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## A cell-based, infectious-free, platform to identify inhibitors of lassa virus ribonucleoprotein (vRNP) activity

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### ABSTRACT

The mammarenavirus Lassa (LASV) is highly prevalent in West Africa where it infects several hundred thousand individuals annually resulting in a high number of Lassa fever (LF) cases, a febrile disease associated with high morbidity and significant mortality. Mounting evidence indicates that the worldwide-distributed prototypic mammarenavirus lymphocytic choriomeningitis virus (LCMV) is a neglected human pathogen of clinical significance. There are not Food and Drug Administration (FDA) licensed vaccines and current anti-mammarenavirus therapy is limited to an off-label use of ribavirin that is only partially effective and can cause significant side effects. Therefore, there is an unmet need for novel antiviral drugs to combat LASV. This task would be facilitated by the implementation of high throughput screens (HTS) to identify inhibitors of the activity of the virus ribonucleoprotein (vRNP) responsible for directing virus RNA genome replication and gene transcription. The use of live LASV for this purpose is jeopardized by the requirement of biosafety level 4 (BSL4) containment. We have developed a virus-free cell platform, where expression levels of reporter genes serve as accurate surrogates of vRNP activity, to develop cell-based assays compatible with HTS to identify inhibitors of LASV and LCMV mammarenavirus vRNP activities.

### 1. Introduction

Mammarenaviruses cause chronic infections of rodents with a worldwide distribution and human infections occur through mucosal exposure to aerosols, or by direct contact of abraded skin with infectious materials (Buchmeier et al., 2007). Several mammarenaviruses cause severe disease in humans and pose an important public health problem in their endemic regions (Bray, 2005; Geisbert and Jahrling, 2004). Thus, Lassa virus (LASV), a mammarenavirus highly prevalent in West Africa, is estimated to infect several hundred thousand individuals annually resulting in a high number of Lassa fever (LF) cases, a disease associated with high morbidity and significant lethality in patients who develop severe symptoms (Gunther and Lenz, 2004; Richmond and Baglole, 2003). Increased travelling has resulted in the importation of cases of LF into non-endemic metropolitan areas across the world including the US (Freedman and Woodall, 1999; Isaacson, 2001). In addition, mounting evidence indicates that the worldwide-distributed mammarenavirus lymphocytic choriomeningitis virus (LCMV) is a neglected human pathogen of clinical relevance (Bonthius, 2009, 2012a, 2012b), which also poses a threat to immune compromised individuals (Macneil et al., 2012; Palacios et al., 2008). There are no Food and Drug Administration (FDA)-licensed mammarenavirus vaccines and current

anti-mammarenavirus therapy is limited to an off-label use of ribavirin that is only partially effective and can cause significant side effects (Bausch et al., 2010; Hadi et al., 2010). The broad-spectrum inhibitor favipiravir (T-705) (Gowen et al., 2013; Mendenhall et al., 2011a; Safronetz et al., 2015) and the mammarenavirus glycoprotein (GPC)-mediated fusion inhibitor ST-193 (Cashman et al., 2011) have shown promising results in animal models of arenaviral hemorrhagic fever (HF) disease. Nevertheless, the development of additional anti-mammarenavirus drugs can facilitate the implementation of combination therapy against LASV and other human pathogenic mammarenaviruses, an approach known to counteract the emergence of drug resistant variants often observed with mono therapy strategies (Domingo, 2006). Likewise, the identification of novel inhibitors of mammarenavirus multiplication can serve as tool compounds for the generation of new knowledge in virus biology by uncovering previously unexplored pathways and specific host cell factors contributing to different steps of the virus life cycle.

Mammarenaviruses are enveloped viruses with a bi-segmented negative strand (NS) RNA genome (Buchmeier et al., 2007). Each genome segment, large (L) and small (S) uses an ambisense coding strategy to direct the synthesis of two proteins in opposite orientation, separated by a non-coding intergenic region (IGR). The S RNA encodes the viral

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nucleoprotein (NP) and the viral glycoprotein precursor (GPC) whose processing by cellular signal peptidase and Site 1 Protease (S1P) generates a 58-amino acid stable signal peptide (SSP) and the mature virion surface glycoproteins GP1 and GP2 that together with SSP form the GP complex that mediates cell entry via receptor-mediated endocytosis. The L RNA encodes the viral RNA dependent RNA polymerase (L polymerase), and the matrix Z protein. Advances in mammarenavirus molecular genetics have opened new approaches for the development of screening strategies to identify inhibitors of mammarenavirus multiplication (Cai et al., 2018; Emonet et al., 2011a; Miranda et al., 2018; Welch et al., 2016). However, the use of these approaches with live LASV are complicated by the requirement of biosafety level 4 (BSL4) containment. The viral trans-acting factors (NP and L) and cis-acting regulatory sequences required for the formation of a functional virus ribonucleoprotein (vRNP) complex responsible for directing viral genome replication and gene transcription of the mammarenavirus genome have been defined (Emonet et al., 2011a; Sarute and Ross, 2017). We used this knowledge to engineer cell lines constitutively expressing synthetic functional vRNPs for LCMV (LCMV/vRNP) and LASV (LASV/vRNP) encoding reporter genes (LCMV/vRNP: *Gaussia luciferase [Gluc]* and *green fluorescent protein [GFP]*; LASV/vRNP: *Gluc* and *ZsGreen [ZsG]*) whose expression levels serve as an accurate surrogate of vRNP activity. We used this cell-based, infectious-free, platform to develop a quantitative high-throughput screening (HTS) on a fully automated robotic screening system (Inglese et al., 2006) (Kalypsys), and used the multiplexed vRNA-cytotoxicity assay to identify inhibitors of LCMV and LASV vRNP activities.

We screened the LCMV/vRNP and LASV/vRNP cell lines against four libraries representing a total of 7163 unique small molecule compounds. We confirmed 16 compounds with  $EC_{50} < 10 \mu\text{M}$  in the *Gluc* assay and greater than 10-fold selectivity between *Gluc* and cytotoxicity assays, in either cell line. These compounds included several known ionophore antibiotics and inhibitors of the mitochondrial Electron Transport Chain (mETC) complex I and III. We selected 11 of the 16 confirmed hits to examine their effects on multiplication of live LCMV in cultured cells. All 11 tested compounds exhibited anti-LCMV activity in the context of a bona fide LCMV infection. Consistent with their identification in the screen as inhibitors of the LCMV and LASV vRNP activities, all tested compounds specifically inhibited the activity of a LCMV minigenome (MG) in a cell-based transfection assay. The mETC complex I and III inhibitors Antimycin A and Mubritinib (TAK165), respectively, exhibited the highest anti-LCMV activity with low (~70 nM) half maximal effective concentration ( $EC_{50}$ ) and high (> 700) selectivity index (SI;  $SI = CC_{50}/EC_{50}$ ) values. Our results support the use of LCMV/vRNP and LASV/vRNP cell lines as a reliable platforms for HTS to identify inhibitors of the activity of the vRNP responsible for directing the biosynthetic processes of replication and gene transcription of the viral genome, with the benefit that the infectious-free nature of this platform overcomes the need of BSL4 containment required for handling live LASV.

## 2. Materials and methods

### 2.1. Reagents

The Pierce *Gaussia luciferase (Gluc)* Glow assay kit and the alamarBlue Cell Viability Reagent were purchased from ThermoFisher (Waltham, MA). Cell culture media and selection were purchased from ThermoFisher.

### 2.2. Cells and viruses

Vero E6 (ATCC CRL-1586), A549 (ATCC CCL-185), HEK293T (ATCC CRL-3216) cells were maintained in Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum (FBS), 2 mM L-Glutamine, 100 mg/ml streptomycin, and 100 U/ml penicillin. HEK293 cells stably

expressing the monomeric red fluorescent protein (mRFP) and the blasticidin (Bls) resistant gene (*bsr*) (HEK293-RFP) (GenTarget Inc) were maintained in the same medium as HEK293T cells but with the addition Bls (10  $\mu\text{g}/\text{ml}$ ). LCMV/vRNP cells were maintained in DMEM 10% FBS, 5  $\mu\text{g}/\text{ml}$  puromycin, 100 mg/ml streptomycin, and 100 U/ml penicillin. LASV/vRNP cells were maintained in DMEM 10% FBS, 100  $\mu\text{g}/\text{ml}$  zeocin, 10  $\mu\text{g}/\text{ml}$  Bls, 100 mg/ml streptomycin, and 100 U/ml penicillin. The recombinant (r)LCMV expressing GFP (rLCMV/GFP-P2A-NP, refer to as rLCMV/GFP) (Miranda et al., 2018), ZsGreen (ZsG) (rLCMV/ZsG-P2A-NP, refer to as rLCMV/ZsG) (Iwasaki et al., 2018), and a single cycle infectious rLCMV expressing ZsG (rLCMV $\Delta$ GPC/ZsG-P2A-NP, refer to as rLCMV $\Delta$ GPC/ZsG) (Iwasaki et al., 2018) have been described.

