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Assay development and high-throughput antiviral drug screening against Bluetongue virus

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ABSTRACT

Bluetongue virus (BTV) infection is one of the most important diseases of domestic livestock. There are no antivirals available against BTV disease. In this paper, we present the development, optimization and validation of an in vitro cell-based high-throughput screening (HTS) assay using the luminescent-based CellTiter-Glo reagent to identify novel antivirals against BTV. Conditions of the cytopathic effect (CPE)-based assay were optimized at cell density of 5000 cells/well in medium containing 1% FBS and a multiplicity of infection at 0.01 in 384-well plate, with Z'-values $\geq$0.70, Coefficient of Variations $\geq$5.68 and signal-to-background ratio $\geq$7.10. This assay was further validated using a 9532 compound library. The fully validated assay was then used to screen the 194 950 compound collection, which identified 693 compounds with >30% CPE inhibition. The 10-concentration dose response assay identified 185 structures with $IC_{50} \leq 100\,\mu M$, out of which 42 compounds were grouped into six analog series corresponding to six scaffolds enriched within the active set compared to their distribution in the library. The CPE-based assay development demonstrated its robustness and reliability, and its application in the HTS campaign will make significant contribution to the antiviral drug discovery against BTV disease.

1. Introduction

Bluetongue virus (BTV) is a non-enveloped, multi-layered, double-stranded RNA virus in the genus Orbivirus, family Reoviridae, one of the largest families of viruses including major human pathogens (e.g., rotavirus) and other vertebrate, plant and insect pathogens. BTV is transmitted by biting midges of *Culicoides*, including *C. variipennis* and *C. imocola* (Mellor, 2000). BTV replicates in both vertebrate and insect hosts, causing diseases in wild ruminants and economic important animals including sheep, goat, cattle and other domestic animals around the world (Mellor, 2000; Mellor and Wittmann, 2002), with $3 billion/year loss worldwide (MacLachlan and Osburn, 2006; Mellor et al., 2008). Due to its economic significance, BTV has been the subject of extensive molecular, genetic and structural studies and now represents one of the well-characterized viruses.

There are no antiviral drugs and no effective control measures developed against BTV. Currently, vector control and vaccination using BTV virus-like particles are two main prevention strategies being implemented to prevent the possible BTV epidemics (Blair et al., 2000; Travanty et al., 2004). However, such strategies have been demonstrated to be insufficient due to the changes of vector habitats and new virus strains. The 1998–2001 outbreak of BTV in the Mediterranean Basin was the greatest epizootic of the disease on record, showing that BTV had extended its range northwards into areas of Europe that were never affected before and has persisted in many of these locations (Meiswinkel et al., 2008). This epidemic has been linked to the recent distribution extensions of its major vector, *C. imicola*, the involvement of novel *Culicoides* vector(s), and an apparent ability of the virus to overwinter in the absence of adult vectors (Mellor et al., 2008; Mellor and Wittmann, 2002). Thus, it is urgent to develop effective antiviral drugs against BTV infection to avoid possible epidemics.

Developing robust and reliable biological assay(s) is critical for antiviral drug discovery. Traditional antiviral drug discovery assays include cytopathic effects (CPE)-based assay, genomic and/or subgenomic replicon-based assay, and plaque forming assay (Puig-Basagoiti et al., 2005; Severson et al., 2007, 2008). CPE is evident in BTV infected vertebrate cells, such as HeLa, BSR, and HEK 293T, including both the biochemical and morphological hallmarks of apoptosis (caspase-3 activation, DNA fragmentation, membrane blebbing and cellular shrinkage) (Li et al., 2007). BTV-induced CPE

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and/or apoptosis could be monitored using different CPE and apoptosis detection methods, which make the development of a robust and reliable assay possible. Here, we present the development of a robust and reliable CPE-based high-throughput screening (HTS) assay for antiviral drug discovery against BTV infection, and the identification of novel antiviral compounds that protect host cells from BTV infection.

2. Methods and materials

2.1. Cell culture

BSR cell line, a derivative cell line from baby hamster kidney (BHK) cells, was maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 5% fetal calf serum (FCS), 100 U/ml of penicillin, and 100 μg/ml of streptomycin (Sigma–Aldrich Chemical Co., St. Louis, MO). Unless otherwise noted, cells were harvested and re-suspended in assay medium, DMEM with 100 μg/ml streptomycin, 100 IU penicillin/l and 1% FBS. After plating and/or compound treatment, cells were incubated at 37 °C, 5% CO_2 and 80–95% humidity for the time period as noted.

2.2. BTV culture

The BTV-10 stocks were propagated in BSR cells as previously described (Li et al., 2007). Briefly, BSR cells were grown in T75 flasks to 90% confluent, and then infected with BTV-10 virus at multiplicity of infection (MOI) of 0.01. After 72 h incubation, cells were harvested and centrifuged. The cell pellet was re-suspended in 20 ml media, and sonicated. The cell debris were then centrifuged at 1500 × *g* for 10 min, and the supernatants were aliquoted (1 ml each tube) and saved at −80 °C. The BTV stocks from the cells were titrated in BSR cells using the $TCID_{50}$ assay. The final titer was at 1×10^8 $TCID_{50}$/ml.

