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A Sensitive *In Vitro* High-Throughput Screen to Identify Pan-Filoviral Replication Inhibitors Targeting the VP35-NP Interface

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Abstract

The 2014 Ebola outbreak in West Africa, the largest outbreak on record, highlighted the need for novel approaches to therapeutics targeting Ebola virus (EBOV). Within the EBOV replication complex, the interaction between polymerase co-factor, viral protein 35 (VP35), and nucleoprotein (NP) is critical for viral RNA synthesis. We recently identified a peptide at the N-terminus of VP35 (termed NPBP) that is sufficient for interaction with NP and suppresses EBOV replication, suggesting that the NPBP binding pocket can serve as a potential drug target. Here we describe the development and validation of a sensitive high throughput screen (HTS) using a fluorescent polarization assay. Initial hits from this HTS include the FDA approved compound tolcapone, whose potency against EBOV infection was validated in a non-fluorescent secondary assay. High conservation of the NP/VP35 interface among filoviruses suggest that this assay has the capacity to identify panfiloviral inhibitors for development as antivirals.

Keywords

Ebola virus; nucleoprotein; VP35; fluorescence polarization assay; high-throughput screening

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SUPPORTING INFORMATION The Supporting Information is available free of charge via the Internet at <http://pubs.acs.org/>.

INTRODUCTION

Filoviruses, including *Ebolavirus* and *Marburgvirus*, belong to the family *Mononegavirales*, which includes non-segmented negative-sense RNA viruses¹. They are causative agents of severe hemorrhagic fever and associated with case fatality rates up to 90% in human patients². The most recent 2014 outbreak in West Africa was the largest in history with over 28,000 confirmed cases and over 11,000 deaths related to EBOV infection according to the WHO³. While there are many promising vaccine candidates⁴⁻⁵ and a monoclonal antibody⁶⁻⁷ mediated therapy that look promising, currently no approved efficacious and safe treatments are available against EBOV infection (CDC. Treatment of Ebola Hemorrhagic Fever. Available from: <http://www.cdc.gov/vhf/ebola/treatment>). For example, ZMapp, a mixture of three monoclonal antibodies that showed initial efficacy against Ebola infection in rhesus macaques⁸, did not show high potency as expected in a randomized and controlled trial in human subjects⁹. Thus, a potent and safe treatment is urgently needed. The most advanced approaches for anti-filovirus therapy, with demonstrated effectiveness in non-human primates (NHPs), utilize RNA interference (RNAi), mixtures of monoclonal antibodies, or repurposed influenza, herpes, respiratory syncytial (RSV), or hepatitis C (HCV) virus inhibitors¹⁰. However, the efficacy of these agents remains unproven; furthermore, many were developed for other viruses and may suffer from limited potency against filoviruses. In addition, the biologics (antibodies, RNAi) are expensive, difficult to store and administer in the field, and act in a viral species-specific manner, which will necessitate stockpiling multiple agents to cover all known virulent filoviruses, or reducing their effectiveness in outbreaks caused by new viral species.

The viral replication complex is an attractive target for drug development because of its essential role in viral pathogenesis. Small molecules that inhibit the formation and/or functions of the viral replication complex are desirable as these would restrict viral infection and spread. The EBOV viral genome encodes for seven open reading frames, of which nucleoprotein (NP), viral protein 35 (VP35), and L (the RNA-dependent RNA polymerase), are required for viral replication in the cytoplasm¹¹⁻¹², along with VP30 for transcription. VP35 is thought to bridge the interaction between L and NP, which encapsidates the viral genome¹¹⁻¹³. We have previously identified a minimal NP-binding peptide region in EBOV VP35, called NPBP (residues 20–48), that is required for interaction with NP¹⁴. This interaction between VP35 NPBP and NP is critical for viral replication as mutations of residues in VP35 NPBP results in diminished activity in the minigenome assay and overexpression of NPBP leads to suppression of EBOV viral replication in HeLa cells, suggesting the therapeutic potential of targeting the interaction between NPBP and NP¹⁴. The availability of the structure of EBOV VP35 NPBP/NP complex along with our studies on the VP35 NPBP/NP interaction provide a framework to design an assay to guide structure-based antiviral development.