### 2.3. Generation of LCMV/vRNP cell line

We transfected HEK293T cells (6 well plate format,  $10^6$  cells/well) with pCAGGS expression plasmids for LCMV NP (0.8  $\mu\text{g}$ ) containing a C-terminal HA epitope tag, and the viral L polymerase (1.0  $\mu\text{g}$ ), together with the plasmid hPol1-LCMV/SMG-Gluc/Pur-GFP (1.0  $\mu\text{g}$ ) using Lipofectamine 2000 (2.5  $\mu\text{l}/\mu\text{g}$  DNA). Plasmid hPol1-LCMV/SMG-Gluc/Pur-GFP directed intracellular synthesis, mediated by the human RNA polymerase I promoter (hPol-I), of a LCMV-like RNA (a.k.a. minigenome-MG) of the small (S) vRNA segment (antigenome polarity) expressing *Gluc* instead of the viral glycoprotein (GPC) open reading frame (ORF) and the gene of resistance to puromycin fused to the N-terminus of the green fluorescent protein (Pur-GFP) instead of the viral NP ORF. After overnight transfection, cells were trypsinized, re-suspended in 10% FBS DMEM and serially 10-fold diluted in 10  $\text{cm}^2$  culture plates. After 24 h, media was changed to 10% FBS DMEM containing 5  $\mu\text{g}/\text{ml}$  of puromycin to select for cells expressing Pur-GFP. Media was changed every 2–3 days to remove dead cells. Puromycin resistant colonies were collected by trypsinization using cloning rings and directly transferred to individual wells (24 well plate format) for cell expansion. Cells in the 24 well plates were further selected in the presence of puromycin (5  $\mu\text{g}/\text{ml}$ ). Amplified individual clones resistant to puromycin were selected by the level of GFP and *Gluc* expression as determine of viral replication and transcription activity. Selected clones were confirmed to express reporter genes by LCMV L and NP by treatment with ribavirin, a nucleoside analog known to inhibit arenavirus viral replication and transcription. Clones exhibiting the most optimal levels of Pur-GFP and *Gluc* expression that show dose-dependent inhibition by ribavirin were amplified, aliquoted and frozen.

### 2.4. Generation of LASV/vRNP cell line

We seeded HEK293-RFP cells (GenTarget Inc) in 12 well plate pre-coated with poly-L-lysine ( $3.5 \times 10^5$  cells/well) and cultured them 16 h at 37 °C/5%  $\text{CO}_2$  in DMEM containing 10% FBS. We transfected HEK293-RFP cells with pCAGGS expression plasmids for LASV NP (0.8  $\mu\text{g}$ ) and L (1.0  $\mu\text{g}$ ) together with plasmid hPol1-LASV/SMG-Gluc/ZeoR-P2A-ZsG (1.0  $\mu\text{g}$ ) using Lipofectamine 2000 (2.5  $\mu\text{l}/\mu\text{g}$  DNA). Plasmid hPol1-LASV/SMG-Gluc/ZeoR-P2A-ZsG directed intracellular synthesis, mediated by the human RNA polymerase I (hPol-I), of a LASV-like RNA (a.k.a. minigenome-MG) of the S segment (genome polarity) where the GPC ORF was replaced by the *Gluc* reporter gene, and the NP ORF by the *Sh ble* gene that confers resistance to Zeocin (ZeoR) fused to the N-terminus of the ZsG reporter gene, separated by the 2A peptide sequence derived from porcine teschovirus (PVT1: P2A) (Szymczak-Workman et al., 2012). After 5 h transfection, cells were washed once with DMEM and fed with DMEM containing 2% FBS. After 48 h we subjected transfected cells to Zeo selection (300  $\mu\text{g}/\text{ml}$ ) to select for cells expressing ZeoR-ZsG. Selection was done for six days, changing the medium after 3 days to remove dead cells. Colonies of cells resistant to Zeo were collected by trypsinization using cloning discs and individual colonies transfer to a 24 well plate for further

selection with Zeo (100 µg/ml). Selected cell clones were screened for ZsG expression response to ribavirin, a validated inhibitor of mammarenavirus vRNP activity. Clones that exhibited optimal ribavirin dose-dependent inhibition of ZsG expression were selected. We analyzed selected clones by fluorescence-activated cell sorting (FACS) to remove outliers (highest and lowest) ZsG expressing cells in the clonal population, from the majority of the ZsG expressing cells. Sorted cell clones were amplified under continued Zeo selection (100 µg/ml) and frozen aliquots made at low passage.

## 2.5. Expression of NP and L in the LCMV/vRNP cell line

Expression of NP and L mRNAs was assessed by RT-PCR. Total RNA was isolated from parental and LCMV/vRNP HEK293T cells using TRI Reagent (TR118, Molecular Research Center) according to the manufacturer's instruction. Total RNA (1 µg) was reverse transcribed to cDNA using SuperScript™ IV First-Strand Synthesis System (Thermo Fisher Scientific, Waltham, MA) using random hexamers for priming. PCR was done using specific primers for LCMV NP (forward 5'-TTAAGAATTCA CCATGGCCTTGCTAAGGAAGTTAA-3'; reverse 5'-AATTGGTACCGCG AGTGTACACAACATTTGGGCC -3') and LCMV L (forward 5'-AATCCCG GGGATGGATGAAATCATCTC; -3'; reverse 5'-AATTGCATGCCAATTTG TTAGTGTCTCTAT -3'). Expression of NP was detected by indirect immunofluorescence (IIF) and Western blot (WB) assays. For IIF, cells were fixed with 4% paraformaldehyde (PFA) in PBS, permeabilized and blocked with 0.1% triton X-100 in PBS containing 3% bovine serum albumin (BSA). After blocking, cells were incubated with an anti-HA monoclonal antibody (Sigma) and probed with a FITC-conjugated rabbit anti-mouse secondary antibody (Dako). For WB, LCMV/vRNP and parental HEK293T cells (3x10<sup>6</sup> cells/well; 6-well plate format) were collected and lysed with 400 µl of lysis buffer (10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 100 mM NaCl, 1% NP-40, complete cocktail of protease inhibitors; Roche). A 20 µl aliquot of total cell lysates was separated on a 12% SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad). Membranes were incubated with an anti-HA monoclonal antibody (Sigma) and probed with a secondary HRP-conjugated anti-mouse antibody (GE Healthcare). LCMV NP expression was detected using a chemiluminescent kit (Denville Scientific). Cellular GAPDH was detected using a polyclonal antibody to GAPDH (Abcam).

## 2.6. Expression of NP and L in the LASV/vRNP cell line

Expression of NP and L mRNAs was assessed by RT-PCR. Total RNA was isolated from parental and LASV/vRNP HEK293mRFP cells using TRI Reagent (TR118, Molecular Research Center) according to the manufacturer's instruction. Total RNA (1 µg) was reverse transcribed to cDNA using SuperScript™ IV First-Strand Synthesis System (Thermo Fisher Scientific, Waltham, MA) using random hexamers for priming. Semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was done using specific primers for LASV NP (forward 5'-CCAA AGAAGAGCTCTCCTGA-3'; reverse 5'-TCAAGTCAACCTGCCCTGT-3'), LASV L (forward 5'-ACATCATTTGGGCCCCACTTACTA-3'; reverse 5'-GGAGAGCAATTCAGAGGCACTTAG-3'), and GAPDH (forward 5'-TGA CATCAAGAAGGTGGTGAAGCAG-3'; reverse 5'-ATTGTCATACCAGGAA ATGAGCTTGAC-3'). Expression of NP was assessed by IIF. Parental and LASV/vRNP HEK293mRFP cells were seeded onto poly-L-lysine coated glass coverslips and allow to grow for 20 h prior fixation with 4% PFA. Cells were permeabilized and blocked with 0.1% triton X-100 in PBS containing 3% BSA. After blocking, cells were incubated with a mouse monoclonal to LASV-NP, followed by a secondary goat anti-mouse antibody conjugated to Alexa Fluor 647 (A-21248, ThermoFisher) allowing the LASV-NP to be visualized using Far Red wavelengths. Nuclei were visualized by DAPI staining.

## 2.7. siRNA knockdown of LCMV and LASV L polymerases

LCMV and LASV vRNP cells were reverse transfected with 15 pmol of siRNA to LCMV or LASV L polymerases, respectively, using 1 µl of Lipofectamine RNAiMAX (10 nM final siRNA concentration). Cells were examined for GFP (LCMV/vRNP) or ZsG (LASV/vRNP) expression after 60 h treatment and compared to non-transfected control cells. Anti-LCMV L and anti-LASV-L siRNAs were designed using Block-it™ RNAi Designer software (thermoFisher Scientific) and selected siRNA molecules were synthesized by Invitrogen.

