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High-throughput, luciferase-based reverse genetics systems for identifying inhibitors of Marburg and Ebola viruses ☆

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ABSTRACT

Marburg virus (MARV) and Ebola virus (EBOV), members of the family *Filoviridae*, represent a significant challenge to global public health. Currently, no licensed therapies exist to treat filovirus infections, which cause up to 90% mortality in human cases. To facilitate development of antivirals against these viruses, we established two distinct screening platforms based on MARV and EBOV reverse genetics systems that express secreted Gaussia luciferase (gLuc). The first platform is a mini-genome replicon to screen viral replication inhibitors using gLuc quantification in a BSL-2 setting. The second platform is complementary to the first and expresses gLuc as a reporter gene product encoded in recombinant infectious MARV and EBOV, thereby allowing for rapid quantification of viral growth during treatment with antiviral compounds. We characterized these viruses by comparing luciferase activity to virus production, and validated luciferase activity as an authentic real-time measure of viral growth. As proof of concept, we adapt both mini-genome and infectious virus platforms to high-throughput formats, and demonstrate efficacy of several antiviral compounds. We anticipate that both approaches will prove highly useful in the development of anti-filovirus therapies, as well as in basic research on the filovirus life cycle.

1. Introduction

Marburg virus (MARV) and Ebola virus (EBOV) are members of the family *Filoviridae*, and cause severe and often fatal hemorrhagic fevers in humans (Feldmann and Geisbert, 2011; US Centers for Disease Control and Prevention, 2013). The genomes of filoviruses are ~19 kb, single-stranded, negative sense RNA molecules encoding a total of 7 genes (NP, VP35, VP40, GP, VP30, VP24, L) separated by intergenic regions of varying lengths and flanked by untranslated regions (UTRs) at the 3′ and 5′ ends (Feldmann et al., 2013) (Fig. 1A).

Since the identification of MARV in 1967 and EBOV in 1976, significant filovirus research has focused on developing antiviral therapies. However, despite promising vaccine candidates, no licensed prophylactics currently exist for preventing or treating filovirus infections. Screening libraries of chemical inhibitors has proven to be an excellent approach, including treatments that target RNA replication using subgenomic (mini-genome) systems (Hoenen et al., 2011; Jasenosky et al., 2010). However, as such systems do not recapitulate the entire viral life cycle, antiviral effects must be confirmed using infectious virus. Previous efforts using infectious virus have utilized GFP or RFP-expressing EBOV (Ebihara et al., 2007; Towner et al., 2005) and MARV (Schmidt et al., 2011; Schudt et al., 2013), sometimes in conjunction with high-content imaging (Panchal et al., 2010, 2012). During the preparation of this manuscript, a novel EBOV expressing Firefly luciferase (fLuc) was reported (Hoenen et al., 2013). Although not adapted to a high-throughput format, this system allows for rapid quantification of viral replication by measuring intracellular fLuc expression.

Here, we present two complementary methods (dual-platform) that could substantially improve the ability to screen for potential MARV and EBOV inhibitors. The first platform utilizes a mini-genome replicon that expresses the Gaussia luciferase (gLuc) reporter gene, a protein that, unlike fLuc, is secreted directly into the cell-culture media making for easy, repetitive, and non-destructive quantitation of mini-genome replication. Further, this platform is non-infectious and can be handled in BSL-2 laboratories common in most university and pharmaceutical industry settings. The second platform uses recombinant infectious MARV and EBOV

☆ The findings and conclusions in this report are ours and do not necessarily represent the views of the Centers for Disease Control and Prevention.

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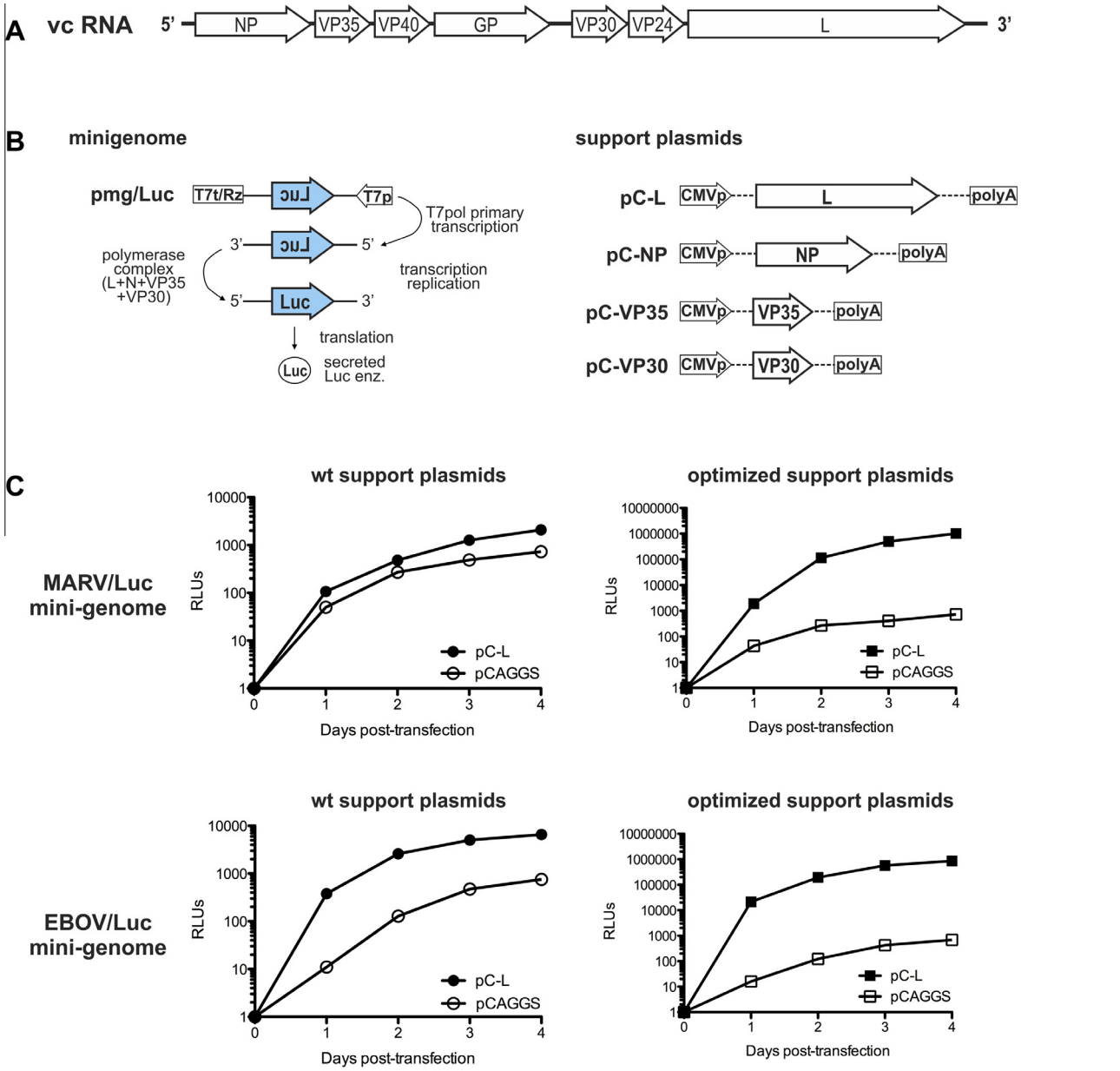