2.3. Virus titration using the $TCID_{50}$ assay

Virus titers were determined in BSR cell monolayers grown in 96-well plates. BSR cells were seeded at 15 000/well in a 96-well plate, and incubated for 2 h at 37 °C, 5% CO_2 and 80–95% humidity. A volume of 100 μl of 10-fold serially diluted virus was inoculated into each well in quadruplicate format. Plates were further incubated at 37 °C, 5% CO_2 and 80–95% humidity for 72 h, after which, CPE was observed microscopically at 40× magnification. Virus titers were expressed as $TCID_{50}$ unit per ml of solution.

2.4. Control and compound preparation

Carrier control consisted of dimethyl sulfoxide (DMSO) diluted in complete assay media to 0.6% and 5 μl of which was dispensed to both cells and virus control wells of 384-well black clear-bottom tissue culture treated plates (Corning). Test compounds were diluted in media to 60 μM with a DMSO concentration of 0.6%. A total volume of 5 μl was dispensed to each well in the assay plate with a final assay volume of 30 μl resulting in final concentrations of 0.1% DMSO and 10 μM test compounds. Compound dilution and addition were performed with a Beckman Coulter FX liquid handler. After brief centrifugation, assay plates were transferred to the cell plating facility. For 10-concentration dose response assay, the compounds were added to assay media by a two-fold serial dilution, ranging from 20 μM to 0.04 μM.

2.5. Cell plating

For the validation assay and large scale HTS assay, cells were plated in 384-well plates at a cell density of 5000 cells/well (or otherwise noted) in 15 μl assay media using a WellMate bulk dispenser (Matrix, Inc.). Plates were then incubated for 2–4 h at 37 °C, 5.0% CO_2 and 80–95% humidity incubators (Thermo-Scientific).

2.6. Virus addition

Cells were infected with BTV at MOI of 0.01, or otherwise noted in the text. The BTV stock (1×10^8 $TCID_{50}$/ml) was first diluted in assay media containing 5000 $TCID_{50}$/ml of the virus. The diluted virus (10 μl) was added to the compound wells and the virus only control wells. Media only (mock infection) was added to the cell control wells. All additions were carried out using a Matrix WellMate housed in a class II Biosafety Cabinet within the Bio-Safety Level 2 laboratory. The plates were incubated for another 72 h, at 37 °C, 5% CO_2 and 80–95% humidity.

2.7. CPE assay and endpoint read

The CellTiter-Glo buffer and the lyophilized CellTiter-Glo substrate (Promega Inc., Madison, WI) were thawed and equilibrated to room temperature prior to use. The homogeneous CellTiter-Glo reagent solution was reconstituted after mixing the lyophilized enzyme/substrate and the buffer reagent according to the manufacturer's instructions. Meanwhile, the assay plates were also equilibrated to room temperature for 15 min. An equal volume (30 μl) of CellTiter-Glo reagent was added to each well. Plates were further incubated for 15 min at room temperature to stabilize luminescent signals. Luminescence was measured using a Perkin Elmer Envision multi-label reader with an integration time of 0.1 s.

2.8. Compound clustering

In order to identify common structural elements within the identified actives hits, the compound sets were clustered using cheminformatics tools available in LeadScope (LeadScope, Inc., Columbus, OH). A hierarchical cluster analysis was performed using a Tanimoto index threshold of 0.7, applying the complete linkage method. For each cluster, a representative substructure was identified as a common motif shared by all members of a cluster. Enrichment ratios were computed for the cluster substructures in order to predict whether the substructure may be considered privileged or not.

2.9. Data analysis

During the HTS campaign, 32 control wells containing cells only and 32 wells containing cells and virus were included on each assay plate. These controls were used to calculate *Z′*-value for each plate and to normalize the data on a per plate basis. All raw data were imported to Activity Base Data Management software (IDBS, Alameda, CA) for the determination of *Z′*-value, signal-to-background (S/B, fold decrease) ratio and percent inhibition for assayed compounds. Results were expressed as percent inhibition of CPE where 100% inhibition of CPE was equal to the mean of the cell only control. Compounds showing greater than average CPE inhibition plus three times standard deviation of % inhibition of viral-induced CPE of all compounds were considered "hits" and tested in 10-concentration dose response assay. Statistical calculations of *Z′*-values were made as follows:

$$Z' = 1 - ((3\mathrm{StdDev_S} + 3\mathrm{StdDev_B})/|\mathrm{Mean_S} - \mathrm{Mean_B}|)$$

Here $\mathrm{Mean_S}$ is the mean luminescent signals from cell control, $\mathrm{StdDev_S}$ is the standard deviation of the luminescent signals from cell control, $\mathrm{Mean_B}$ is the mean luminescent signals from BTV infection control, and $\mathrm{StdDev_B}$ is the standard deviation of the luminescent signals from virus infection control. $\mathrm{S/B} = \mathrm{Mean_S}/\mathrm{Mean_B}$

(Ghosh et al., 2005; Zhang et al., 1999). Each assay validation step was performed in triplicate with the exception of the validation screening of 9532 compounds which was performed in duplicate. The screening of NIH Molecular Libraries Small Molecule Repository (MLSMR) with 194 950 compounds and dose response assay were carried out once.