## 2.8. Multiplexed vRNP-cytotoxicity assay

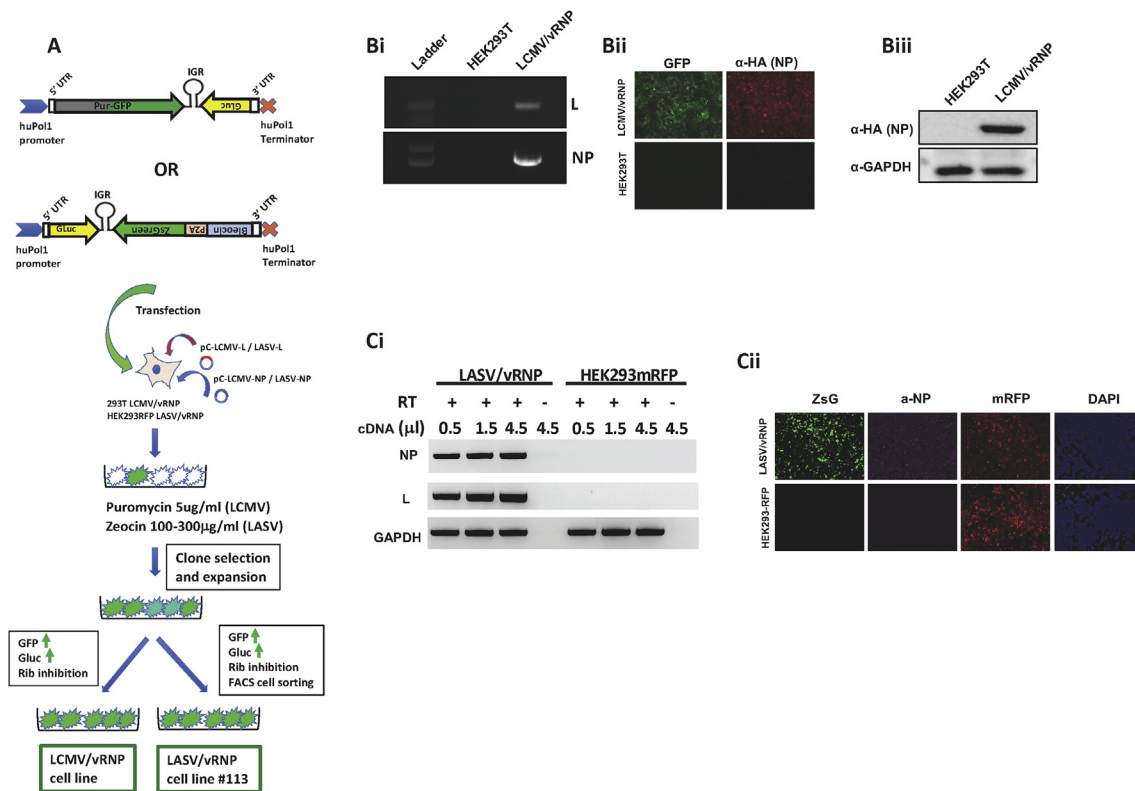
LASVvRNP and LCMV/vRNP cells were seeded into 1,536-well white, solid bottom, tissue culture treated microtiter plates (Greiner Bio-One) at 2,000 cells/well in 4 µl of growth media. The plates were incubated for 16–20 h at 37 °C with 5% CO<sub>2</sub>, followed by addition of 23 nl of compounds in DMSO to each well via a pintool (Kalypsis). The cells were incubated with compounds for 48 h at 37 °C with 5% CO<sub>2</sub>. Then, 0.5 µl of alamarBlue Viability reagent was dispensed to each well using a MultiDro Combi (ThermoFisher). The plates were incubated for 1 h at 37 °C with 5% CO<sub>2</sub>, and the fluorescence signal read at excitation of 570 nm and emission of 590 nm. Gluc reporter expression was then assayed by dispensing 4 µl/well of Gluc substrate in 2x lysis buffer, followed by 5 min incubation at room temperature before the luminescence signal was read.

## 2.9. Compound libraries and qHTS

A quantitative HTS (qHTS) was done on a fully automated robotic screening system (Inglese et al., 2006) (Kalypsys). LASV/vRNP and LCMV/vRNP cell lines were screened against four libraries using the multiplexed vRNA-cytotoxicity assay (Buchmeier et al., 2007). The library of pharmacologically active compounds (LOPAC), a collection of 1,280 small molecules with characterized biological activities, was purchased from Sigma-Aldrich (St. Louis, MO, USA) (Bray, 2005). The National Center for Advancing Translation Sciences (NCATS) Pharmaceutical Collection (NPC) of approved and investigational drug collection was generated in house (Huang et al., 2011). At the time of screening, the NPC library consisted of 2,816 small molecule compounds, 52% of which were drugs approved for human or veterinary use by the United States Food and Drug Administration (FDA), 22% were drugs approved in Europe, Canada or Japan, and the remaining 25% were compounds that entered clinical trials or were research compounds commonly used in biomedical research (Geisbert and Jahrling, 2004). The mechanism interrogation plate (MIPE) library of approved and investigational drugs (Gunther and Lenz, 2004). NPACT library of natural products. Compounds from the LOPAC, NPC and NPACT libraries were screened at 5 concentration points at 1:5 dilutions ranging from 92 nM to 57.5 µM. Compounds from the MIPE library were screened at 11 concentration points at 1:3 dilution ranging from 1 nM to 57.5 µM. Selectivity values were calculated as CC<sub>50</sub> from the alamarBlue assay divided by EC<sub>50</sub> from the GLuc assay. For compounds where no cytotoxicity was observed at the highest concentration tested of 57.5 µM, a value of 100 µM was used as CC<sub>50</sub> value for selectivity calculations.

## 2.10. Determination of compounds EC<sub>50</sub> and CC<sub>50</sub> in LCMV cell-based infection assay

Cell viability was assessed using the CellTiter 96 AQueous One Solution reagent (Promega). Cells were seeded on a 96-well clear bottom plate (2.0 x 10<sup>4</sup> cells/well) and 20 h later treated with 3-fold serial dilutions of each compound (four replicates for each compound concentration). At 48 h after drug treatment, CellTiter 96 AQueous One solution reagent (Promega) was added to each well and the plate



**Fig. 1.** Generation of LCMV/vRNP and LASV/vRNP cell lines. **A.** Schematic of the generation of LCMV/vRNP and LASV/vRNP cell lines. We generated LCMV and LASV MG reporter constructs hPol1-LCMV/SMG-Gluc/Pur-GFP and hPol1-LASV/SMG-Gluc/ZeoR-P2A-ZsG as described in material and methods. HEK293T (LCMV), or HEK293-RFP (LASV) cells were transfected with hPol1 constructs expressing LCMV and LASV MGs, respectively, together with pCAGGS expression plasmids for the corresponding viral trans-acting factors NP and L polymerase. Transfected cells were selected in the presence of puromycin (LCMV/vRNP) or Zeocin (LASV/vRNP). Selected cells clones expressing high levels of Gluc and either GFP (LCMV/vRNP) or ZsG (LASV/vRNP) were examined for reporter gene expression sensitivity to ribavirin treatment to select those with optimal response dynamic range. IGR, intergenic regions. UTR, untranslated regions. **B.** Expression of LCMV NP and L polymerase in LCMV/vRNP cells. **Bi.** Detection of LCMV NP and L mRNAs by RT-PCR. **Bii.** GFP expression directed by vRNP and detection of LCMV NP by IIF. **Biii.** Detection of NP by Western blot. **C.** Expression of LASV NP and L polymerase in LASV/vRNP cells. **Ci.** Detection of LASV NP and L mRNAs by RT-PCR. **Cii.** ZsGreen expression directed by vRNP and detection of LASV NP by IIF.

incubated for 15 min at 37 °C and 5% CO<sub>2</sub>. The absorbance was measured at 490 nm by using an enzyme-linked immunosorbent assay (ELISA) reader (SPECTRA max plus 384, Molecular Devices, Sunnyvale, CA). Values were normalized with vehicle control group (DMSO) which was adjusted to 100%. The 50% cytotoxic concentrations (CC<sub>50</sub>) were determined using GraphPad Prism. For determination of compounds half maximal effective concentrations (EC<sub>50</sub>), cells were plated on a 96-well clear bottom black plate (2.0 × 10<sup>4</sup> cells/well) and incubated for 20 h at 37 °C and 5% CO<sub>2</sub>. Cells were infected (multiplicity of infection, MOI = 0.01) with rLCMV/GFP-P2A-NP and treated with 3-fold serial dilutions of each compound (four replicates for each compound concentration). At 48 h post-infection (pi) cells were fixed with 4% paraformaldehyde and GFP expression levels were determined by fluorescence using a fluorescent plate reader (Synergy H4 Hybrid Multi-Mode Microplate Reader, BioTek, Winooski, VT). Mean values were normalized to infected and vehicle (DMSO) treated control group that was adjusted to 100%. GFP expression levels were also normalized for total cell protein in the lysate (Pierce BCA Protein Assay Kit, Thermo Scientific, #23227). EC<sub>50</sub> values were determined using GraphPad Prism (GraphPad Software, San Diego, CA).