Fig. 1. Construction and optimization of filovirus gLuc mini-genomes. (A) Schematic of the basic filovirus genome in anti-genome (viral complementary) sense, depicting 7 viral genes separated by intergenic regions and flanked by 5′ and 3′ UTRs. (B) Schematic of mini-genome and viral support protein plasmids used to drive the gLuc mini-genome systems. The gLuc reporter gene was cloned in genomic (viral) sense between 5′ and 3′ UTRs of MARV (Genbank FJ750958) or EBOV (Genbank NC_002549). Solid lines indicate filovirus UTRs, and dashed lines indicate pCAGGS plasmid sequence. (C) Codon optimization of gLuc mini-genomes. BSR-T7/5 cells were transfected with MARV or EBOV gLuc mini-genome plasmid and corresponding wild-type (circles, left graphs) or codon-optimized (squares, right graphs) support plasmids pC-NP, pC-VP35, pC-VP30, and pC-L. Parallel transfections were carried out using empty expression vector (pCAGGS) containing no virus polymerase sequence as a control (open symbols). Data is representative of 3 independent experiments, with mean and standard error of the mean (SEM) of luciferase expression from 3 wells displayed for each time point in relative light units (RLUs).

that also express gLuc. We show that these gLuc-expressing filoviruses can be reliably rescued and propagated to high titers, and that luciferase expression is a faithful representation of virus growth. Although restricted to BSL-4 containment, these gLuc-reporter viruses can serve as a stand-alone option for screening inhibitors of all stages of the viral life cycle. Both platforms can be easily adapted to high-throughput (96-well) formats. Finally, we demonstrate the utility of both platforms by screening several known and unknown filovirus inhibitors, and find that 6azaU, a compound that inhibits *de novo* pyrimidine biosynthesis (Handschumacher, 1960), inhibits both MARV and EBOV mini-genome replication and infectious virus growth.

2. Materials and methods

2.1. Cell culture and biosafety

BSR-T7/5, BHK-21, and Vero-E6 cells were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS) and 1% penicillin–streptomycin (Invitrogen). BSR-T7/5 cells were selected with G418 (1 mg/mL Geneticin; Invitrogen) every other passage. Mini-genome experiments were conducted under BSL-2 conditions. All work with infectious virus, including rescue from cDNA, was done in a BSL-4 laboratory in accordance with select agent regulations. Work with plasmids

containing full-length genome sequences was performed in accordance with approved Institutional Biosafety Committee (IBC) protocols.

2.2. Plasmid construction and terminal sequencing of viral genomes

Support plasmids, mini-genomes, and infectious viruses expressing gLuc were derived from previous constructs expressing GFP, and have been described previously by our group (Albarino et al., 2013; Towner et al., 2005). Codon-optimized genes for EBOV were obtained from a commercial source (GenScript) and subcloned into the standard Pol II expression plasmid, pCAGGS. All support plasmids were codon-optimized for rodents.

2.3. Chemicals

Imatinib methanesulfonate salt (Gleevec) was purchased from LC Laboratories. Ribavirin, chloroquine diphosphate, and 6-azauridine were purchased from Sigma Life Science (Sigma–Aldrich). 2′CMC, 2′CMU, 2′OMC, 2′OMU, and 7-deaza-adenosine were purchased from Carbosynth Limited. All chemicals were diluted to a concentration of 100 mM prior to use, per manufacturers' instructions.