3. Results

3.1. BTV infection induces apoptosis and CPE in BSR cells

BTV infection induces apoptosis and/or CPE in a number of mammalian cells, including Vero, HeLa, BHK/BSR (Li et al., 2007; Mortola et al., 2004; Nagaleekar et al., 2007; Schwartz-Cornil et al., 2008). Characteristics of apoptosis and CPE, including cell shrinkage, membrane blebbing, and DNA fragmentation, have been shown and the apoptotic pathway has been elucidated (Li et al., 2007; Mortola et al., 2004; Nagaleekar et al., 2007). The quantitative analysis of the CPE induced by BTV infection was shown here using 96-well plate. BSR cells were seeded with a density of 15 000 cells/well, and infected with BTV at different MOIs, 0.5, 1.0 and 10, respectively. At different hours post-infection (h.p.i.), BTV-induced CPE was determined using the CellTiter-Glo Luminescent Cell Viability kit, a homogeneous method of determining the number of viable cells in culture based on quantitation of the ATP present, which signals the presence of metabolically active cells. The single step "add–mix–measure" format resulting in cell lysis and the generation of luminescent signals is also ideal for future large scale HTS of various compound libraries. While there was no significant decrease of cell viability in BTV infected cell at 24 h.p.i., a two-fold decrease of cell viability in BTV infected cells was observed in all three MOIs at 48 h.p.i. The decrease of cell viability was much more apparent at 72 h.p.i., with 9.7-, 12.0- and 11.7-fold decreases, i.e. S/B ratio, for MOI of 0.5, 1.0 and 10, respectively (Fig. 1). This result showed that BTV infection induced CPE can be quantitated, especially at 72 h.p.i. with remarkable high S/B ratios. Meanwhile, this single step CPE-based assay will be suitable for future HTS assays, although further optimizations were necessary to improve this CPE-based assay into a primary HTS assay.

3.2. DMSO sensitivity analysis

For large scale HTS, all compounds to be tested will be dissolved in DMSO solution. It has been shown that high DMSO concentrations (>1%) have dramatics effect on membrane permeability, cell viability and virus infection in MDCK cells (Irvine et al., 1999; Noah et al., 2007). Thus, it is necessary to evaluate the effect of DMSO on cell growth and cell viability of BSR cells. After being seeded in 96-well plates at 15 000 cells/well, BSR cells were treated with DMSO at different concentrations, ranging from 0.02% up to 2% (Fig. 2). After 72 h incubation, cell viability was determined using the CellTiter-Glo reagent. The effect of DMSO on cell viability was compared between the cell viability in DMSO treated cells relative to cell viability in cells that were not treated with DMSO. When BSR cells were treated with DMSO at 1% or lower concentration, there were no decreases on the cell viability. In contrast, there was a slight increase of cell viability, ranging from 5% to 12%, when cells were treated with DMSO concentration below 1%. However, when cells were treated with 2% DMSO, there was 28.9% decrease of cell viability, a considerable decrease comparing with cells without DMSO treatment (Fig. 2). This result supported that the designated 0.6% DMSO concentration for our HTS campaign will be suitable for the screening. Meanwhile, there should be no more than 1% DMSO added to the cell culture for the entire screening when using this assay.

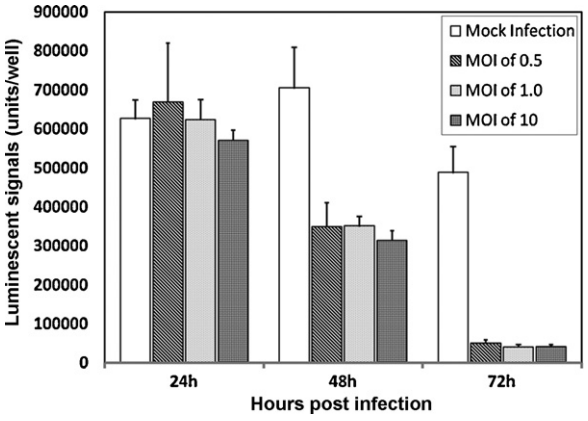


Fig. 1. BTV infection induced CPE in BSR cells. Cell viability at different MOIs was determined at 24, 48 and 72 h post-BTV infection using CellTiter-Glo reagent and expressed as luminescent signals (units per well). Mock infection: cells were mock infected with DMEM media. MOI: multiplicity of infection. The experiment was repeated for three times and each data point represents the average value of eight replicates.