### 2.11. Data and statistical analysis

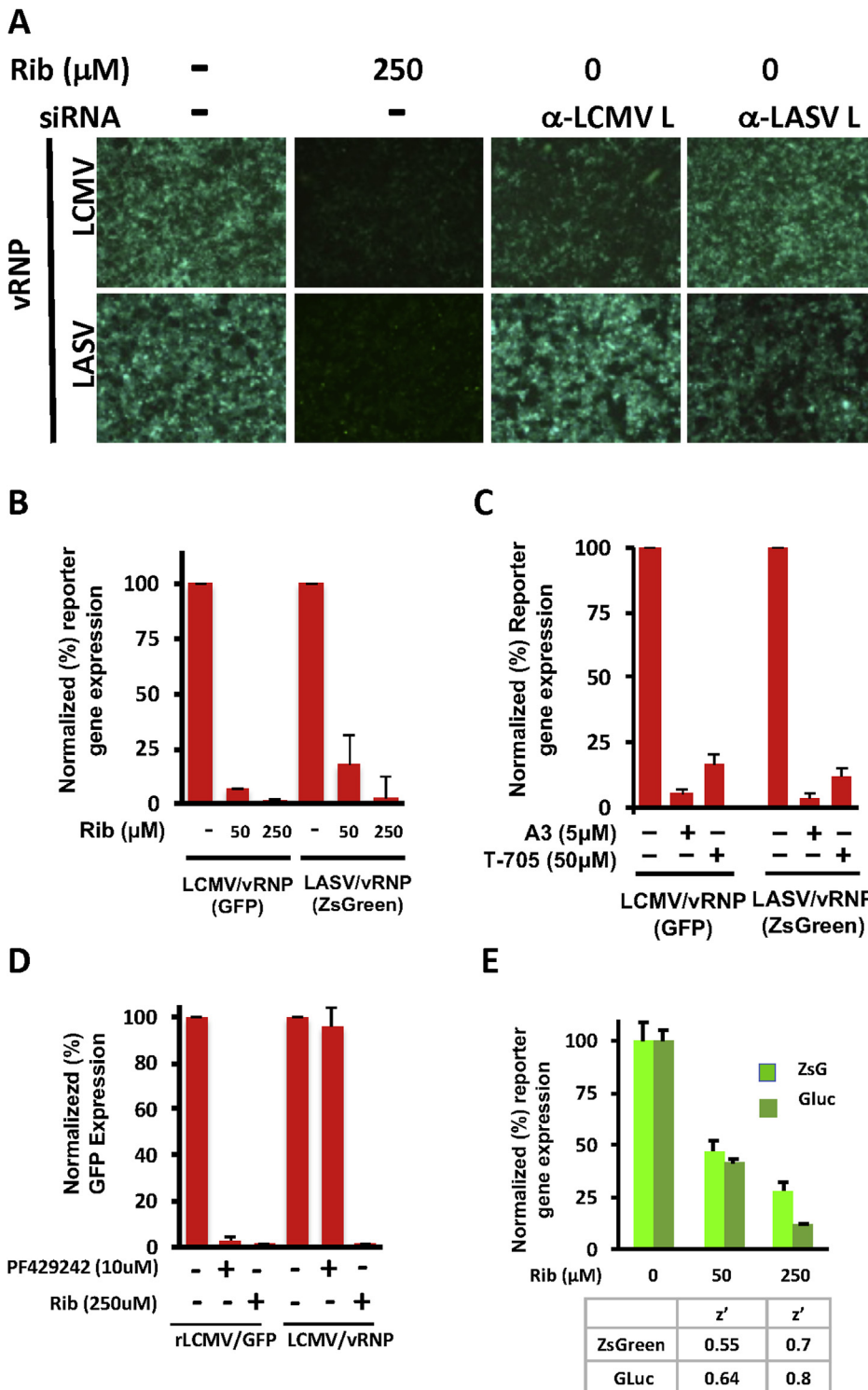
The primary screen data were analyzed using customized software developed internally (Wang et al., 2010). All data were presented as mean ± standard error of mean (SEM).

### 2.12. Determination of production of LCMV infectious progeny

A549 cells (1.25 × 10<sup>5</sup> cells/well, 24 well plate format, triplicates) were infected (MOI = 0.01). After 90 min adsorption, the virus inoculum was removed, cells washed twice with DMEM 2% FBS, and fresh complete medium containing the indicated compounds and concentrations were added. At the indicated hours pi, tissue culture supernatants (TCS) were collected and viral titers determined by focus forming assay (Battagay, 1993). Briefly, 10-fold serial dilutions (triplicates) of virus samples were used to infect Vero cell monolayers in a 96 well plate (2 × 10<sup>4</sup> cells/well). At 20 h pi, cells were fixed with 4% paraformaldehyde (PFA) in PBS. Foci of rLCMV/GFP-infected cells were determined directly by epifluorescence and further validated by immunofluorescence using a rat monoclonal antibody (VL4) to NP conjugated to Alexa fluor 568.

### 2.13. LCMV MG assay

LCMV MG assays were performed as previously described (Iwasaki and de la Torre, 2018; Iwasaki et al., 2015a; Iwasaki et al., 2015b). HEK293T cells were cultured on poly-L-lysine coated 12 well plates (4.5 × 10<sup>5</sup> cells/well) the day before transfection. Cells were transfected with a set of plasmids containing 0.5  $\mu$ g of T7 polymerase (pCAGGS T7), 0.5  $\mu$ g of pT7MG-GFP, 0.3  $\mu$ g of pCAGGS NP, and 0.3  $\mu$ g of pCAGGS L using Lipofectamine 2000 (2.5  $\mu$ l/ $\mu$ g of DNA) (Thermo Fisher Scientific). After 5 h, the transfection mixture was replaced with fresh medium containing test compounds followed by 48 h incubation at



**Fig. 2. Functional Characterization of LCMV/vRNP and LASV/vRNP cell lines.** A. GFP and ZsG expression levels by LCMV/vRNP and LASV/vRNP, respectively, were reduced by transfection of siRNA specifically targeting the corresponding viral L polymerase. LCMV vRNP and LASV vRNP cells were reverse transfected with 15 pmol of siRNA to LCMV or LASV L polymerase using Lipofectamine 2000. Cells were examined for GFP (LCMV/vRNP) or ZsG (LASV/vRNP) expression after 60 h treatment and compared to untransfected control cells. B. Inhibitory effect of ribavirin treatment on GFP (LCMV/vRNP) and ZsG (LASV/vRNP) expression levels. C. Inhibitory effect of A3 or T-705 treatment on GFP (LCMV/vRNP) and ZsG (LASV/vRNP) expression levels. D. Effect of PF429242 or ribavirin on GFP expression levels in cells infected with rLCMV/GFP or in LCMV/vRNP cells. E. Reproducibility of LASV/vRNP performance. LASV/vRNP cells were seeded into a 96-well plate (4x10<sup>4</sup> cells/well) and 8 h later ribavirin was added at the indicated final concentration. At 48 h post-seeding, TCS were collected and cells fixed (4% PFA). Expression levels of Gluc and ZsG were determined in TCS and fixed cells, respectively, and normalized (%) by assigning the value of 100% to reporter gene expression levels obtained in non-treated cells (0 μM ribavirin). Results correspond to average and SD of 14 replicates. Z' scores were determined as  $Z' = 1 - [(3\sigma_{c+} + 3\sigma_{c-}) / (3\mu_{c+} - 3\mu_{c-})]$  where  $\sigma_{c+}$  and  $\sigma_{c-}$  corresponded to standard deviations of Max (0 μM ribavirin) and Min (50 and 250 μM ribavirin) signals, and  $\mu_{c+}$  and  $\mu_{c-}$  to the mean activities of Max (0 μM ribavirin) and Min (50 and 250 μM ribavirin) signals.

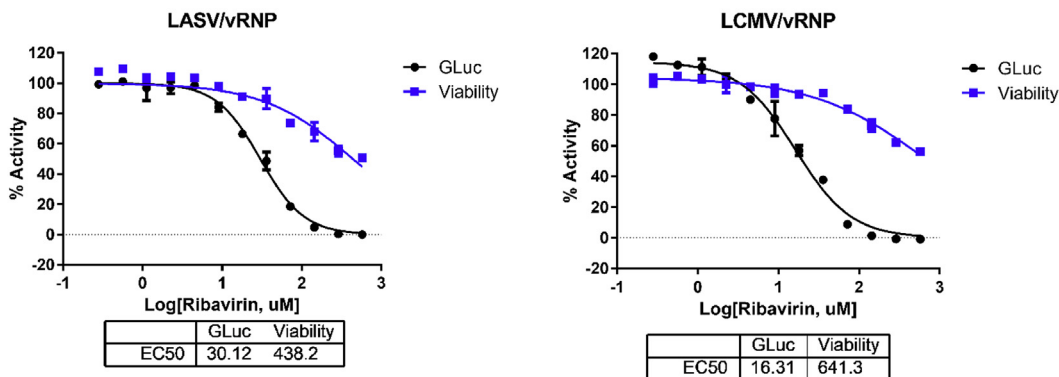
37 °C and 5% CO<sub>2</sub>. At 48 h post-transfection, whole cell lysates were harvested to determine levels of GFP. Briefly whole cell lysates were prepared with 0.2 ml lysis buffer (50 mM Tris pH 7.4, 1 mM EDTA, 0.5% NP-40, 150 mM NaCl) and GFP levels were measured in equivalent amounts of the clarified lysate using a Synergy H4 reader.

2.14. LASV MG assay

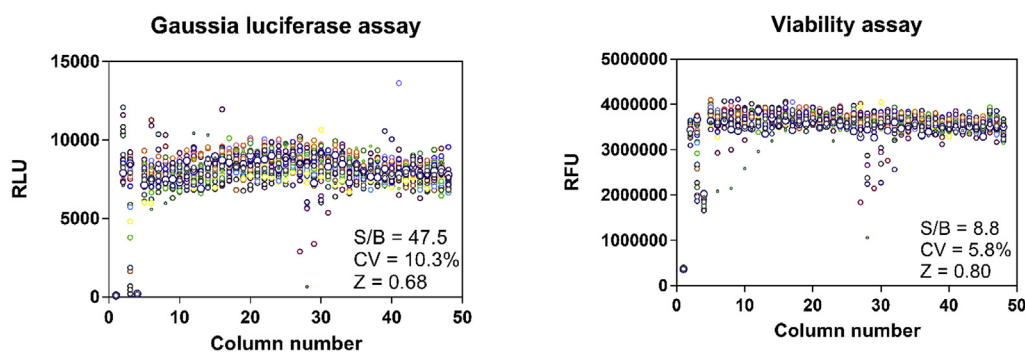
HEK293T cells were cultured on poly-L-lysine coated 24 well plates (2.5 x 10<sup>5</sup> cells/well) the day before transfection. Cells were transfected

with pCAGGS T7 (0.25 μg), pT7MG-ZsGreen (0.25 μg), pCAGGS LASV NP (0.15 μg) and pCAGGS LASV L (0.3 μg) using Lipofectamine 2000 (2.5 μl/μg of DNA) (Thermo Fisher Scientific). After 5 h, the transfection mixture was replaced with fresh medium containing test compounds followed by 48 h incubation at 37 °C and 5% CO<sub>2</sub>. At 48 h post-transfection, whole cell lysates were harvested to determine levels of ZsGreen. Briefly whole cell lysates were prepared with 0.2 ml lysis buffer (50 mM Tris pH 7.4, 1 mM EDTA, 0.5% NP-40, 150 mM NaCl) and ZsGreen levels were measured using a Synergy H4 reader using equivalent amounts of the clarified lysate normalized for total cell