2.4. Mini-genome expression and high-throughput analysis

Luciferase mini-genome experiments were carried out using the same conditions as previously reported for a MARV GFP mini-genome (Albarino et al., 2013). For high-throughput compound screening experiments, BSR-T7/5 cells were split, washed, and mixed with LT1 transfection reagent plus plasmids. 4×10^4 cells were plated in 100 μL media per well, and compounds added at 2× concentration in an additional 100 μL media for a total 200 μL of media. For gLuc quantification, 5 μL of supernatant was harvested and transferred to a 96-well black flat-bottom polystyrene plate (Corning Costar). 50 μL of luciferase reagent (1:100 mixture of Luciferase Assay Substrate and Luciferase Assay Buffer; Promega) was added to each well at a rate of 300 μL/s, and luminescence from the resultant reaction was read after a 2 s delay using a monochromator-based multi-mode microplate reader (BioTek Synergy™ H1MD, 10 ms integration, 100 gain/sensitivity). To determine cell viability, supernatants were removed and 50 μL of CellTiter-Glo® Luminescent Cell Viability reagent added to each well (Promega). Cells were lysed with gentle rocking for 10 min at room temperature before transfer to a 96-well black flat-bottom polystyrene plate. ATP content was determined by reading luminescence.

2.5. Calculating CC_{50}, IC_{50}, and SI_{50}

The range of compound concentrations used in the high-throughput mini-genome experiments was converted to a log scale, and both luciferase expression and ATP content data were graphed and subjected to 4-parameter, non-linear regression analysis using GraphPad Prism software. The resulting CC_{50} (ATP content) was divided by the IC_{50} (luciferase expression) to determine the SI_{50} of each compound.

2.6. Rescue and characterization of infectious gLuc viruses

To generate infectious gLuc viruses, BHK-21 cells were transfected with full-length gLuc-containing genomes and support plasmids, and the supernatants were applied to Vero-E6 cells following established protocols (Albarino et al., 2013). As previously reported, all viruses used in these studies have a passage history of 1 passage in BHK-21 cells and 2 passages in Vero-E6 cells. Aliquots of working viral stocks were fully sequenced, and small aliquots were assayed for luciferase activity. To characterize gLuc viruses, T25 flasks of 70% confluent Vero-E6 cells were infected at an MOI of 0.1 using 0.5 mL of a 1:5 virus dilution in media. Virus was allowed to adsorb for 1 h with gentle rocking. After adsorption, monolayers were washed 3 times with PBS to eliminate any residual virus. Supernatant aliquots were taken daily to determine luciferase expression and viral titer by a standard $TCID_{50}$ assay.

2.7. High-throughput analysis of inhibitors using infectious gLuc viruses

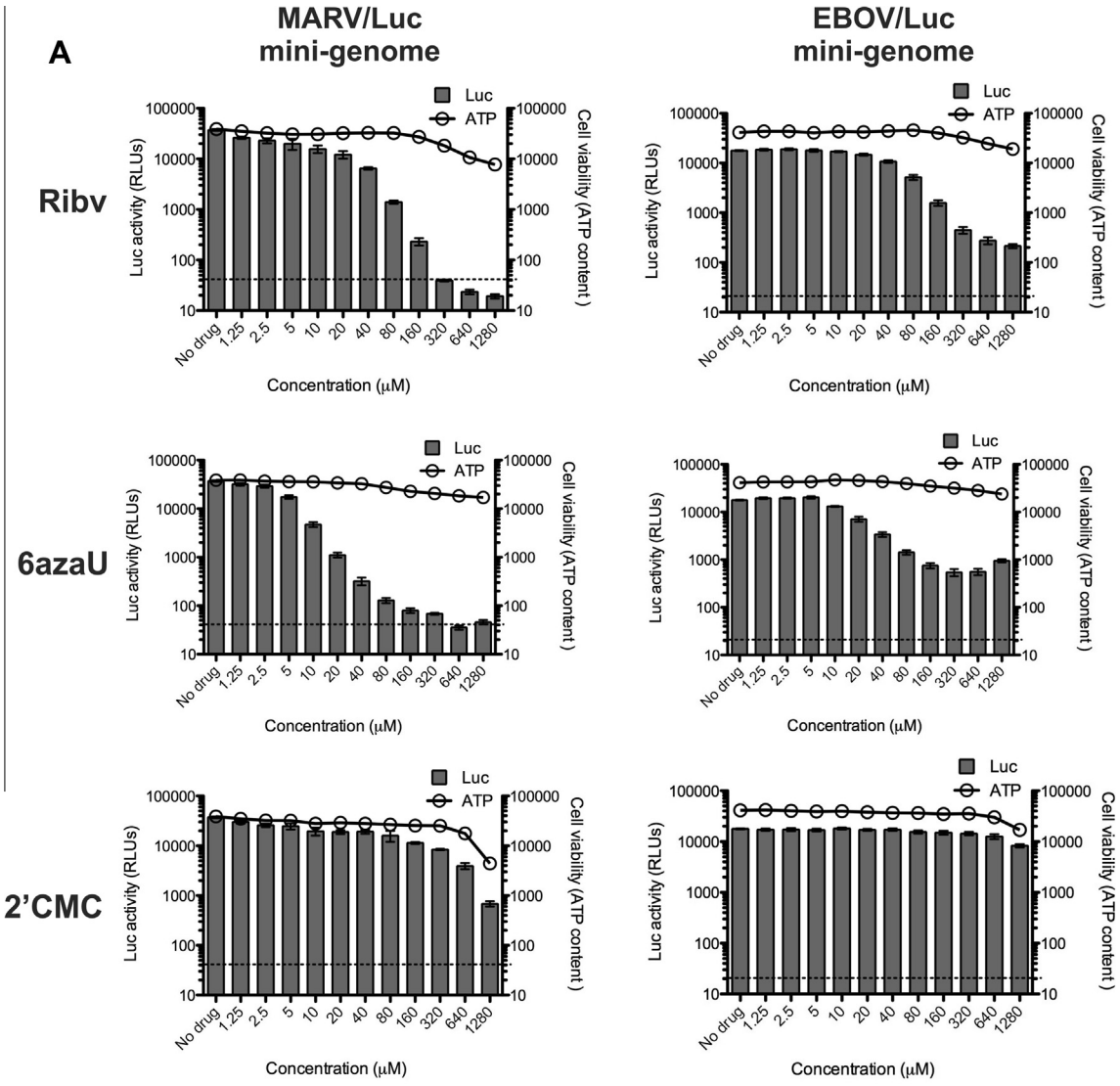
To validate infectious gLuc viruses as a high-throughput system, Vero-E6 cells were plated in 96-well plates overnight, and the next day, media was removed and cells pre-treated for 1 h with 100 μL media containing various concentrations of inhibitors. Virus was added in an additional 100 μL media (final MOI of 0.05). After 1.5 h, cells were washed 3 times with PBS and re-supplemented with media containing inhibitors. 48 h post-infection, 5 μL of supernatant was tested for luciferase expression and ATP content as above.