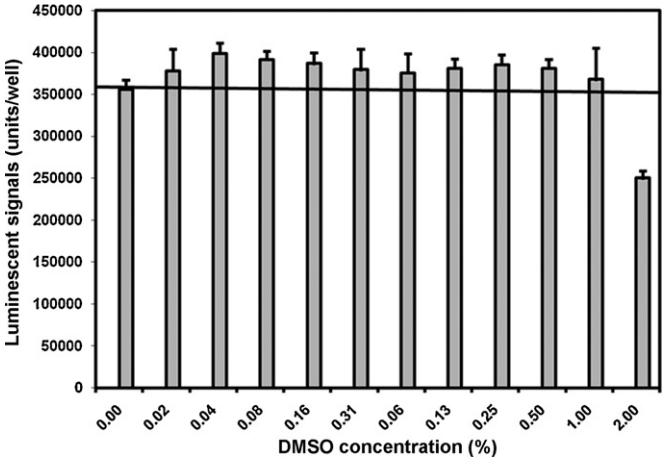


Fig. 2. The effect of DMSO on the cell viability at 72 h post-seeding. Cells were seeded and treated with different concentrations of DMSO (percentage). Cell viability was expressed by luminescent signals (units per well). Line indicated the level of luminescent signals from the cells with no DMSO treatment. The experiment was repeated for three times and each data point represents the average value of 16 replicates.

3.3. CPE-based assay optimization

3.3.1. Optimization of cell density

The seeding cell density is important to avoid possible cell overgrowth, to obtain a maximum and consistent luminescent signal strength and good S/B ratio (minimum > 5) in each well. To optimize the seeding cell density, BSR cells were seeded in 384-well plates at different density—2500, 5000, 7500 and 10 000 cells/well, respectively. Cell viability were evaluated and analyzed at different time post-seeding using CellTiter-Glo reagent (Fig. 3a). At cell density of 7500 and 10 000 cell/wells, cell growth reached a plateau at 48 h post-seeding and luminescent signals decreased at 72 h post-seeding. At cell density of 2500 and 5000 cells/well, cell growth did not reach a plateau at 72 h post-seeding and luminescent signals were increased from 48 to 72 h, and the 5000 cells/well density showed the maximal luminescent signals, i.e. cell viability, and was chosen as the optimal cell density for all subsequent experiments.

3.3.2. Optimization of FBS concentration

Cell growth and/or viability are also affected by the FBS concentration in the cell culture media. Traditional cell culture medium

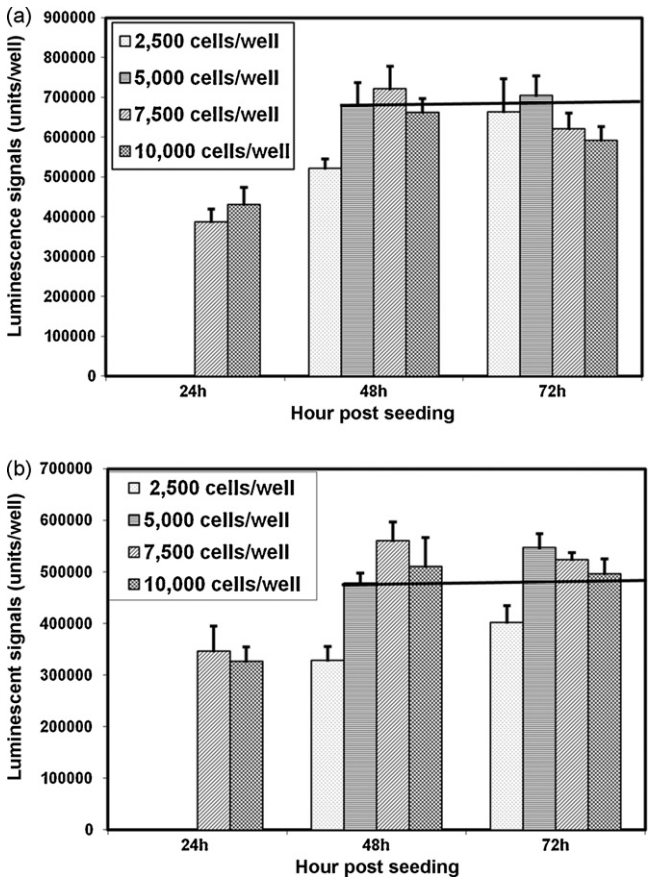


Fig. 3. The effect of seeding cell density and FBS concentrations in DMEM media on the BSR cells. Cell viability was expressed by luminescent signals (units per well). Cells were seeded in 384-well plates at different cell densities with different FBS concentration in DMEM media, 5% FBS concentration in (a) and 1% FBS concentration in (b). Cell viabilities were measured at different time post-seeding and were expressed as luminescent signals per well using CellTiter-Glo reagent. At 24 h post-seeding, data for cell density at 2500 and 5000 cells/well were not shown in both (a) and (b). The experiment was repeated for three times and each data point represents the average value of 16 replicates.

contains 5% or higher FBS which keeps the cells grow rapidly, however, the risk of overgrowth, which may lead to the decrease of cell viability, is high. Also, some compounds and viruses are known to bind to FBS and therefore would show up as false negatives in a screen. Two different FBS concentrations, 5% and 1% in DMEM media, were evaluated at different seeding cell densities. At both concentrations, seeding density at 7500 and 10 000 cells/well showed similar pattern and the decreases of cell viability were observed at 72 h post-seeding. At 5000 cells/well seeding density, although cells were still multiplying at 72 h for both 1% and 5% FBS, the cell growth at 1% FBS concentration was more active when compared with cell growth at 5% FBS. Thus, we chose 1% FBS concentration and 5000 cells/well cell density as the optimal cell culture conditions for all subsequent experiments.