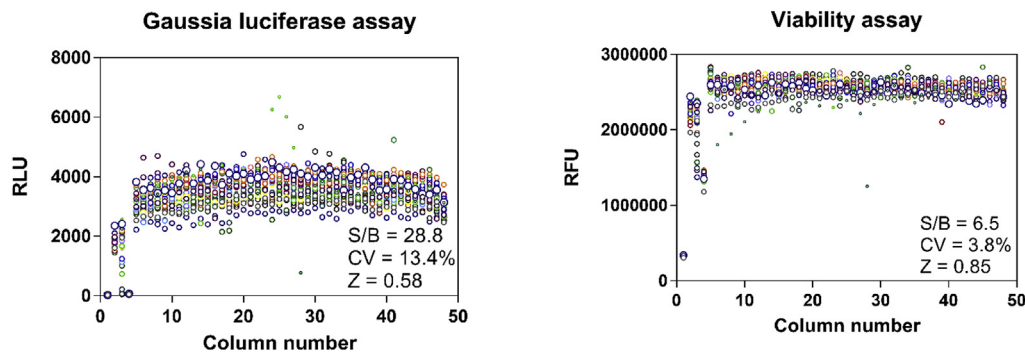
**a. Ribavirin concentration response**



**b. DMSO plate test in LASV/vRNP**



**c. DMSO plate test in LCMV/vRNP**



**Fig. 3. Assay performance in 1,536-well plate format.** A. Dose response of ribavirin. B. DMSO plate test of HEK293-LASV-MG cells. C. DMSO plate test of HEK293T-LCMV-MG cells. For DMSO plate tests, column 1 was media only with DMSO, column 2 and 5–48 was cells with DMSO, column 3 was cells with ribavirin titration, and column 4 was cells with 575  $\mu$ M ribavirin.

protein in the lysate (Pierce BCA Protein Assay Kit, Thermo Scientific, #23227). Mean values were normalized to vehicle (DMSO) treated control group (V) that was adjusted to 100%.

**3. Results**

**3.1. Generation and characterization of cell lines expressing functional LCMV and LASV vRNPs**

We aimed to engineer an infectious-free cell platform that accurately recreated RNA replication and gene transcription biosynthetic processes of LASV in infected cells to enable screening protocols to

identify inhibitors of LASV vRNP activity without the requirement of BSL4 containment. We first explore the feasibility of this approach using the LCMV system as it would allow us to compare results obtained with the virus-free cell platform and live virus infection in BSL2. We constructed the plasmid hPol1-LCMV/SMG-Gluc/Pur-GFP that directed intracellular synthesis, mediated by the human RNA polymerase I promoter (hPol1), of a LCMV-like vRNA (a.k.a. minigenome-MG) of the S segment (antigenome polarity, cRNA) where the NP ORF was replaced by the Pur resistant gene fused to the N-terminus of GFP (Pur-GFP) and the GPC ORF by the Gluc reporter gene (Fig. 1A). We transfected HEK293T cells with the hPol1-LCMV/SMG-Gluc/Pur-GFP plasmid together with polymerase II expression pCAGGS plasmids

**Table 1**  
Summary of hits identified as exerting an inhibitory effect on the activity of both LCMV and LASV vRNPs.

| Sample ID       | LASV/vRNP                    |                            |                                  |             | LCMV/vRNP                  |                                  |             |         |  |
|-----------------|------------------------------|----------------------------|----------------------------------|-------------|----------------------------|----------------------------------|-------------|---------|--|
|                 | Sample Name                  | EC50 ( $\mu$ M) GLuc assay | CC50 ( $\mu$ M) AlamarBlue assay | Selectivity | EC50 ( $\mu$ M) GLuc assay | CC50 ( $\mu$ M) AlamarBlue assay | Selectivity |         |  |
| NCGC00263158-01 | Amuvatinib                   | 1.98                       | 7.03                             | 3.55        | 2.22                       | 28.00                            |             | 12.59   |  |
| NCGC00017338-05 | Antimycin A                  | 0.03                       | 0.06                             | 1.78        | 0.03                       | NA                               |             | 2911.46 |  |
| NCGC00163818-04 | Azoxystrobin                 | 7.03                       | 22.24                            | 3.16        | 4.98                       | 62.68                            |             | 12.59   |  |
| NCGC00253624-01 | Buparvaquone                 | 7.69                       | 34.35                            | 4.47        | 6.85                       | NA                               |             | 14.59   |  |
| NCGC00015339-05 | Dequalinium chloride hydrate | 1.53                       | 15.34                            | 10.00       | 1.09                       | NA                               |             | 92.07   |  |
| NCGC00351592-01 | Manassantin A                | 0.02                       | 0.97                             | 50.12       | 0.02                       | 24.32                            |             | 1000.02 |  |
| NCGC00179454-04 | Monensin sodium salt         | 0.27                       | 2.11                             | 7.94        | 0.19                       | 5.95                             |             | 31.62   |  |
| NCGC00346582-01 | Mubritinib                   | 0.02                       | 0.05                             | 2.51        | 0.02                       | NA                               |             | 6518.06 |  |
| NCGC00167550-06 | Narasin                      | 0.22                       | 2.22                             | 10.00       | 0.11                       | 4.44                             |             | 39.81   |  |
| NCGC00015810-09 | Papaverine hydrochloride     | 4.32                       | 24.32                            | 5.62        | 3.43                       | NA                               |             | 29.12   |  |
| NCGC00094258-02 | Papaverine hydrochloride     | 3.06                       | 12.19                            | 3.98        | 1.93                       | NA                               |             | 51.77   |  |
| NCGC00015802-18 | Pimozide                     | 8.63                       | NA                               | 11.59       | 24.32                      | NA                               |             | 4.11    |  |
| NCGC00015901-02 | Rotenone                     | 0.02                       | 0.15                             | 7.08        | 0.02                       | 24.32                            |             | 1412.54 |  |
| NCGC00017358-03 | Rotenone                     | 0.04                       | 0.24                             | 6.31        | 0.05                       | 4.32                             |             | 89.12   |  |
| NCGC00263555-02 | Spautin-1                    | 4.85                       | 19.31                            | 3.98        | 8.63                       | NA                               |             | 11.59   |  |
| NCGC00178679-02 | Valinomycin                  | 0.61                       | 1.22                             | 2.00        | 0.15                       | 1.93                             |             | 12.59   |  |
| NCGC00186050-01 | Ziprasidone                  | 2.17                       | NA                               | 46.14       | 9.68                       | NA                               |             | 10.33   |  |
| NCGC00263539-01 | Ziprasidone                  | 3.85                       | NA                               | 25.95       | 4.85                       | NA                               |             | 20.61   |  |
| NCGC00160337-02 | Tryptanthrin                 | 1.93                       | 19.31                            | 10.00       | 1.22                       | 12.19                            |             | 10.00   |  |

encoding LCMV NP and L, the minimal trans-acting viral factors required for LCMV genome replication and gene expression (Hass et al., 2004; Lee et al., 2000) (Fig. 1A). At 24 h post-transfection we plated cells into 10-cm dishes at low density and subjected them to selection in the presence of Pur (2.5  $\mu$ g/ml) to select cell clones that expressed a functional LCMV vRNP as determined by high levels of GFP (> 95% GFP + cells and > 20,000 fluorescent units over parental control cells) and Gluc (> 100-fold induction over parental control cells) (Fig. 1A). We used a similar approach to generate the hPol1-LASV/SMG-Gluc/Zeo-P2A-ZsG construct where Gluc substituted for the GPC ORF, and the NP ORF was replaced for a Zeo-P2A-ZsG (Fig. 1A). We selected Zeo-P2A-ZsG over the Pur-GFP we used with LCMV, based on evidence supporting the superior performance of Zeocin as selection agent for development of human cell lines (Lanza et al., 2013), and the stronger mean intensity fluorescence of ZsG compared to GFP. This hPol1-LASV/SMG-Gluc/Zeo-P2A-ZsG directs intracellular synthesis of an LASV S MG RNA specie of genome polarity where Zeo-P2A-ZsG is in antisense polarity with respect to the hPol1 promoter, which prevented the possibility that the hPol1 could direct the synthesis of RNA species that, despite the lack of capping and polyadenylation, could result in low levels of Zeo-P2A-ZsG expression and introduce a confounding factor during cell line selection. To engineer the cell line expressing the functional LASV vRNP we used commercially available (GenTarget Inc) HEK293 cells stably expressing the monomeric red fluorescent protein (mRFP) and the blasticidin resistant gene (bsr) (HEK293-RFP). This approach facilitates rapid identification of compounds that may significantly interfere with Pol-II-mediated expression and thereby could affect expression of the viral NP and LP with the consequent effect on vRNP activity. ZsG and mRFP have different excitation and emission spectra (Shaner et al., 2005), and therefore their expression levels in the same sample can be determined without mutual interference. We transfected HEK293-RFP cells with hPol1-LASV/SMG-Gluc/Zeo-P2A-ZsG together with the Pol-II pCAGGS expression vectors for LASV NP and L, and used zeocin treatment (100  $\mu$ g/ml) to select cells expressing a functional LASV vRNP as determined by high levels of ZsG (> 95% ZsG + cells and > 15,000 FU over non-expressing vRNP control cells) and Gluc (> 100-fold induction over parental control cells).