Here we describe the development of a binding assay using fluorescence polarization as a platform for high throughput screening of small molecules that target the EBOV replication complex. We used the highly specific interaction between VP35 NPBP and NP to build a fluorescence polarization assay that detects fluorescein-labeled NPBP binding to NP. The assay was miniaturized, optimized, and validated prior to use in a high throughput screening

format. Our results highlight the high sensitivity of the assay and its potential to identify pan-filoviral inhibitors.

RESULTS AND DISCUSSION

The EBOV replication complex is minimally comprised of NP, VP35, and the polymerase L, where VP35 bridges the interaction between NP and L^{15–16} (Figure 1A). The minimal region of VP35 required to bind to the N-terminal domain of NP (NP_{NTD}), referred to as the NP binding peptide (NPBP), is highly conserved in filoviruses. Examination of the VP35 NPBP/ NP_{NTD} complex structure (PDB: 4YPI) reveals a large number of intermolecular interactions and surface complementarity between VP35 NPBP and NP_{NTD} that drives specificity and affinity (Figure 1B)¹⁴. Furthermore, VP35 NPBP binding to NP_{NTD} prevents oligomerization of NP¹⁴ and NP-RNA binding, which may be part of a critical mechanism to regulate viral RNA synthesis.

In order to identify inhibitors of this target site, we developed a fluorescence polarization (FP) assay to measure the interaction between NPBP and NP_{NTD}. The FP assay has a number of advantages compared to other biophysical and biochemical methods. It does not consume large amounts of proteins or probes, it is free of radioactive labels, homogeneous, and lacks a washing step that could disrupt the interaction between probe and receptor. The FP assay is both, sensitive and robust, and the FP signal is independent of probe concentrations providing useful information about biomolecular interactions. The 29 residue (~3.2 kDa) VP35 NPBP peptide was synthesized alone and with fluorescein isothiocyanate (FITC) conjugation at the N-terminus (Genscript, NJ). NP_{NTD} was expressed in *E. coli* and purified as previously described¹⁴. Upon excitation with polarized light, free FITC-NPBP emits depolarized light because of its fast molecular rotation (Figure 1C). Binding of the 48 kDa protein receptor, NP_{NTD}, restricts the mobility of FITC-NPBP (Figure 1C), and as a result, bound FITC-NPBP emits relatively more polarized light upon excitation. In addition, fluorescence polarization increases with increasing NP_{NTD} concentration, reflecting increased proportions of bound FITC-NPBP (Figure 2A). The dissociation constant, K_D , calculated from the titration curve is $32 \text{ nM} \pm 3 \text{ nM}$, consistent with K_D determined by isothermal titration calorimetry¹⁴, suggesting that FITC conjugation to the VP35 NPBP does not interfere with the NP-NPBP interactions. Titration of unlabeled NPBP into 1 nM FITC-NPBP + 20 nM NP_{NTD} (black, Figure 2B) demonstrates that NPBP displaces FITC-NPBP from NP_{NTD}, with an IC_{50} of $262 \text{ nM} \pm 68 \text{ nM}$. Two truncated VP35 NPBP constructs, NPBP mut1 (28–46) and NPBP mut2 (26–48), are also able to outcompete FITC-NPBP for NP_{NTD} with IC_{50} values higher than that of NPBP (Figure 2B). This is consistent with our ITC data showing that the K_D values of NPBP mut1 and mut2 are higher than that of NPBP¹⁴. However, it is important to note that at high concentrations both NPBP mutants reduced FP signal to the free-probe level similar to the effect of excess wild-type NPBP.