3.3.3. Optimization of BTV infection

The optimization of BTV infection was carried out to achieve the relative highest luminescent signals at the lowest MOI. In the previous experiment, at MOI of 0.5, the luminescent signals were decreased to a level similar to that obtained with an MOI of 1.0 and 10. Further observation showed that at even lower MOIs, CPE was also obvious (results not shown). Thus, we chose five low MOIs, 0.0025, 0.005, 0.01, 0.02 and 0.04 to infect BSR cells seeded at optimized cell density (5000 cells/well). After cells were incubated for 48 and 72 h, cell viabilities were evaluated using the CellTiter-Glo

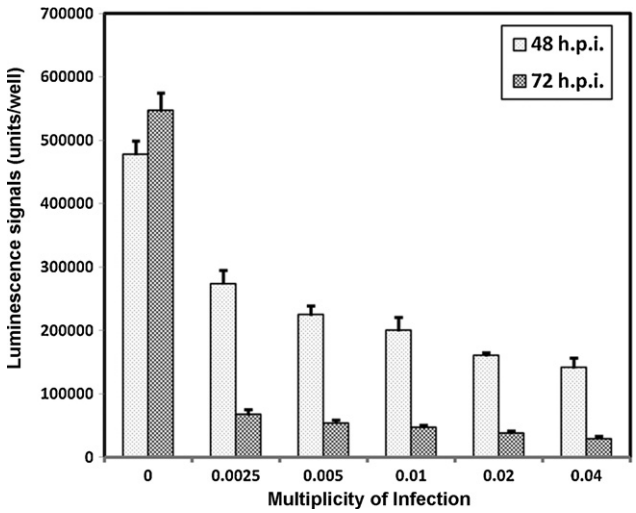


Fig. 4. Cell viability at 48 and 72 h post-BTV infection at different MOIs. All cells were infected by BTV at different MOIs as noted. Cell viability, expressed as luminescent signals (units per well), was measured at 48 and 72 h.p.i. using CellTiter-Glo reagent. The experiment was repeated for three times and each data point represents the average value of 16 replicates.

reagent. At 48 h.p.i, cell viability was 1.7, 2.1, 2.4, 3.0 and 3.4 folders lower for MOIs of 0.0025, 0.005, 0.01, 0.02 and 0.04, respectively, when compared with that of the mock infected cells. At 72 h.p.i., the fold decreases (S/B ratios) were 6.3, 8.5, 11.4, 14.4 and 20.9 for MOIs of 0.0025, 0.005, 0.01, 0.02 and 0.04, respectively (Fig. 4). The luminescent signals at MOI of 0.01 was comparable to that at MOI of 0.02 and 0.04 with good S/B ratio (11.4), which fit our standard to achieve relative high signal difference with lowest MOI. Thus, MOI of 0.01 was selected for all subsequent experiments including the primary HTS assay.

3.3.4. Z′-Value determination

The Z'-value analysis is designed to evaluate if an assay is robust enough for screening on an HTS platform. The Z'-value evaluates the assay's signal dynamic range, the data variation associated with the sample measurement, and the data variation associated with the reference control measurement. To determine the Z'-value, the CPE-based assay in 384-well plates was run at three different days and three different assays, at the optimized conditions including cell density at 5000 cells/well, 1% FBS media, BTV infection at MOI of 0.01 and final luminescent signals read at 72 h.p.i. The 384-well plate was divided into four quadrants, with the BTV infected cells as positive controls (S) in Quadrants I and IV, and the mock infected cells as negative controls (B) in Quadrants II and III on the plate. The Z'-value analysis was performed repeatedly to demonstrate reproducibility. We obtained an average Z'-value of 0.70 ± 0.01 under the optimized assay condition using the formula in Section 2.9 (Zhang et al., 1999). This demonstrated that the assay was robust and suitable as a primary HTS assay. Furthermore, according to our experience in HTS setting, the Z'-value would be higher once this assay was fully automated.

3.4. HTS assay validation

After the CPE-based assay was optimized and Z'-value determined in the laboratory, the assay was transferred to and adapted by the HTS Center at Southern Research Institute. The assay was first repeated in 384-well plates using automated liquid handling equipment which was also used for the screening of large scale compound library in Section 3.5. Integration of the assay onto a HTS platform was evaluated at each individual step of the procedure,

Table 1
Summary of the validation screenings and HTS of MLSMR against BTV.

	N	*Z′*	CV (cell)	CV (virus)	S/B ratio	Number of hits	Hit rate
10K validation repeat I	9532	0.70 ± 0.06	5.94 ± 1.71	19.87 ± 3.19	7.56 ± 0.43	22 (>21.7%) 5 (>50%)	0.23% 0.05%
10K validation repeat II	9532	0.71 ± 0.05	4.52 ± 1.07	13.70 ± 2.41	4.49 ± 0.22	135 (>21.7%) 10 (>50%)	1.41% 0.10%
200K primary screening	194950	0.70 ± 0.06	4.63 ± 1.31	10.50 ± 3.54	3.88 ± 0.56	693 (>30%) 147 (>50%)	0.36% 0.07%