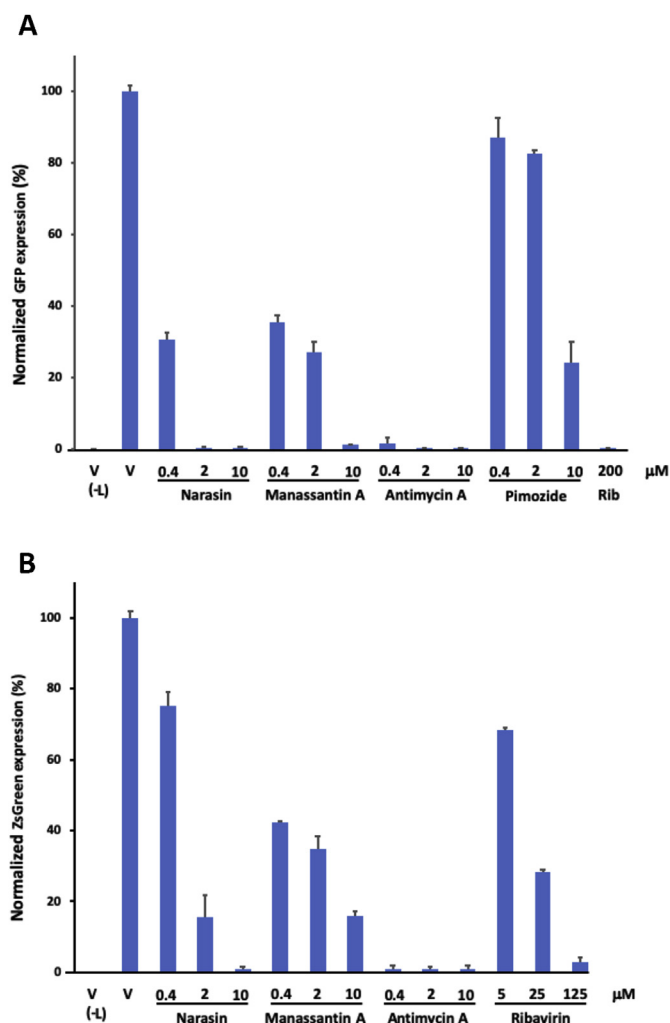
We confirmed the expression of NP and L mRNAs and proteins of LCMV and LASV in LCMV/vRNP and LASV/vRNP, respectively, cell lines (Fig. 1B). Detection of LCMV NP and L mRNAs was done RT-PCR (Fig 1Bi), and expression of LCMV NP assessed by IIF (Fig 1Bii) and WB

(Fig 1Biii) using an antibody to the C-terminal HA epitope tag present in LCMV NP. Detection of LASV NP and L mRNAs was done by RT-PCR (Fig 1Ci), and expression of LASV NP assessed by IIF (Fig 1Cii) using a mouse monoclonal antibody to LASV NP.

We characterized cell clones expressing LCMV and LASV functional vRNP to identify those with optimal properties for their use in the development of assays for HTS to identify inhibitors of viral RNA genome replication and gene transcription. For this we used the following criteria: 1) L polymerase dependent vRNP-directed reporter gene expression, confirmed by reduced expression levels of GFP or ZsG caused by treatment with siRNA that specifically target LCMV or LASV L polymerase, respectively (Fig. 2A). 2) Sensitivity to the inhibitory effect of ribavirin, a validated inhibitor of LCMV and LASV RNA replication and transcription (Fig. 2B), as well as to A3 and favipiravir (T-705) (Fig. 2C), two described inhibitors of the activity of LCMV and LASV vRNP (Mendenhall et al., 2011b; Ortiz-Riano et al., 2014). As predicted, we found that multiplication of a rLCMV expressing GFP (rLCMV/GFP), but not GFP expression directed by LCMV/vRNP, was inhibited by PF429242, an inhibitor of S1P mediated processing of arenavirus GPC (Urata et al., 2010) (Fig. 2D). We also confirmed that mRFP expression levels in LASV/vRNP cells were not significantly affected by ribavirin, A3 or T-705 (favipiravir). Growth properties of LASV/vRNP and LCMV/vRNP cell lines were similar to those exhibited by their respective parental cell lines and sustained high expression levels of reporter genes over 20 passages in cultured cells. We also included as criteria for cell clone selection their viability upon freeze-thaw steps, and reproducibility in assay performance from batch-to-batch.

To assess the feasibility of using the vRNP-cell lines as platforms to implement HTS to identify inhibitors of LCMV and LASV vRNP genome replication and gene transcription, we evaluated the performance of LCMV/vRNP and LASV/vRNP cell lines in a 96-well plate format assay. For that, we seeded LCMV/vRNP and LASV/vRNP cells in 96-well plates (black with optically clear bottom, Greiner Bio) at different densities and at different post-seeding times treated cells with ribavirin (0–250  $\mu$ M), which is known to inhibit both LCMV (Ortiz-Riano et al., 2014) and LASV (Gunther et al., 2004) multiplication in a dose-dependent manner. At different times (12, 24 and 48 h) post-treatment with ribavirin, we determined expression levels of Gluc in tissue culture supernatants and GFP (LCMV/vRNP) or ZsG (LASV/vRNP) in cells. We used DAPI staining to estimate cell biomass and avoid differences in cell numbers as confounding factor of values of vRNP activity. We observed





**Fig. 4.** Effect of selected hits on GFP expression directed by LCMV and LASV MGs in transiently transfected cells. HEK293T cells were transfected with pCAGGS T7, T7-S-MG-GFP for LCMV or LASV, and Pol-II pCAGGS expression vectors for the L and NP trans-activating factors for LCMV (Fig. 4A) or LASV (Fig. 4B). Transfections were done in the presence of the indicated compound concentrations and at 48 h post-transfection we prepared cell lysates to measure levels of GFP expression. For the four (narasin, manassantin A, antimycin A and pimozide) and three (narasin, manassantin A, antimycin A) tested hits in the LCMV (Fig. 4A) and LASV (Fig. 4B), respectively, MG systems pT7MG-GFP, pCAGGS NP, and pCAGGS L. At 5 h post-transfection, fresh medium containing selected drug hits was added and cells were incubated for 48 h. GFP expression levels were determined by lysing the cells and measuring GFP expression in samples with equivalent amounts of total protein. GFP expression values were normalized to values obtained with VC-treated control cells.

the expected dose-dependent inhibitory activity of ribavirin on LCMV and LASV vRNP-directed expression of both Gluc and GFP (LCMV) or ZsG (LASV). We obtained optimal results for both LCMV/vRNP and LASV/vRNP cell lines (14 replicates/condition) using the following conditions: 1) seeding  $4 \times 10^4$  cells/well, 2) compound (ribavirin) addition at 8 h after cell seeding, and 3) assay read out at 48 h after starting ribavirin treatment. The assay had a signal to background ratio (S/B) > 100 for Gluc and > 15 for GFP (LCMV) or ZsG (LASV) readouts, as determined by comparing signals obtained with vRNP-expressing lines and control cells lacking the corresponding vRNP. We observed an inhibition > 99% of LCMV and LASV vRNP activities at 250 μM ribavirin and selected this ribavirin concentration to generate the minimal signal of the assay. To evaluate the assay signal dynamic range and data

variation associated with sample measurement we determined the Z' factor values for Gluc and ZsG-based readouts of the LASV vRNP activity in ribavirin-treated LASV/vRNP cells. We used as maximal (Max) and minimal (Min) reporter signals the values obtained at 0 and 50 or 250 μM ribavirin, respectively, after subtracting background signals (Fig. 2E). Under the optimized assay conditions we consistently observed a Z' ≥ 0.5 for Gluc and ZsG-based readouts in the presence of 50 or 250 μM ribavirin, these Z' factor values are compatible with the assay being adapted to HTS (Iversen et al., 2006; Zhang et al., 1999).

At the highest concentration tested (250 μM), ribavirin had a minimal effect on cell viability, which prevented us from assessing the response of our cell-based assay to compound induced toxicity. To address this issue, we examined the dose-dependent effect of DMSO on cell viability and vRNP-directed reporter gene expression. Determining the DMSO tolerance of our assay was also critically important as primary screens will be done at single dose (10 μM) compound prepared in DMSO, which would require our assay to be compatible with the compound contributed % of DMSO in the final volume. We found that at concentrations ≤ 0.5% DMSO did not have any significant effect on the viability of LCMV/vRNP or LASV/vRNP cell lines, whereas subsequent increases in the % of DMSO resulted in dose-dependent cell toxicity as determined by the CellTiter-Fluor cell viability assay, which correlated with reduced levels of vRNP directed expression of GFP (LCMV/vRNP) or ZsG (LASV/vRNP) and Gluc (both LCMV/vRNP and LASV/vRNP).