2.8. Virus titration

Viruses were titrated using a standard 50% tissue culture infective dose ($TCID_{50}$) protocol (Krahling et al., 2010; Neumann et al., 2002). Briefly, VeroE6 cells were seeded in 96-well plates, and infected 24 h later using 10-fold dilutions (10^{-1} to 10^{-8}) of the virus samples. Each virus dilution was applied into a plate column; therefore, each dilution was counted in 8 replica wells. Five days later, cells were fixed with formalin, permeabilized with Triton 0.1% and stained with either a rabbit anti-MARV or anti-EBOV polyclonal antibody, followed by counter-staining with anti-rabbit Alexafluor 498 or 594 nm secondary antibodies (Molecular Probes). The $TCID_{50}$/ml was determined using the Reed and Muench method.

3. Results

To facilitate the screening of antivirals against EBOV and MARV, we first created a recombinant system in which gLuc expression is controlled by mini-genome RNA replication and transcription. The gLuc reporter gene was cloned between the viral 3′ and 5′ UTRs in the negative-sense orientation into a standard T7 plasmid. Support plasmids were created by cloning the open reading frames (ORFs) of MARV and EBOV NP, VP35, VP30 and L into a standard Pol II expression plasmid (Fig. 1B). Mini-genome activity was tested in BSR-T7/5 cells stably expressing T7 RNA polymerase by transfecting MARV or EBOV mini-genome plasmids, along with their corresponding support plasmids. Thus, gLuc expression relies on mini-genome replication (Fig. 1B), and gLuc is secreted from cells, allowing for easy collection of supernatants at various times post-transfection to quantify luciferase production (Fig. 1C). In these experiments, gLuc expression was relatively low over background (3-fold in MARV and 10-fold in EBOV), and plateaued four days post-transfection (Fig. 1C, left panels). The relatively high signal from the negative control that contains no polymerase support plasmid is most likely due to positive-sense transcripts started on cryptic RNApol II promoters present in many plasmid backbones and/or related to the enzymatic nature of the reporter gene. Indeed, this is not an unusual problem and it has been addressed using different approaches by others (Groseth et al., 2005; Jasenosky et al., 2010). However, it should be noted that multiple groups have successfully used MARV and EBOV mini-genome systems supported by wild-type plasmids in multiple



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Mini-genome	Ribv (80 μM)	6azaU (40 μM)	2'CMC (160 μM)
MARV/Luc	96	99	32
EBOV/Luc	71	81	19

Reduction of Luc signal (%)

C

Mini-genome	Ribv	6azaU	2'CMC
MARV/Luc	287, 15, 19	89, 5, 17	$4x10^6$, $2x10^{15}$, <1
EBOV/Luc	374, 46, 8	221, 14, 16	$4x10^5$, $1x10^6$, <1

CC50 (uM), IC50 (uM), SI50

Fig. 2. High-throughput screen of RNA replication inhibitors using gLuc mini-genomes. (A) BSR-T7/5 cells were trypsinized, transfected with mini-genomes plus codon-optimized support plasmids, and plated in 96-well plates with compounds at indicated concentrations. 5 μL of supernatant was assayed for luciferase expression of EBOV (right panels) and MARV (left panels) mini-genome plasmids. Data is representative of 2 independent experiments; mean and SEM of luciferase expression from 4 wells is depicted as bars (left *Y*-axis). Cell monolayers were lysed immediately after luciferase quantification and assayed for ATP content (circles, right *Y*-axis). Background luciferase activity observed in transfected cells lacking virus polymerase activity (e.g., transfected with the empty vector control pCAGGS) is denoted by a dotted line (——). (B) Reduction of luciferase signal by each compound at the highest tested non-toxic concentration. Numbers represent percentage of reduction compared to non-treated cells replicating mini-genomes. (C) 50% cytotoxic concentration (CC_{50}), 50% inhibitory concentration (IC_{50}), and 50% selectivity index (SI_{50}) of each compound were calculated as described in methods.