To determine assay sensitivity and to evaluate the potential of FP assays as a tool for HTS, we carried out a small scale FP assay. We measured the FP signals for a series of samples of free FITC-NPBP (red) and bound to NP_{NTD} (blue) and determined a ~190 mP window between positive and negative samples (Figure 3A and Table S1). A similar value was also obtained for bound FITC-NPBP with NP_{NTD} (magenta) and in the presence of unlabeled

NPBP (purple) (Figure 3B and Table S1). The Z'-factor is a statistical parameter used to assess the quality of a HTS assay and it is calculated using equation (4) and with control data¹⁷. A Z' factor between 0.5 and 1 indicates that the HTS assay is optimized sufficiently to enable the statistically significant identification of inhibitors. The Z'-factor values for both experiments are above 0.7 ($Z' = 0.81$ and 0.72 for in Figure 3A and 3B, respectively) demonstrating the robustness of the FP assay.

To further optimize the FP assay parameters, an optimal read height (distance between the microplate and instrument optics) was obtained at which data is collected producing maximum signal. Control samples described previously were collected at read heights (height from the reading plane to the detector) varying between 6 to 11.75 mm. At a read height above 8 mm, desirable maximum Z' values (0.86) were obtained (Figure S1A); and thus, an 8.5 mm read height was maintained for FP assays.

The effect of probe (FITC-NPBP) concentrations was also determined while keeping the NP_{NTD} protein concentration range constant (Figure S1B). FITC-NPBP probe concentrations over a range of 1 to 10 nM did not significantly affect the observed binding K_D . Similarly, the concentration of NP_{NTD}, protein receptor, was optimized. At NP_{NTD} concentration above 20 nM, Z' values remain above 0.8. (Figure S1C and S1D). Taken together, high-throughput screening experiments were performed with 1 nM probe (FITC-NPBP) and 20 nM protein receptor (NP_{NTD}).

The FPA was then transferred to a HTS format. A pilot screen was performed on a chemically diverse library of 640 FDA approved drugs to determine utility of the assay in a HTS format. Z' values are above 0.5 (0.51 and 0.67 for each assay plate). The separation between positive and negative controls (blue and magenta, respectively) is ~170 mP (Figure 3C). In addition, the majority of test wells (green) show mP values consistent with the negative control, with relatively few compounds showing significant inhibition/depolarization. These results demonstrate the robustness of the FPA in a scaled up HTS format and some selectivity for inhibition of the FITC-NPBP/NP interaction.

To determine the reproducibility of the FPA, a second HTS was performed on the same FDA library. In this second screen, the assay again showed Z' values above 0.5 (0.78 and 0.79 for each assay plate). Individual mP values for each well were then converted to Z-scores (number of standard deviations from the average negative control). Negative Z-scores indicate a response less than the average negative control potentially due to inhibition. Plotting the Z-scores for these duplicate sets against each other illustrates excellent reproducibility in the negative and positive control wells (Figure 3D, blue and magenta). In addition, the Z-scores for the screening wells demonstrate a strong linear correlation between both screening sets ($R^2 = 0.74$). Based on these results, we conclude that the FPA shows a high level of reproducibility between screening sets.

We implemented an FPA-based counter screen using two host proteins to isolate compounds that are potentially false positives. Nrf2 (nuclear factor erythroid-derived 2-like 2) is a transcription factor, which regulates expression of proteins that protect cells from oxidative stress¹⁸. The degradation of Nrf2 is controlled by Keap1 (Kelch-like ECH-associated protein

1), a substrate adaptor protein for the Cul3 E3 ubiquitin ligase. The interaction between Nrf2 and Keap1 have been previously characterized to involve the Nrf Neh2 domain residues ETGE and the Keap1 Kelch domain¹⁹. In the counter screen, FITC-labeled Nrf2 peptide containing ETGE residues (FITC-ETGE) was used as the probe and the Keap1 Kelch domain, as the protein receptor. Screening the previously described library in the counter screen, a total of seven compounds were identified as initial hits in both FITC-NPBP/

NP_{NTD} and FITC-ETGE/Kelch screens. These compounds were considered false positives and were excluded from downstream confirmation (Table S2).