The antiviral assay against BTV was validated twice using the 10K compound library. The primary HTS against BTV was carried out once via the screening of a 194950 compounds from NIH MLSMR. Results from the validations and primary screening were summarized and compared in the table. Number of hits and hit rates were expressed in two data sets. One set was expressed as the mean inhibition of CPE plus three times the standard deviation, which varied from assay to assay (>21.7% for the 10K screenings I and II, and 30% for the 200K screening). Another set was expressed as the 50% inhibition of the virus induced CPE.
N: number of compound screened; *Z′*: average *Z′*-value from each plate screened; CV: coefficient of variation; S/B ratio: signal-to-background ratio.

from loading the plates with cells to the final data analysis. The examination of intra- and inter-run variations, including well to well, plate to plate, and day to day variability, were carefully evaluated by running three *Z′* plates on three separate days for a total of nine plates, determined that the assay was sufficiently robust to screen a large compound library. Our results showed that the average *Z′*-value from these repeats were 0.75 ± 0.05, average coefficient of variations (CV) were 5.68 ± 0.62% for the cell control and S/B ratio of 7.10 ± 1.72. This results demonstrated that the quality control parameters for the assay met the requirements, including CV < 10%, *Z′*-values > 0.5, S/B > 5, and inter-run reproducibility.

The final validation step was the screening of a small compound set of 9532 compounds on two different time and statistically comparing the results for reproducibility. The 9532 compounds were screened at 10 μM concentration in two duplicate screens at different time. Quality control was assessed at several different points within the assay process. The values for the cell control and the BTV infection control wells were reviewed to determine the viability of each plate of compound data. Values from the duplicate screening were also compared to determine consistency. The average *Z′*-value from each plate, CV, S/B, and hit rates for compounds that inhibited CPE above 21.7% and 50%, were showed in Table 1. The 21.7% inhibition of CPE equaled to average CPE inhibition plus three times standard deviation of % inhibition of all compounds from the duplicated screenings. The reproducibility and signal window robustness of the duplicate screenings were demonstrated with similar *Z′* values (0.70 vs 0.71), CVs from both cell controls (5.94 vs 4.52) and virus controls (19.87 vs 13.70), and S/B ratios that averaged from each plate (7.56 vs 4.49) (Table 1). Overall, we observed an average hit rate of 0.2–1.4% and 0.05–0.1% for compounds that inhibited 21.7% and 50% of BTV-induced CPE, respectively. The Pearson's product moment correlations were high when comparing the duplicate screens, with a correlation coefficient of 0.80 with a *P* value of 0.23 indicating that there was no difference between the duplicate screenings.

3.5. Primary screening MLSMR compound library

After the reproducibility and robustness of the CPE-based assay were demonstrated and validated, 194950 compounds from MLSMR were screened. Compounds from the MLSMR collection are generically grouped into the following four categories (a) specialty sets (SS), such as known drugs and toxins, (b) targeted libraries (TL) such as kinase or GPCR inhibitors, (c) natural products (NP), and (d) diversity compounds (DC), which represents general chemical diversity. These compounds were selected based on their physicochemical properties with cutoffs applied, including MW < 1000, cLogP < 5, HBA < 20 and HBD < 10. All compounds were screened once at 10 μM using the validated CPE-based assay in six separate runs under the exact optimized experimental conditions. The hits identified were based on a 30.04% inhibition of virus induced CPE which equaled to average inhibition plus three times standard deviation of % inhibition of all compounds from the entire MLSMR screen. The assay robustness, including the average *Z′*-value and S/B ratio were graphically illustrated in Fig. 5. The average *Z′*-value for the entire screening was 0.70 ± 0.06 (Fig. 5a), which matched to the *Z′*-values obtained from the duplicated validation screenings. The average CVs were 4.63 ± 1.31 for cells only and 10.50 ± 3.54 for virus infection only (results not shown). The S/B ratio was 3.88 ± 0.56 (Fig. 5b) for the entire screening including the cell control, virus infection control and the virus infection plus drug treatments. The percentage of inhibition of virus induced CPE from each compound in the screening was plotted in Fig. 6. Out of the 194950 compounds screened, we identified 693 hits with over 30% CPE inhibition, with