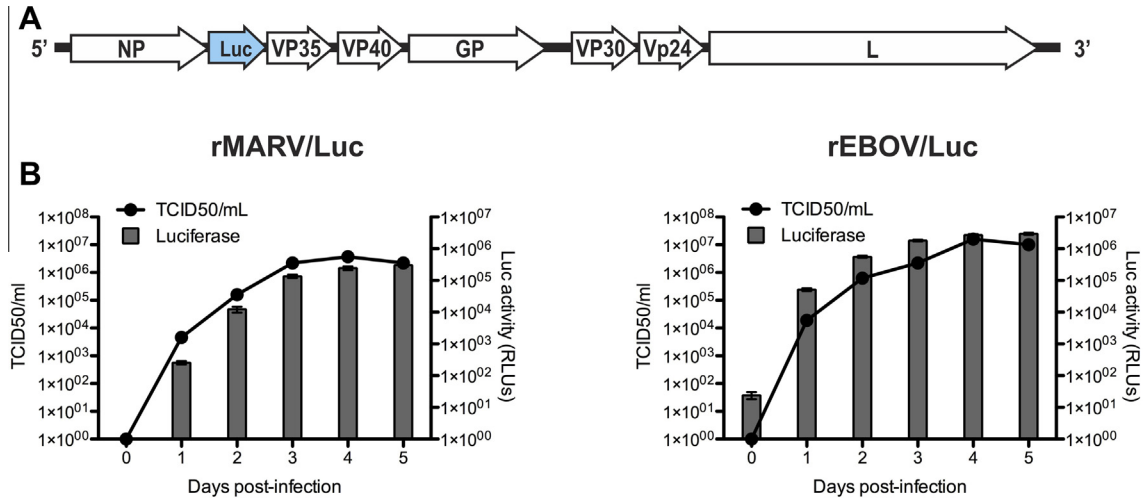


Fig. 3. Rescue and characterization of infectious rMARV/Luc and rEBOV/Luc viruses. (A) Schematic representation of gLuc-expressing filovirus genomes. (B) Characterization of rMARV/Luc and rEBOV/Luc viruses. Vero-E6 cells were infected with rMARV/Luc or rEBOV/Luc at an MOI of 0.1, and supernatants collected up to 5 d post-infection and frozen at −80 °C. After thawing, samples were divided and used to determine luciferase expression (bars, right *Y*-axis, duplicate reads) or viral titer using $TCID_{50}$ assay (circles, left *Y*-axis). Note the accelerated luciferase and virion production (~1 log and ~2 logs increase, respectively) in rEBOV/Luc at 1 d post-infection compared to rMARV/Luc.

mammalian cell types including hamster-derived cells (Boehmann et al., 2005; DiCarlo et al., 2007; Groseth et al., 2005, 2007; Hoenen et al., 2011; Jasenosky et al., 2010; Moller et al., 2005; Muhlberger et al., 1998, 1999; Prins et al., 2010; Schumann et al., 2009; Trunschke et al., 2013; Wenigenrath et al., 2010).

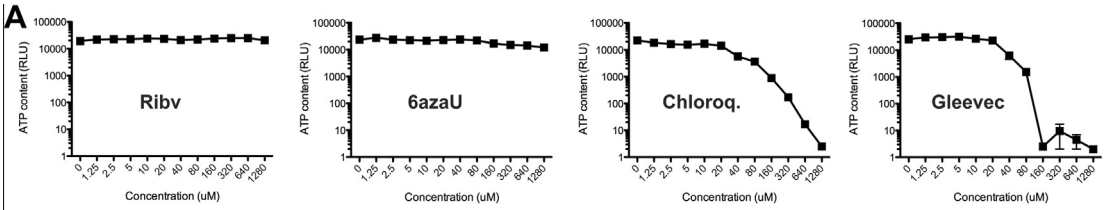
To improve signal, wild-type viral genes in support plasmids were codon-optimized for rodents to match the species origin of our target cells. Plasmids were then co-transfected with the original gLuc mini-genomes, and sampled as before. This approach resulted in an approximately 2000-fold increase of signal-to-background ratios, and high, sustained gLuc expression easily detectable in <5 μL of harvested supernatant starting 24 h post-transfection in both EBOV and MARV systems (Fig. 1C, right panels; Supporting Fig. 1).