Of the initial hits, 4 compounds (Figure 4) were further characterization. Trequisin showed no concentration dependent inhibitory effect on FP (blue, Figure 4A). Epinephrine-(+)-L(-) (Figure 4B) and 5-aminosalicylic acid (Figure 4C) disrupted the NP-NPBP interaction at high concentrations with a decrease in FP. However, fluorescence intensity also increased with increasing compound concentration, indicating that intrinsic compound fluorescence likely interferes with FP measurements (green, Figure 4B and 4C). Tolcapone (Figure 4D) showed a more dramatic dose-dependent inhibitory effect with an IC₅₀ of 2 μ M $\pm$ 0.2 μ M, while fluorescence intensity remained relatively the same.

As noted above, a potential limitation of the fluorescence polarization assay is that intrinsic fluorescence of compounds may dominate the FP signals and lead to false positive due to interference. Therefore, we developed an orthogonal non-fluorescence-based secondary assay using biolayer interferometry (BLI) to confirm the initial hits in order to eliminate high background issues associated with autofluorescence. Association of NP_{NTD} to biotinylated NPBP (Bio-NPBP) bound to streptavidin pins was measured in the presence of unlabeled NPBP, Trequisin, epinephrine-(+)-L(-), 5-aminosalicylic acid, or tolcapone. Figure 5A shows the overlay of a complete cycle, including dissociation, association, and baseline. Tolcapone is the most effective inhibitor of VP35 NPBP/ NP_{NTD} interaction (Figure 5B), consistent with the FP assay data (Figure 4).

We next tested tolcapone against a recombinant EBOV that expresses GFP (EBOV-GFP)²⁰ in Vero cells. Vero cells were initially treated with tolcapone at 10 μ M or DMSO only in the absence of EBOV to rule out effects simply due to cytotoxicity. We found the cell monolayers were still intact four days post treatment (Figure 5C). Therefore, cells were treated with 10 μ M tolcapone or diluent (DMSO) alone and infected with EBOV-GFP at an MOI of 0.01 or 2 PFU/cell (Figure 5C). Viral titers in the presence of DMSO or tolcapone were quantified by focus-forming assay 3 days post infection (Figure 5C– 5D). Tolcapone reduced EBOV titer by >100-fold at a MOI of 0.01 and by >5-fold at a MOI of 2. Taken together, these data demonstrate that the FPA HTS is able to identify inhibitors of EBOV replication.

The FPA described above can also be used to identify pan-filoviral inhibitors. The EBOV VP35 residues encompassed by NPBP is highly conserved among members of the *Filoviridae* family (Figure 6A). When we test binding of the EBOV VP35 NPBP corresponding peptide from three different filoviruses to EBOV NP_{NTD}, we find that all filoviral VP35 peptides bind with similar affinities (Figure 6B–E), suggesting that NP binding is a conserved function of VP35. Addition of each of the four compounds identified

from the FPA HTS for EBOV VP35 NPBP/ NP_{NTD} on the homologous proteins from MARV showed an inhibition pattern similar to that observed for EBOV VP35 NPBP/ NP_{NTD} (compare Figure 6F to Figure 5B). Taken together, these results suggest that the FP assay can be applied to targeting panfiloviral protein interactions and that inhibitors that interrupt EBOV NP-NPBP interactions will have similar impact on NP-NPBP interactions across species.

CONCLUSIONS

The fluorescence polarization assay described here is a robust and reliable assay that can identify inhibitors of Ebola viral replication and potentially function in a panfiloviral manner. From a pilot screen using FPA in a HTS format, we identified an FDA approved drug, tolcapone, whose potency against EBOV infection was confirmed in a cell-based assay. Since it is an existing drug, future studies of tolcapone will involve evaluation of its pan-filoviral activity and confirmation of activity in filoviral animal infection models to determine its safety and efficacy *in vivo*. Novel inhibitors discovered in the FPA will undergo chemical optimization of potency, selectivity, and drug-like properties to advance them toward therapeutics.

