227		63.9	4	> 10.2	41.2	> 100	10.3	0
05-022821		100	1.3	NA	40.4	NA	30.9	NA
05-022923		66.1	4.1	> 100	> 100	> 100	> 24.5	0
05-024039		100	4.1	7.3	> 100	> 100	> 24.2	13.8
05-026054		64.3	9.3	>100	>100	>100	10.7	0
05-026708		100	8.8	57.2	> 100	> 100	> 11.4	> 1.8

(Continued)

Development and Validation of HTS for Inhibitors of SARS CoV

Table 2. (continued)

ARB #	Structure	%CPE	EC ₅₀	EC ₉₀	IC ₅₀	IC ₉₀	SI ₅₀	SI ₉₀
05-029388		64.3	2.5	> 10.2	41.6	61	16.6	< 0.6
05-037787		51.5	9.6	>100	>100	>100	>10.4	0
05-049606		57.3	2.3	> 100	> 100	> 100	> 43.9	0
05-049631		57.9	9.8	> 100	> 100	> 100	> 10.2	0
05-053242		81.4	2.1	> 100	> 100	> 100	> 47.9	0
05-061600		54.9	9.7	> 100	> 100	> 100	> 10.3	0
05-074532		51.9	4.4	> 100	> 100	> 100	> 22.6	0
05-089276		50.1	4.5	>100	>100	>100	>22.2	0
05-090643		56.8	3.3	> 100	> 100	> 100	> 30.8	0
05-090830		89.7	6.9	> 100	> 100	> 100	> 14.4	0
05-097826		63	2.5	5.9	51.2	> 100	20.3	> 16.8

CPE = cytopathic effect.

are given in **Figure 5**. These 3 compounds belong to 3 distinct chemical series—namely, tricyclic methanopyridodiazocinones, 5-arylidenerhodanines, and spirocyclic imidazothiazolesulfonamides. The structures of these active compounds 1–3 are shown

in **Figure 5**. Although each of these 3 compounds exhibited an SI₅₀ value > 53, 2 of these (compounds 1 and 3) also displayed significant SI₉₀ values of 56 and > 9, respectively, indicating that these are excellent candidates for animal studies. It is interesting to

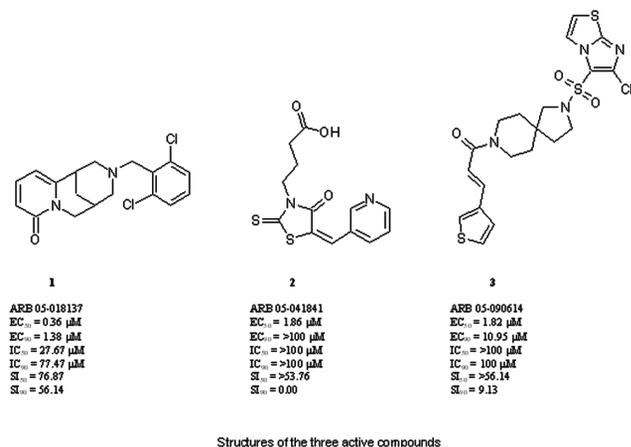


FIG. 5. Structures of candidate drugs.

note that in the NINDS library of compounds examined, there were 34 analogs of the active compound 1. All of these analogs differ from 1 only in the substitutions on the phenyl ring of the benzyl group in 1. Of these 34 compounds, 7 displayed significant inhibition potencies at 5 μg/mL in the single-dose assay and were tested in the dose-response assay. Although the EC₅₀ values of 6 of these analogs were in the range of 0.36 to 4.7 μg/mL, none of these compounds met our criteria for a “highly active” compound due to poor SI₅₀ ratios (< 50) resulting from high cellular toxicity. The identification of the 3 compounds from 3 distinct chemical classes with high efficacy and selectivity indicates that these can serve as attractive leads for pursuing structure-activity relationships and for lead optimization to develop novel therapeutic agents.

In summary, an automated HTS for the rapid identification of potential inhibitors of SARS CoV has been developed and validated in a BSL3 setting. This cell-based assay uses the luminescence signal generated by the amount of ATP present in the samples to measure the inhibition of virus-induced cytopathic effects on the cells. Screening of the Microsource, Prestwick, and NINDS libraries resulted in identification of several compounds that effectively inhibited the CPE of SARS CoV in vitro, making them excellent candidates for further evaluation.

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