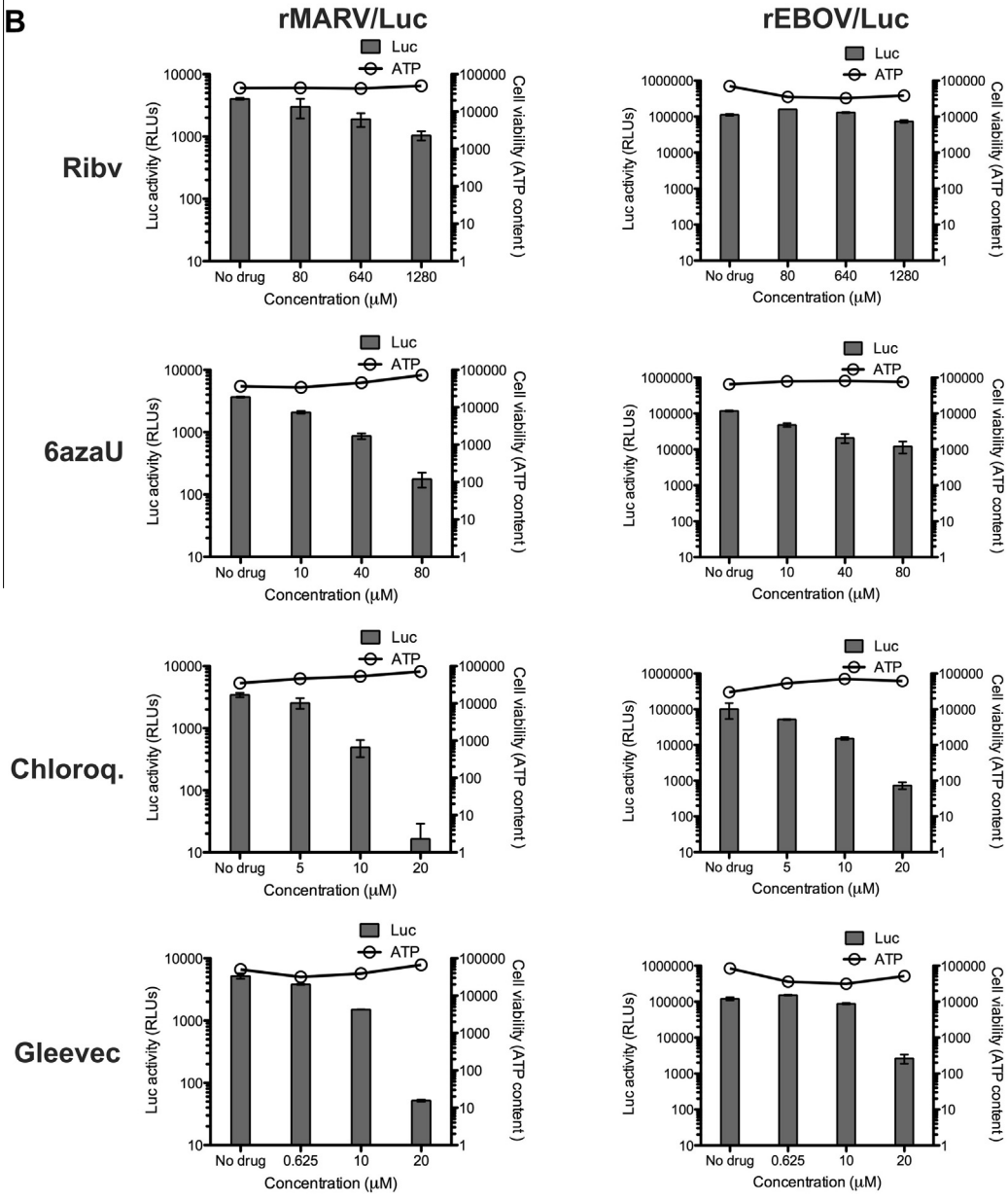
Previous efforts to screen anti-filovirus compounds have reportedly been hindered by the lack of high-throughput quantitative reporter systems that can be used in a BSL-2 setting [see (Hoenen et al., 2011) for review]. To address this limitation, we evaluated the potential of the codon-optimized gLuc mini-genome systems to function in a high-throughput 96-well format. In our initial experiments, BSR-T7/5 cells were grown in 12-well plates (low throughput) and transfected with either EBOV or MARV mini-genome plasmids and sampled 24 h and 48 h post-transfection to measure gLuc expression. *Z′* values, a measure of well-to-well variability, were calculated and found to be 0.52 for both EBOV and MARV. Assays with *Z′* values >0.5 are considered higher quality, with replicate variability decreasing as *Z′* values approach 1 (Zhang et al., 1999). To adapt this system to a high-throughput format, BSR-T7/5 cells were trypsinized, washed, mixed with the appropriate plasmids and transfection reagent, and plated in a 96-well plate. Interestingly, MARV mini-genome replication lags slightly behind EBOV mini-genome replication regardless of the format (see Fig. 1C, right panels, for low-throughput example). For this reason, we chose 48 h post-transfection as the standardized sampling time for future work using high throughput mini-genome systems. *Z′* values were re-calculated for the 96-well format and were found to not significantly change for either MARV (0.48) or EBOV (0.56).

To test the high-throughput mini-genome gLuc reporter systems, we screened ribavirin (Ribv) and several nucleoside analogs, each with known antiviral activities against other viruses. BSR-T7/5 cells were transfected with gLuc mini-genomes and optimized support plasmids and plated in a 96-well format in growth media containing each compound at various concentrations (Fig. 2; Supporting Fig. 1). The 50% cytotoxicity concentration (CC_{50}; determined by cellular ATP content), inhibitory concentration (IC_{50}; determined by luciferase expression), and selectivity index (SI_{50}) were then calculated (see Section 2). In both MARV and EBOV systems, several compounds inhibited viral replication at concentrations that showed low cellular toxicity (Fig. 2). 80 μM Ribv reduced luciferase signal by 96% for MARV and 71% for EBOV, and 40 μM 6-azauridine (6azaU) reduced luciferase signal by 99% for MARV and 81% for EBOV (Fig. 2B). Specific signal reduction by Ribv corresponds to SI_{50} of 19 for MARV and 8 for EBOV, while SI_{50} of 6azaU was 17 for MARV and 16 for EBOV (Fig. 2C). Several compounds, such as 2′-C-methylcytidine (2′CMC), showed negligible SI_{50} indices in both systems, with decreases in luciferase signal due to cell death (Fig. 2A). 2′-O-methylcytidine (2′OMC), 2′-O-methyluridine (2′OMU), 2′-C-methyluridine (2′CMU), and 7-deaza-adenosine were tested in the MARV mini-genome system, but showed negligible inhibition (data not shown).