Extension of this assay to other NNSVs, such as human respiratory syncytial virus, as well as Nipah and Hendra viruses, would likely yield a class of inhibitors that can serve as biological probes or therapeutic leads. Because the interaction between viral nucleoprotein and co-factor VP35 of the replication complex is conserved across negative-sense RNA viruses, we envision that this FPA can be used as a tool to screen for small molecule inhibitors of replication of other RNA viruses.

METHODS

Protein Expression and Purification

All filoviral proteins and Keap1 Kelch were purified using methods previously described^{14, 21–22}. Protein purity was assessed by Coomassie staining of SDS-PAGE. All peptides, including VP35 NPBP, FITC-NPBP, Bio-NPBP, Nrf2 ETGE, and FITC-ETGE were purchased from GenScript (Piscataway, New Jersey).

Fluorescence polarization assay development

Parameter settings—FP assays were performed on Synergy NEO HTS and Cytation5 plate reader from BioTek (Winooski, Vermont) operating with Gen5 Software. Excitation and emission wavelengths were set to 485 and 529 nm, respectively, with a bandpass of 20 nm for both. Autogain function was selected. 25 reads were collected per well and the read height was set to 8.5 mm. On Synergy NEO, parallel fluorescence and FP signal from a probe-only sample were set to 50000 RFU and 20 mP, respectively. On Cytation5, parallel fluorescence from a probe-only sample was set to 50000 RFU and G factor was optimized following manufacturer guidance (set to 1.26 for most experiments) in order to achieve probe only fluorescence polarization signal of ~20 mP.

Titration experiments—FITC-NPBP at a final concentration of 1 nM was added to protein receptor samples (NP_{NTD}) prepared with a series of 2-fold dilutions with buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 2 mM TCEP, and 2% (v/v) DMSO in a 384-well plate (Corning). After a 10-min incubation, FP signals were measured. FP in millipolarization units (mP) were plotted against protein concentration (μM) using Origin software (San Clemente, California). Data were fitted using Equation (2).

Competition experiments—Competitor samples were prepared with a series of 2-fold dilutions. Protein receptor and FITC-NPBP were then added to competitors sequentially. Final protein receptor and FITC-NPBP concentrations used were 20 and 1 nM, respectively. Samples were incubated in a 384-well plate at room temperature for 30 min before data collection. FP signals were plotted against competitor concentration and fitted using Equation (3) in Origin.

Z'-factor determination—FP signals of a total of 24 samples of both positive and negative controls were measured and plotted using Origin. Z' values were calculated using Equation (4)¹⁷.

Data analysis—Fluorescence polarization is calculated automatically by Gen5 software using the following equation:

$$FP = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} \quad \text{Equation (1)}$$

where $I_{\parallel}$ is the parallel fluorescence intensity and $I_{\perp}$ is the perpendicular fluorescence intensity. Titration curves of the protein receptor were fitted using the following equation in Origin:

$$F = (F_{\text{bound}} - F_{\text{free}}) \times \frac{(K_D + L_{\text{tot}} + R_{\text{tot}}) - \sqrt{(K_D + L_{\text{tot}} + R_{\text{tot}})^2 - 4R_{\text{tot}}L_{\text{tot}}}}{2R_{\text{tot}}} + F_{\text{free}} \quad \text{Equation (2)}$$

Where F_{bound} is the FP when FITC-NPBP is saturated with NP, F_{free} is the FP of free FITC-NPBP, K_D is the dissociation constant, L_{tot} the total ligand/probe concentration, and R_{tot} the total receptor concentration. Competition data were fitted using:

$$F = F_{\text{free}} + \frac{F_{\text{bound}} - F_{\text{free}}}{K_D \times \frac{C_{\text{tot}} + K_2}{K_2 \times L} + 1} \quad \text{Equation (3)}$$