### 3.2. Assay performance in 1,536-well format

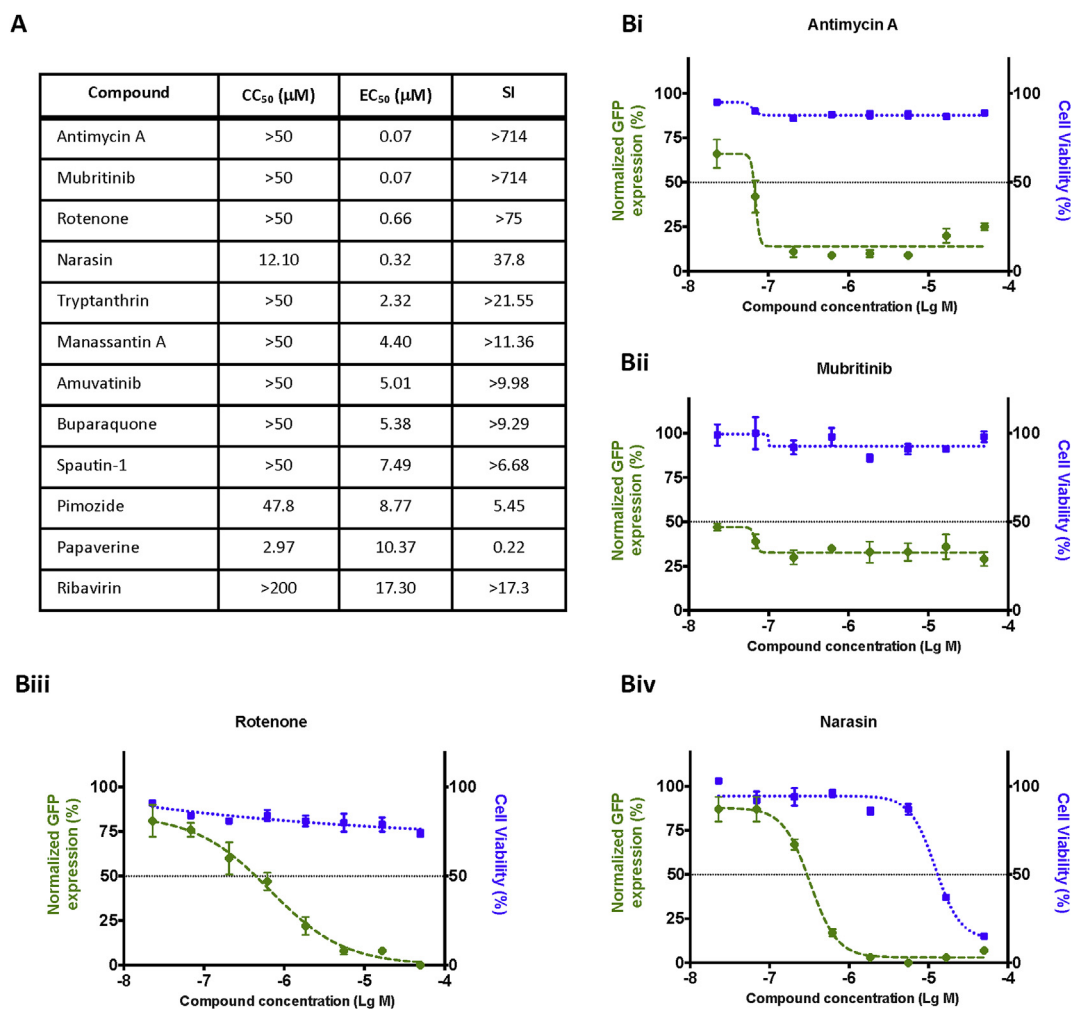
The activity of LCMV and LASV vRNP was assayed by determining expression levels of the Gluc reporter gene. Optimal biological response required a 48 h incubation period, and therefore compounds with cytotoxic effects could present as false positives. To identify and eliminate cytotoxic compounds during the primary screen, a multiplexed assay was developed by using alamarBlue Viability Reagent as a readout for cytotoxicity before lysing the cells to assay for Gluc activity. The multiplexed assay was tested in 1,536-well format and was found to have robust performance in both LASV/vRNP and LCMV/vRNP cell lines (Fig. 3).

### 3.3. qHTS and confirmation

A total of 7,163 unique small molecule compounds were screened in qHTS format against the LASV/vRNP and the LCMV/vRNP cell lines using the multiplexed vRNP assay. The data has been deposited into PubChem under AID #1347082 for LASV luciferase data, AID #1347083 for LASV viability data, AID #1347086 for LCMV luciferase data, and AID #1347085 for LCMV viability data. A total of 67 unique compounds showing greater than 3-fold selectivity in Gluc activity versus alamarBlue activity in either of the LASV or LCMV cell lines were cherry picked for confirmation. Confirmation was done as 11-point 1:3 titration in duplicates in each of the cell lines using the multiplexed vRNP assay. The confirmation data can be found in PubChem as AID #1347087 for LASV luciferase data, AID #1347081 for LASV viability data, 1347084 for LCMV luciferase data, and AID #1347088 for LCMV viability data. Upon confirmation, 16 compounds showed EC<sub>50</sub> < 10 μM in the luciferase assay, with a greater than 10-fold selectivity between luciferase and alamarBlue assays, in either cell line (Table 1). Three compounds were identified in separate libraries and tested twice. These 16 compounds were further cherry picked as 10 mM DMSO solutions and tested in follow up assays.

### 3.4. Functional confirmation of vRNPs as targets of selected hits

To further confirm that identified hits targeted the activity of the vRNP, we used our transient transfection based LCMV and LASV MG system (Ortiz-Riano et al., 2014; Emonet et al., 2011b; Yun et al., 2013)



**Fig. 5. Dose-response of selected hits on LCMV multiplication.** A. Summary of CC<sub>50</sub>, EC<sub>50</sub>, and SI values of hits. B. Graphic display of dose-response results from selected hit compounds with SI > 30. A549 cells were infected with rLCMV-GFP (MOI = 0.01) and treated with serial dilutions of test compounds. At 48 h pi, cells were PFA-fixed and fluorescence levels determined. Cell viability was determined after 48 h treatment with the indicated compounds and concentrations. Data were normalized to vehicle control (VC) group (DMSO) which was adjusted to 100%.

to examine the effect of selected hits on viral genome replication and gene expression directed by the LCMV or LASV MG expressing either GFP (LCMV MG) or ZsGreen (LASV MG) from the NP locus. We transfected HEK293T cells with T7-S-MG-GFP (LCMV) or T7-S-MG-ZsGreen (LASV) together with Pol-II pCAGGS expression vectors for the T7 RNA polymerase, and L and NP trans-active factors for LCMV (Fig. 4A) or LASV (Fig. 4B). Transfections were done in the presence of the indicated compound concentrations and at 48 h post-transfection we prepared cell lysates to measure levels of GFP and ZsGreen expression. For the four (narasin, manassantin A, antimycin A and pimozide) and three (narasin, manassantin A, antimycin A) tested hits in the LCMV (Fig. 4A) and LASV (Fig. 4B), respectively, MG systems we observed a dose-dependent inhibitory effect on the MG activity.

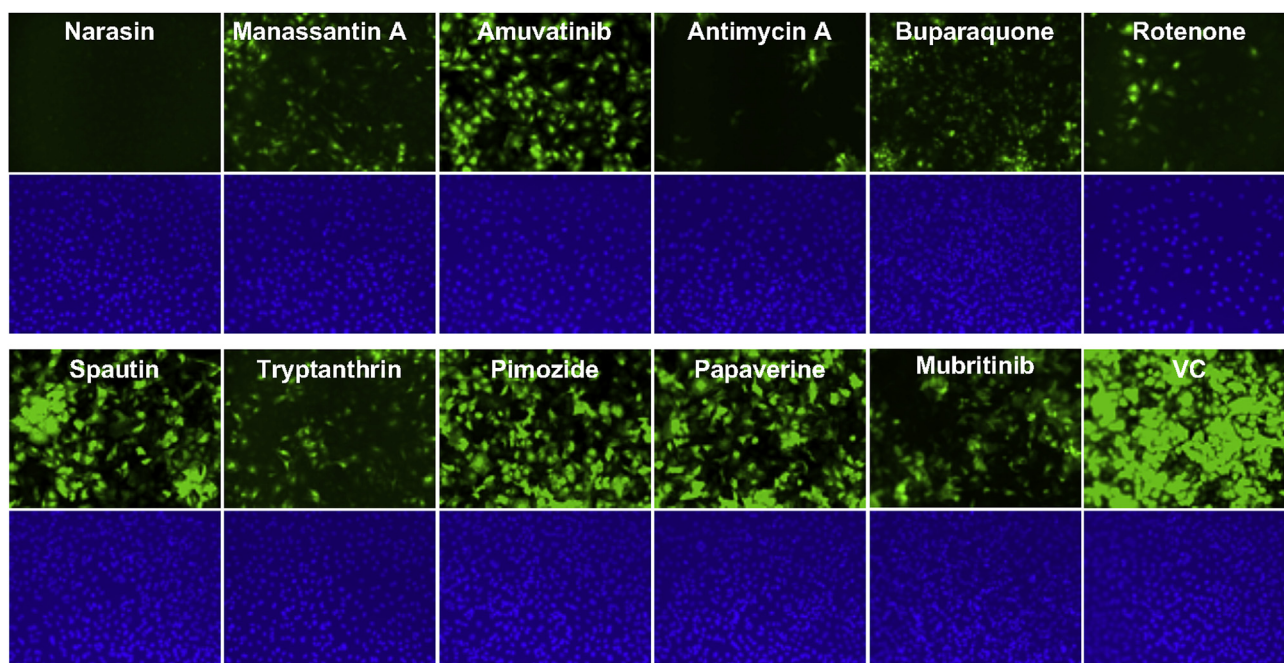
### 3.5. Hit validation in the context of LCMV infection

We selected 11 out of the 16 hits with the ability to inhibit the activity of both LASV and LCMV vRNP to examine their effects on multiplication of live LCMV (Fig. 5). For this we treated A549 cells with 3-fold serial dilutions of each compound starting 2 h prior to infection (MOI = 0.01) with rLCMV/GFP and maintained compounds throughout the course of infection (48 h) when we determined GFP expression levels. We also examined the dose-dependent effect of these selected hits on cell viability using the CellTiter 96 AQueous One