Next, we tested candidate compounds on infectious MARV and EBOV to determine whether compounds that inhibit mini-genome replication also inhibit infectious virus. Previous approaches used wild-type, GFP-expressing or Firefly luciferase-expressing EBOV in the presence of candidate antivirals, and measured viral growth by standard $TCID_{50}$ assay, qRT-PCR (following RNA extraction), GFP, or luciferase signal. However, each of these approaches has various drawbacks such as lengthy multi-step assays, limited dynamic range or required destruction of infected cell monolayers (Hoenen et al., 2013). For these reasons, we constructed recombinant viruses expressing gLuc to allow a rapid, real-time assessment of viral replication over an 8-log range from repeated small aliquots (1–5 μL) of infected cell supernatants.

Briefly, full-length genomes of MARV and EBOV containing the gLuc reporter gene, rMARV/Luc and rEBOV/Luc, respectively, were cloned in viral complementary orientation into a standard T7 transcription plasmid, similar to that used to generate GFP-expressing filoviruses (Albarino et al., 2013; Towner et al., 2005) (Fig. 3A). Using optimized ratios of support plasmids and plasmids containing the full-length genomes, gLuc-containing viruses were rescued and stocks grown to high titers. For each virus, we measured viral growth kinetics by standard $TCID_{50}$ assay, and compared these





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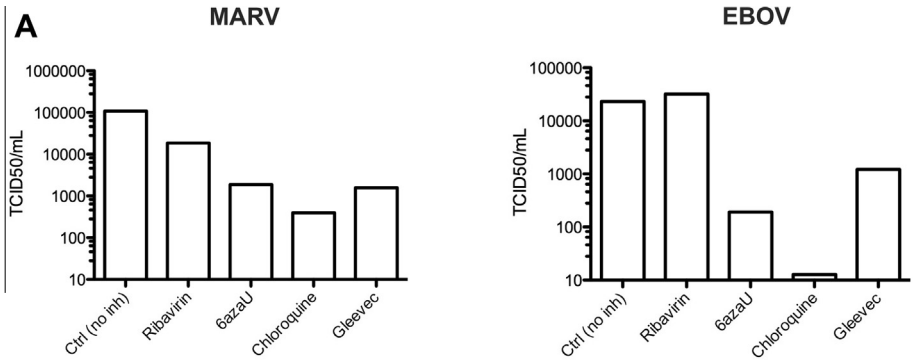
	Ribavirin (80 1280 μM)		6azaU (80 μM)	Chloroq. (20 μM)	Gleevec (20 μM)
rMARV/Luc	26	73	95	99.5	99
rEBOV/Luc	0	35	90	99.3	98

Reduction of Luc signal (%)

Fig. 4. High-throughput screen of candidate antiviral compounds using rMARV/Luc and rEBOV/Luc viruses. (A) Uninfected Vero-E6 cells were treated with compounds at the indicated concentration and assayed for cytotoxicity. (B) Vero-E6 cells in 96-well plates were pre-treated with compounds at indicated concentrations for 1 h, and then infected with rMARV/Luc or rEBOV/Luc at an MOI of 0.05. 48 h post-infection, 5 μL supernatant samples were assayed for luciferase expression (bars); cells were lysed immediately afterwards to determine ATP content (circles). Mean and SEM of 4 wells are displayed. (C) Reduction of luciferase signal compared to untreated cells was calculated for each compound at the indicated concentrations.



B

	Ribv (1280 μM)	6azaU (80 μM)	Chloroq. (20 μM)	Gleevec (20 μM)
MARV	83	98	99.9	99
EBOV	0	99	99.9	95

Reduction of viral titer (%)

Fig. 5. Confirmation of candidate antiviral compound activity using wild-type MARV and EBOV. (A) Vero-E6 cells were pre-treated with compounds at the highest concentrations used in Fig. 4, and infected with 0.5 MOI wild-type MARV or EBOV. Supernatants were collected 48 h post-infection, and viral titers were determined by $TCID_{50}$ assay. (B) Percent reduction in viral titers after treatment with indicated compounds compared to untreated cells.

values to luciferase production measured at the same time points. Our data show that luciferase activity closely mirrors virus production in rMARV/Luc. From 0 to 1 d post-infection, luciferase production increased 200-fold while $TCID_{50}$ values increased 2000-fold. From 1 to 2 d post-infection, the respective increases for both methods were 80-fold (Fig. 3B, left panel). For rEBOV/Luc, luciferase expression and virus production were nearly identical: luciferase levels increased 5000-fold and 20-fold from day 0 to 1 and 1 to 2, respectively, while TCID50 values increased 10,000-fold and 30-fold over the same interval (Fig. 3B, right panel).

After confirming that luciferase expression paralleled virus production, we tested compounds for antiviral activity using infectious gLuc viruses. Ribv and 6azaU, which effectively inhibited mini-genome replication, were tested, along with several compounds previously shown to be effective anti-filoviral agents (Garcia et al., 2012; Madrid et al., 2013). Initially, uninfected Vero-E6 cells were treated with each compound using a wide range of concentrations, and assayed for cell viability to determine the maximum concentration tolerated without toxicity (Fig. 4A). Then, cells were pre-treated with concentrations below this value and infected at a low MOI with either rMARV/Luc or rEBOV/Luc viruses. Supernatants were collected 48 h post-infection and luciferase production was measured. The data show 6azaU, chloroquine, and Gleevec (Imatinib methanesulfonate salt) all significantly reduced signal by 90–95%, >99%, and 98–99%, respectively (Fig. 4B). Ribv however, had very little effect on virus growth even at 1280 μM, the highest concentration tested (Fig. 4B), contrasting the reduction observed with the mini-genome system (Fig. 2). These results with infectious virus are consistent with previous studies of EBOV in *in vitro* and *in vivo* models (Huggins, 1989; Ignatyev et al., 2000). The ribavirin data were included here to highlight the fact that while mini-genome systems can be very useful for initial antiviral screens at BSL-2, the results do not always translate to that observed with infectious virus. A minor difference between the minigenome and infectious virus assays worth noting is that in the infectious virus assays, cells were pre-treated one hour before virus infection whereas in the minigenome assays, compounds were added at the time of transfection.