Where F_{free} is the FP of free FITC-NPBP, F_{bound} is the FP of FITC-NPBP saturated with protein receptor, K_D is the dissociation constant of FITC-NPBP, K_2 is the dissociation

constant of compound/competitor, and L is the concentration of the compound/competitor. Z' is calculated using the following equation¹⁷:

$$Z' = 1 - \frac{3(\sigma_+ + \sigma_-)}{|\mu_+ - \mu_-|} \quad \text{Equation (4)}$$

where σ_+ and σ_- are the standard deviation of positive and negative controls, respectively, and μ_+ and μ_- are the FP values of positive and negative controls, respectively.

Fluorescence polarization assay (HTS)

Automation and Screening Parameters—The pilot HTS was performed using an FDA compound library diluted in DMSO (initial concentration of 250 μ M). A Caliper LifeSciences SciClone ALH 3000 was then used to transfer 2 μ L of compound (final compound concentration of 10 μ M in the screening wells) or DMSO only (control wells) from these compound plates into black 384-well plates (Corning). A Matrix WellMate was used to add 50 μ L per well of screen/negative control and positive control master mixes prepared in buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 2 mM TCEP. Screening and negative control wells had a final concentration of 10 nM FITC-NPBP peptide probe and 20 nM NP receptor. Positive control wells had a final concentration of 1 μ M unlabeled NPBP in addition to the receptor and probe. Plates were then incubated for 10 minutes at room temperature before being read.

Read Parameters—For HTS, the FPA was performed using a PerkinElmer EnVision 2102 Multilabel Plate Reader equipped with a FITC FP Dual Emission Label. Read parameters on the PerkinElmer Envision were set as follows - read height 9 mm, excitation 480 nm, emission detection 535 nm, excitation energy 100%, 25 flashes per well. In addition, the gain for both P/S detectors was set to 300 with a G-factor of ~0.61. These parameters yielded a probe-only sample with values of ~500,000 RFU and ~20 mP.

Analysis—RFU and mP values were automatically calculated by the Perkin Elmer EnVision software as above (see *Data analysis*). Compounds showing significant inhibition with an mP z-score of <-5 and P and S plane RFU values within a z-score of 5 with respect to the negative control were selected as hits.

Dose Responses—Hit compounds were cherry picked from 2.5 mM compound stock plates. Dilution series were prepared to yield a 100 μ M highest concentration in the assay with serial dilutions down to 0.6 μ M. The FPA was performed, read, and analyzed as above.

FITC-ETGE counter screen

FITC-ETGE studies were carried out for the counter screen following a similar strategy to that described above for FITC-NPBP studies. Briefly, FPA studies were performed on a Cytation5 plate reader using 485 nm excitation and 528 nm emission filters appropriate for FITC labelled Nrf2 ETGE peptide (59-LEETGEFL-68). FP assays were performed in 20 mM Tris pH 7.5, 150 mM NaCl, and 2 mM TCEP and in black nonbinding surface Corning

3650 96-well plates. The binding affinity of FITC-Nrf2 ETGE peptide to the Keap1 Kelch domain was determined by titration experiments. Titration experiments were carried out with 2 nM FITC-ETGE peptide and increasing concentrations of the Keap1 Kelch protein ranging from $> 1.0\text{E-}5$ to $50\text{ }\mu\text{M}$. Experiments were performed at least in duplicate. FP was determined using Gen5 2.07 analysis software by measuring the parallel and perpendicular fluorescence intensity with respect to linear polarized excitation light. Competition assays were established using conditions described above for 1 nM FITC-Nrf2 ETGE peptide, 40 nM Keap1 Kelch, and varying concentrations of unlabeled Nrf2 ETGE peptide ranging from 0.025 to $100\text{ }\mu\text{M}$ in a $100\text{ }\mu\text{L}$ volume per well and analyzed using Origin software.