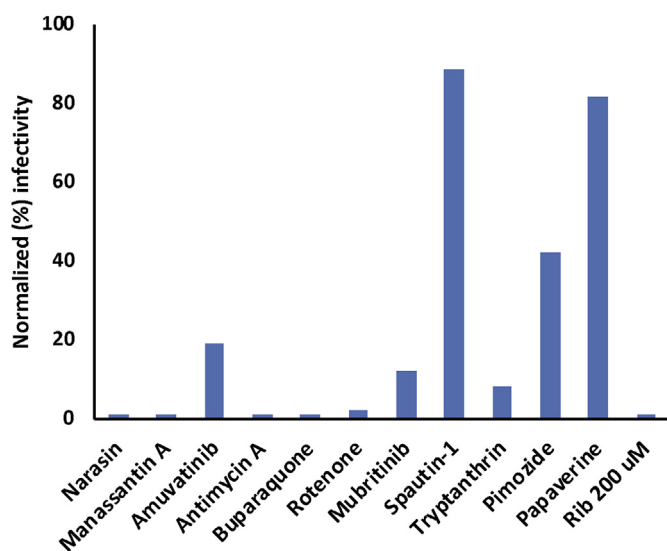
Solution reagent. Levels of GFP expression were normalized assigning a value of 100% to GFP expression levels in vehicle control (VC)-treated (DMSO) and infected cells. Values of cell viability were normalized assigning a value of 100% to VC-treated cells. We used these normalized values to determine compounds EC<sub>50</sub> and CC<sub>50</sub> values and their corresponding selectivity index (SI; SI = CC<sub>50</sub>/EC<sub>50</sub>) values (Fig. 5A). Among the 11 hits evaluated, four exhibited SI > 30 (Fig. 5B). We observed a correlation between EC<sub>50</sub> values and the degree to which the hit interfered with propagation of LCMV in cultured cells (Fig. 6), and production of LCMV infectious progeny (Fig. 7) at 48 h pi.

## 4. Discussion

We have developed cell-based platforms that allow for screening of compounds affecting viral RNA genome replication and/or gene transcription biosynthetic processes directed by the vRNPs of LCMV and LASV. These platforms are based on the generation of human HEK293 cell lines stably expressing LCMV and LASV trans-acting factors, NP and L, required for the formation of a functional vRNP together with a virus-like RNA (MG) encoding for reporter genes whose expression levels serve as accurate surrogates of the vRNP activity. Generation of the LCMV/vRNP and LASV/vRNP cell lines was facilitated by the MG expressing of a fusion gene between a drug resistant gene and the reporter gene. The dynamic range and reproducibility of



**Fig. 6.** Effect of selected hits on LCMV cell propagation. A549 cells were infected (MOI = 0.01) with rLCMV-GFP and treated (5  $\mu$ M) with the indicated compounds. Cells were fixed at 48 h pi and infected cells identified based on GFP expression detected by epifluorescence.



**Fig. 7.** Effect of selected hits on production of infectious LCMV progeny. A549 cells were infected (MOI = 0.01) with rLCMV-GFP and treated (5  $\mu$ M) with the indicated compounds. At 48 h pi tissue culture supernatants (TCS) were collected and titers of infectious virus determined based on foci of rLCMV/GFP-infected cells identified by epifluorescence and further validated by immunofluorescence using a rat monoclonal antibody (VL4) to NP conjugated to Alexa fluor 568. TCS titers were normalized by assigning the value of 100% to TCS titers in VC-treated (DMSO) cells. Results correspond to the average of two biological replicates with SD (not shown) < 10%. At the experimental endpoint (48 h pi) cell viability, as determined by CellTiter 96 AQueous One solution reagent (Promega), was > 75% for all tested compounds with exception of papaverine treatment that resulted in 55% cell viability.

vRNP-directed reporter gene expression in response to known inhibitors of LCMV and LASV RNA synthesis validated the use of the LCMV/vRNP and LASV/vRNP cell lines for the implementation of HTS approaches to identify inhibitors of LCMV and LASV vRNP activities. Our central goal was to generate an infectious-free cell system to identify compounds that modulate LASV RNA synthesis mediated by an intracellularly

reconstituted functional vRNP without the need of BSL4 containment. These compounds would include candidate antiviral drugs and tool compounds for the investigation of the mechanisms regulating vRNP-directed LASV RNA synthesis. To examine the feasibility of this approach we first generated the LCMV/vRNP cell line and confirmed it faithfully recreated the biosynthetic features of the vRNP observed in the context of LCMV-infected cells.

RNA synthesis by viral RNA dependent RNA polymerases is modulated by host cell factors (Ahluquist et al., 2003; Bortz et al., 2011). Accordingly, the activity of the mammarenavirus vRNP likely involves viral protein-protein and protein-RNA interactions, as well as the contribution of host cell factors. Compounds that target the function of viral proteins, and their interactions, required for vRNP activity are expected to offer specificity, but inhibitor-escape mutants have been isolated for virtually any virus for which a specific inhibitor has been developed, which poses a general problem in antiviral therapy (Domingo, 2003). Combination therapy has proven effective to reduce and delay the selection of viral escape mutants, but does not entirely solve this problem (Domingo, 2003). In contrast, intra-host virus evolution is unlikely to result in viral variants able to escape from inhibitors that disrupt cellular functions required for vRNP activity. Moreover, vRNP of related viruses, like LCMV and LASV, are likely to rely on the same host cell factors for their activities, thus compounds targeting these host cell factors provide an opportunity for the development of broad-spectrum antiviral therapeutics. Targeting host cell factors poses the concern of drug-associated toxicity. However, it should be noted that treatment of acute arenaviral HF disease would be of relatively short duration, and clinical benefits may easily surpass potential short-term duration side effects. It should be also noted that compounds that inhibit LASV and LCMV via the same mechanism of action can be used in studies using the well-established mouse model of LCMV infection to gain valuable information to design more effectively studies in animal models of LASV infection, which should minimize the cost and complex logistic of the use of BSL4 containment required to handle live forms of LASV.

Antimycin A, a mETC complex III inhibitor (Ma et al., 2011; Raveh et al., 2013), and the human epidermal factor receptor 2 (HER2/ErbB2) inhibitor mubritinib (TAK165) inhibited both LCMV and LASV vRNPs,

but with different SI. Both compounds had high (> 1000) SI values for LCMV vRNP, but lower SI values for LASV vRNP due to their activity in the AlamarBlue assay (Table 1). We observed a trend where many compounds exhibited significant higher cytotoxicity in LASV/vRNP cells than in LCMV/vRNP cells, which may have critically contributed to consistently lower SI values for LASV/vRNP cells. LCMV/vRNP and LASV/vRNP lines were generated using HEK293T and HEK293-RFP (GenTarget Inc), respectively, as parental cells. In addition, despite their phylogenetic proximity, LCMV and LASV exhibit a significant degree of genetic divergence and have multiple amino acid differences in their NP and L polymerase proteins. It is plausible that differences in the parental cell line combined with differences in amino acid compositions of the components of each vRNP contributed to LCMV/vRNP and LASV/vRNP cell lines exhibiting differences in susceptibility to specific compounds, which impacted SI values obtained for the same compound in LCMV/vRNP and LASV/vRNP cell lines.

Mubritinib has been recently identified as a novel mETC complex I inhibitor (Bacelli IG et al., 2019), which may have contributed to its anti-LCMV activity. Moreover, rotenone, another validated mETC complex I inhibitor (Majander et al., 1996), exhibited also a potent ( $EC_{50} = 0.66 \mu\text{M}$ ;  $SI > 75$ ) anti-LCMV activity. Impaired mETC function can suppress autophagy (Ma et al., 2011), which has been shown to cause reduced production of infectious progeny of the mammarenavirus LASV (Baillet et al., 2019). *De novo* pyrimidine biosynthesis has been also linked to mETC activity via dihydroorotate dehydrogenase (DHODH) (Loffler et al., 1997), and DHODH inhibitors have been shown to be potent broad-spectrum antivirals (Hoffmann et al., 2011). Clinical implementation of inhibitors of mETC function as antiviral agents can raise objections related to their expected potential toxicity. However, the mETC complex I inhibitor biguanide metformin is a widely prescribed antihyperglycaemic for Type II diabetes that has a good safety profile (Bridges et al., 2014). The  $\text{Na}^+$  and  $\text{K}^+$  ionophore antibiotics narasin and valinomycin, respectively, exhibited also strong inhibitory effect on LCMV and LASV vRNP activity. Narasin has been shown to exert antiviral activity against dengue virus (Low et al., 2011), whereas valinomycin was identified as a potent hit in a cell-based screen to identify effective antivirals against severe acute respiratory syndrome coronavirus (SARS-CoV) (Wu et al., 2004).

## 5. Conclusion

We have developed stable human cell lines constitutively expressing functional vRNPs of LCMV and LASV. We used these cell lines for the identification of inhibitors of LCMV and LASV RNA genome replication and gene transcription using HTS approaches. The infectivity-free feature of our LASV vRNP-expressing cell line overcomes the need of BSL4 facilities currently required to handle infectious LASV, which has the potential to accelerate development of antivirals against LASV. Using these vRNP-expressing cell lines in drug repurposing screens we identified 16 compounds that inhibited LCMV and LASV vRNP activity. Importantly, a subset of 11 tested compounds inhibited multiplication of live LCMV in cultured cells. Existing knowledge about the targets of these antiviral compounds would suggest the mETC as a potential target for therapeutics against mammarenaviruses, as well as the merits of exploring ionophore-based compounds as potential antivirals against LASV. Future studies, beyond the scope of the present paper, will examine whether newly identified compounds with the ability to inhibit LCMV and LASV vRNP activity are also active against HF-causing New World arenavirus such as Junin virus, as well as the antiviral activity of these compounds in animal models of mammarenavirus infection.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.antiviral.2019.104667>.

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