To confirm that the effects of the selected compounds were not specific to gLuc-expressing viruses, we re-tested the same compounds on wild-type MARV and EBOV using the highest concentrations shown in Fig. 4. As before, Ribv had no effect on wt EBOV and had only a modest effect on wt MARV (Fig. 5A and B) while 6azaU, chloroquine, and Gleevec all reduced MARV and EBOV growth by >95%, as assessed by $TCID_{50}$ assay (Fig. 5A; % titer reduction summarized in Fig. 5B). Overall, gLuc-expressing filoviruses grew similarly to wild-type viruses, and generally proved a faithful extension of our gLuc mini-genome results, although not always as demonstrated by the ribavirin studies. Importantly, gLuc results were obtained 3 times faster than results from wild-type virus (2–3 days versus 7–9 days, respectively), demonstrating the advantage of screening compounds using a luciferase-based readout rather than traditional titration methods.

As further confirmation that these systems can be used to screen inhibitors of filovirus RNA replication, we tested several small interfering RNAs (siRNAs) in the MARV gLuc mini-genome and rMARV/Luc infectious virus platforms. siRNAs have been shown previously to block filovirus and arenavirus replication (Fowler et al., 2005; Geisbert et al., 2006), including ones that target the 5′ and 3′ UTRs (Groseth et al., 2007; Muller and Gunther, 2007). Here, siRNAs targeting both the 5′ and 3′ UTRs of MARV were co-transfected into BSR-T7/5 cells with the MARV gLuc mini-genome and support plasmids. Two siRNAs targeting the 3′UTR were able to inhibit luciferase expression >90% compared to mock-transfected cells (Supporting Fig. 2A). These siRNAs were also able to inhibit luciferase activity of rMARV/Luc, showing 75–90% reduction over mock-transfected cells (Supporting Fig. 2B). The siRNAs targeting the 5′UTR showed no inhibitory activity (data not shown). These results suggest that both mini- and infectious gLuc systems can be used to screen siRNA inhibitors in addition to antiviral compounds, thus extending their potential utility.

4. Discussion

Together, the platforms presented here form a complementary approach to screen candidate antivirals targeting filoviruses. The gLuc mini-genome platform was used to identify potential

inhibitors of viral replication, and gLuc viruses confirmed in some cases inhibitor activity and identified additional agents inhibiting other stages of the virus life cycle. The gLuc mini-genome platform produces more rapid results (<48 h) than some previous low-containment screening approaches, such as chloramphenicol acetyltransferase (Muhlberger et al., 1998, 1999) or GFP (Groseth et al., 2005) reporter genes. The gLuc infectious virus platform is also rapid, although it remains restricted to BSL-4 containment. rMARV/Luc is the first of its kind, while rEBOV/Luc is similar to a recently described Firefly luciferase-expressing virus (Hoenen et al., 2013). In general, luciferase-expressing viruses are a significant improvement over earlier, less quantitative GFP or RFP-expressing viruses (Ebihara et al., 2007; Schmidt et al., 2011; Schudt et al., 2013; Towner et al., 2005) that require high-content sophisticated imaging techniques (Panchal et al., 2010, 2012). Moreover, both gLuc-expressing platforms presented here improve on previous Firefly or *Renilla* luciferase-based platforms (Filone et al., 2013; Jasenosky et al., 2010; Krahling et al., 2010; Schumann et al., 2009; Wenigenrath et al., 2010) because low volumes (1–5 μL) of supernatant samples can be collected at multiple time points without affecting cell viability. Both mini-genome and infectious virus platforms are easily adapted to a 96-well, high-throughput format without compromising overall robustness (assessed by Z' value).

Using the dual-platform methodology, we have identified 6azaU, a compound that suppresses *de novo* pyrimidine biosynthesis (Handschumacher, 1960), as a potent inhibitor of both MARV and EBOV mini-genomes and infectious viruses. This compound has broad-spectrum antiviral activity *in vitro*, particularly against pathogenic viruses (Crance et al., 2003; Morrey et al., 2002; Pyrc et al., 2006; Smee et al., 1987). We have additionally demonstrated that gLuc viruses can serve as a stand-alone approach for identifying inhibitors of viral life cycle stages besides RNA replication. Chloroquine and Gleevec, two previously identified effective anti-EBOV agents (Garcia et al., 2012; Madrid et al., 2013), reduced virus luciferase signal by >98%, and these results were confirmed using wild-type non-reporter viruses measured by traditional assays. Combining these compounds with one another, or with other therapeutic approaches like siRNA or neutralizing antibodies, may produce an enhanced synergistic effect.

To our knowledge, ours are the first mini-genome and infectious filovirus systems expressing a secreted gLuc reporter [see (Hoenen et al., 2011) for review] and the only description of recombinant MARV expressing luciferase. The possibility of using mini-genomes in a low-containment setting should significantly expand the number of institutions able to conduct anti-filovirus agent screens. Although the compounds we tested may be specific to the strains of MARV and EBOV presented here, the methodologies could easily be adapted to other filoviruses.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2014.03.018.

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