Biolayer interferometry (BLI) binding studies

BLI competition experiments were performed on an Octet RED from *forteBIO*. Streptavidin (SA) pins were soaked in wells with biotinylated NPBP at 50 nM for 15 min, washed with buffer for 5 min and baselined for 1 min. Bio-NPBP loaded SA pins were then moved to wells containing NP_{NTD} at 250 nM premixed with competitors at $10\text{ }\mu\text{M}$. NP_{NTD} alone or compounds was used as the reference to calculate relative response. Buffer contained 20 mM Tris (pH 7.5), 150 mM NaCl, 2 mM TCEP, 2% DMSO, 0.5% BSA, and 0.02% Tween 20. BSA and Tween 20 were used to prevent non-specific binding by NP_{NTD} to the streptavidin pins.

Cell culture

Vero E6 cells were maintained in minimal essential medium (MEM) with 2% FBS, 0.1% gentamicin, 1% nonessential amino acids, and 1% sodium pyruvate and cultured at $37\text{ }^{\circ}\text{C}$ and 5% CO_2 .

Viruses

Wild-type Zaire EBOV was previously modified by addition of the transcriptional cassette encoding enhanced green fluorescent protein (eGFP) between the NP and VP35 genes as described²⁰, generously provided by Dr. J. Towner and Dr. S. Nichol (CDC).

Infectious assay

Vero E6 cells were seeded at 2×10^5 /ml in 24-well plates with minimal essential medium with 10% bovine fetal serum and 0.1% gentamycin and incubated overnight. After changing growth media with 2% fetal bovine serum with gentamycin, cells were treated with either DMSO or Tolcapone in triplicate. At 24 hours post treatment, Vero cells were infected with EBOV-GFP at a MOI of 0.01 or 2 PFU/cell and incubated for 1 hour at $37\text{ }^{\circ}\text{C}$. Media was aspirated from cells, and cells were washed with PBS. 1 mL fresh medium with compounds was then added to the wells, and incubated at $37\text{ }^{\circ}\text{C}$. One hundred microliter aliquots were taken from the supernatant to determine the virus titer using focus-forming assay. One hundred microliters of medium containing DMSO or Tolcapone were added back to each well to maintain the total sample volume. Images were taken to record antiviral effects using the Olympus IX-71 Inverted Fluorescence Microscope, Hamamatsu camera with DCAM driver and HImage Software. All work with EBOV was performed in the biosafety level 4 (BSL-4) facility of the Galveston National Laboratory.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

The EBOV VP35/NP interface is a target for HTS assay development. (A) EBOV RNA synthesis requires viral proteins NP, VP35, and polymerase L. The N-terminal domain (NTD) of NP (blue) binds ssRNA and the VP35 NPBP (VP35 NP binding peptide) (red). The NP C-terminal domain (CTD) is important for viral replication, but the precise function is currently unknown. VP35 oligomerization domain (OD) and interferon inhibitory domain (IID) are labeled as shown. Arrows indicate interactions that are validated at present. (B) Ribbon representation of the crystal structure of the EBOV NP_{NTD} (blue) and VP35 NPBP (red) complex (PDB 4YPI). (C) Schematic of the fluorescence polarization assay based on the NP/NPBP interaction. Outcomes in the polarization assays and the corresponding peptides (NPBP or FITC-NPBP), protein receptor (NP_{NTD}) and competitor are labeled.

Figure 2.

FITC-NPBP binding to NP_{NTD} results in a highly sensitive FPA with a large dynamic range. (A) Titration with the protein receptor, NP_{NTD} , results in an increase in fluorescence polarization of FITC-NPBP peptide. Apparent $K_D = 32 \pm 3$ nM was calculated for FITC-NPBP from non-linear fitting (red) using Origin (San Clemente, California). (B) Competition with unlabeled NPBP (black) and NPBP truncation mutants 28–46 (red) and 26–48 (green) with 1 nM FITC-NPBP in the presence of 20 nM NP_{NTD} resulted in apparent dissociation constants of 0.27 ± 0.07 , 1.39 ± 0.36 , and 6.61 ± 1.47 μM for NPBP and NPBP mutants, respectively.