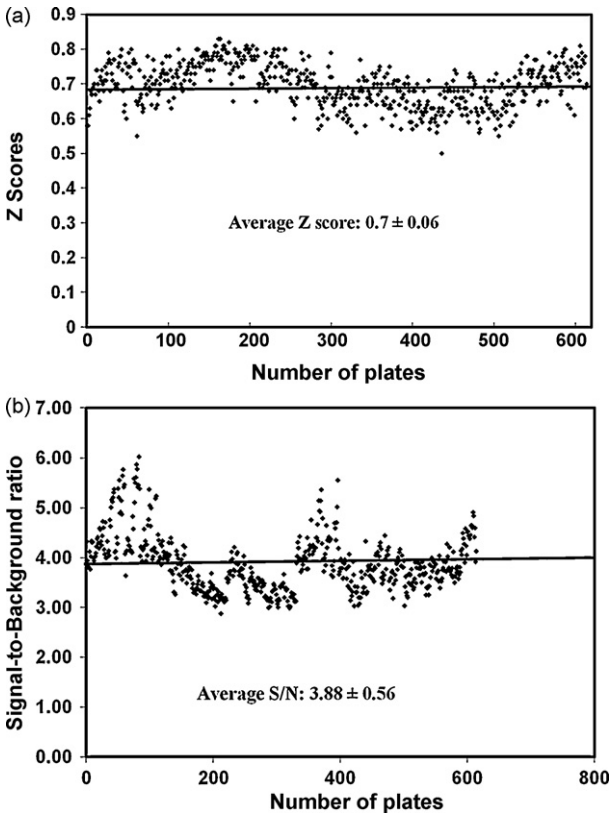
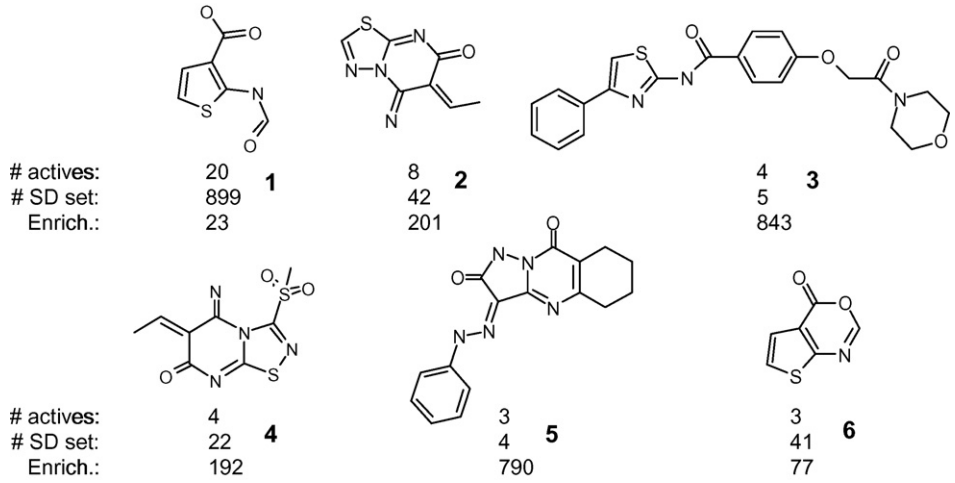


Fig. 5. The average *Z′*-value (a) and average signal-to-background (S/B) ratio from the screening of the 194950 NIH MLSMR collection. Each dot represents the *Z′*-value (a) or S/B (b) from one plate in the primary HTS. The line in the figure represents the average *Z′*-value (a) or S/B (b) obtained by averaging the total of 614 plates from the whole HTS.



Scheme 1. Scaffolds representative of clusters identified from clustering the compounds confirmed in dose response assay. # actives are the total number of active compounds containing the given substructure. # SD set is the total number of compounds containing the given substructure within the entire single dose screened library. Enrich.: meant the computed enrichment ratio.

a hit rate of 0.03%. There were 147 compounds identified with over 50% inhibition of virus induced CPE (Table 1 and Fig. 6).

Six hundred and fifty-eight of these compounds were available for further testing using a 10-concentration dose response assay. Out of the 658 dose response tested hits, an 'active set' of 185 compounds possessing IC_{50} values less than 100 μM was selected for the purpose of structural analysis. In order to identify common structural elements within the 185 actives, this set was clustered. For each cluster, a representative substructure was identified as a common motif shared by all members of the cluster. The enrichment ratio characterizing a particular substructure was defined here as the ratio between the percentage of compounds that contain this substructure within the active set (185 compounds) and the same percentage within the entire library screened (194 942 compounds). Privileged scaffolds contributing to the activity of hits were typically present in higher percentages among active compounds compared to their distribution within the entire screened library. We selected scaffolds representative of hit clusters that showed enrichment ratios greater than 10 and these were listed in decreasing order of cluster sizes in Scheme 1. Privileged scaffolds were likely to be present among non-clustered actives or in small clusters (with members less than 3) as well. Therefore selection of scaffolds further pursued will include clustered hits as well as non-clustered, singleton active structures.

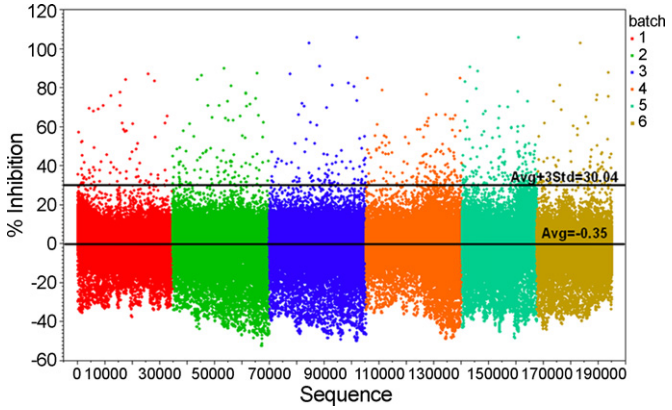


Fig. 6. The percentage of inhibition of virus induced CPE from each compound in the HTS against MLSMR compound library. Each dot represents one compound from the library, and total of six batches were plotted here. The higher line at a 30.04% inhibition of virus induced CPE was calculated as the average inhibition (0.35%, the lower line) plus three times standard deviation of % inhibition of all compounds from the entire MLSMR screen.