Figure 3.

Results from the initial screens reveals that the assay is highly reproducible. (A) FP signals from 22 samples containing 1 nM free FITC-NPBP (red, positive controls) and with 20 nM NP_{NTD} (blue, negative controls) were used to determine the Z'-factor, signal-to-noise (S/N) and signal-to-background (S/B) ratios, and assay window values using 1 nM probe (FITC-NPBP). See Table S1. (B) FP signals of 22 samples of 1 nM FITC-NPBP + 20 nM NP_{NTD} (blue, negative control) and with 1 μM NPBP (magenta, positive control) plotted versus sample wells. (C) Pilot screening using 640 FDA-approved drugs (green). 64 samples of 1 nM FITC-NPBP + 1 μM NPBP with 20 nM NP_{NTD} (magenta) and 64 of nM FITC-NPBP

with 20 nM NP_{NTD} (blue) are used as positive and negative controls, respectively. (D) Results from duplicate screens of the FDA-approved drugs were converted to Z-scores with respect to the negative control. The correlation coefficient (R^2) between the duplicate assays is 0.74.

Figure 4.

The NPBP/NP FPA assay identified several hits that show dose-dependent responses.

Representative data for a non-hit (A) and three hits (B–D) are shown where FP (blue) is plotted as a function of compound concentration for: (A) trequinsin, (B) epinephrine-(+)-L(-), (C) 5-aminosalicylic acid, and (D) tolcapone. Assay conditions contained 1 nM FITC-NPBP + 20 nM NP_{NTD} with 2-fold dilutions of compounds. Fluorescence intensity is plotted in green.

Figure 5.

Validation of compounds identified from FPA with biolayer interferometry (BLI) analysis and infectious assay. (A) BLI analysis of VP35 NPBP binding to NP_{NTD} in the presence of compounds. BLI association and dissociation curves for NPBP (black), epinephrine-(+)-L (-) (red), tolcapone (blue), 5-aminosalicylic acid (cyan), and trequinsin (orange). Baseline curve for DMSO only is shown in gray. (B) Normalized data from (A) at 5 seconds prior to the beginning of dissociation indicated by the arrowhead in (A). 250 nM NP_{NTD} and 10 μ M competitor were used in the assays. (C and D) Tolcapone inhibits the infection of Vero cells by EBOV-GFP. (C) Bright-field (top) and fluorescence (bottom) images of EBOV-GFP-

infected Vero E6 cells treated with DMSO (mock) or Tolcapone at 10 μ M 4dpi. (D) Viral titers were determined by focus-forming assay 3 dpi. Results are in triplicate and plotted as averages and standard deviations. * indicates p value <0.030, ** p value <0.011.

Figure 6.

EBOV NPBP/NP FP assay is a versatile panfiloviral assay that can function with NP proteins from all ebolaviruses and marburgviruses. (A) Multiple sequence alignment of the NPBP regions from *Ebolavirus* (accession AAQ55046), *Restonvirus* (accession AB050936.1), *Sudanvirus* (accession EU338380.1) and *Marburgvirus* (accession CAA78115) using ClustalW. Identical residues are shown in blue and labeled with '*'; strongly conserved in green and with '!'; and weakly conserved in dark blue and with '.'. FPA dose response curves for FITC-NPBP competition with corresponding NPBP sequences from (B) *Ebolavirus* (NPBP), (C) *Restonvirus* (rNPBP), (D) *Sudanvirus* (sNPBP), and (E) *Marburgvirus* (mNPBP). (F) Normalized BLI data for the competitors in B–E in Figure 5 using MARV NP as the protein receptor. 250 nM NP_{NTD} mNP_{NTD} and 10 μM competitor were used in the assays.