4. Discussions

CPE is a general term used to describe the adverse effect on the cells post-viral infection. CPE involves at least two distinct processes: necrosis and apoptosis. Various assays are available to distinguish these two mechanisms through measuring a variety of different markers that indicate the number of dead cells (cytotoxicity), the number of live cells (viability), and the mechanism of cell death (apoptosis). Most conventional cell-based antiviral drug screening assays were developed based on the cell viability assay (ATPase, kinase and cytotoxicity). The introduction of CPE-based assay using ATP/luminescence readout, such as CellTiter-Glo reagent, has been recently reported, which made the HTS against large compound library possible in several different antiviral assays, including the antiviral HTS assays against influenza virus (Noah et al., 2007; Severson et al., 2008), SARS-CoV (Severson et al., 2007), Yellow fever virus (Gong et al., 2008), Crimean-Congo hemorrhagic fever virus (Paragas et al., 2004). Our CPE-based antiviral assay against BTV also showed its feasibility with the "mix and measure" one step protocol, and flexibility with stable luminescent signals. Meanwhile, toxic compounds that reduced the cell viability were also excluded in this CPE-based assay. This CPE-based assay was also easy to be adapted and validated in HTS Center, making it possible to screen large compound libraries, such as MLSMR collection. This assay may also be used for assays based on CPE induced by other reoviruses and arboviruses.

Our assay also demonstrated its robustness with a high degree of reproducibility and low deviation including a reproducible Z'-value over 0.7, high sensitivity and low background with S/B ratios ranging from 3.88 to 7.56. Typically, a S/B ratio greater than 5 is an adequate detection window, and the Z'-value >0.5 are considered robust enough for a HTS assay (Zhang et al., 1999). The duplicated validation screening of the CPE-based assay using the 9532 testing compound library, and the primary HTS assay against MLSMR further demonstrated the assay was sufficiently robust and reproducible for a HTS platform with very similar statistical assay performance values, including Z'-value between 0.70 and 0.71, CV for the cell control between 4.52% and 5.94%, and S/B between

4.49 and 7.56 (Table 1). CV from the virus infected control was higher than 10%, which is natural for the virus infected cells due to the complexity nature when virus infection process. The S/B ratio form one of the validation screenings was 4.49, lower than 5.0 as requirement, however, the consistent Z'-value from the validation screenings indicated that this assay was robust for using in HTS. Indeed, similar Z'-values, CV and S/B ratio were also obtained for these assay parameters in our primary HTS, which further demonstrated that the assay is reproducible and reliable.

All three screenings showed similar hit rates, between 0.05% and 0.10% for the hits that inhibited CPE by >50% (Table 1). Similar hit rates were observed in a number of HTS assays against various viruses, including 0.08% and 0.022% hit rates in two assays for identification of influenza inhibitors, respectively (Noah et al., 2007; Severson et al., 2008), and 0.01% for SARS-CoV HTS (Severson et al., 2007). Although it is possible to increase the hit rate by manipulating the assay conditions, including dosing at a higher compound concentration (i.e. >10 μM) and/or reducing the assay length from 72 to 48 h, we were very confident that these hits identified through the primary HTS were accurate, evidenced by the corresponding hit correlation in the duplicated 10K screening and confirmed by the dose response assay.

Hit compounds with IC_{50} less than 100 μM were clustered to identify common substructures. Among these, Scheme 1 lists substructures with highest enrichment among active hits comparing to their distribution in the entire library screened. The clusters identified include mononuclear heterocylic compounds such as the thiophene and thiazole derivatives **1** and **3**, bicyclic compounds such as the pyrimidines **2**, **4** and **5** fused to a 1,3,4-thiadiazole, 1,2-4-thiadiazole and pyrazole ring systems. Scaffolds **2** and **4** have very similar core scaffolds. The thienooxazinones **6** can be considered as cyclic derivatives of the ortho sustituted thiophene derivatives represented by **1**. Note, scaffold **3** may be considered a small group of closely related analog series where nearly all members in the series were active.

The development of a CPE-based assay against BTV infection and the HTS of the MLSMR library provided a unique model system and identified a number of novel antiviral compounds that protect host cells from BTV infection. BTV is the prototype virus in the genus Orbivirus within the family Reoviridae. BTV is considered to be one of the most important diseases of domestic livestock, and has been a subject of intense molecular study for the last three decades and is now one of the best understood viruses at the molecular and structural levels (Mellor et al., 2008; Roy, 2008). As a member of reoviruses and arboviruses, BTV must be capable and should be sufficiently flexible to allow its replication in evolutionarily distant hosts, which also applied to other reoviruses and arboviruses including members in the families of Togaviridae, Flaviviridae, Bunyaviridae, Rhabdoviridae, and Reoviridae. Since antiviral compounds identified here could protect host cells from BTV-induced CPE responses, these compounds might also protect host cells from other reoviruses and arboviruses infections. Meanwhile, any compound identified using this assay could be used as a chemical probe to further study the virus–host interactions. Thus, our developed HTS assay will be of direct benefit to the drug development and drug design to counter reoviruses and arboviruses diseases, thereby have a positive impact on the widespread burden of these diseases.

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