Lassa and Ebola virus inhibitors identified using minigenome and recombinant virus reporter systems

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13 **Abstract**

14 Lassa virus (LASV) and Ebola virus (EBOV) infections are important global health issues resulting in
15 significant morbidity and mortality. While several promising drug and vaccine trials for EBOV are
16 ongoing, options for LASV infection are currently limited to ribavirin treatment. A major factor impeding
17 the development of antiviral compounds to treat these infections is the need to manipulate the virus
18 under BSL-4 containment, limiting research to a few institutes worldwide. Here we describe the
19 development of a novel LASV minigenome assay based on the ambisense LASV S segment genome,
20 with authentic terminal untranslated regions flanking a ZsGreen (ZsG) fluorescent reporter protein and
21 a *Gaussia princeps* luciferase (gLuc) reporter gene. This assay, along with a similar previously
22 established EBOV minigenome, was optimized for high-throughput screening (HTS) of potential
23 antiviral compounds under BSL-2 containment. In addition, we rescued a recombinant LASV
24 expressing ZsG, which, in conjunction with a recombinant EBOV reporter virus, was used to confirm
25 any potential antiviral hits *in vitro*. Combining an initial screen to identify potential antiviral compounds
26 at BSL-2 containment before progressing to HTS with infectious virus will reduce the amount of
27 expensive and technically challenging BSL-4 containment research. Using these assays, we identified
28 6-azauridine as having anti-LASV activity, and demonstrated its anti-EBOV activity in human cells. We
29 further identified 2'-deoxy-2'-fluorocytidine as having potent anti-LASV activity, with an EC₅₀ value 10
30 times lower than that of ribavirin.

31

1. Introduction

Lassa virus (LASV) and Ebola virus (EBOV) cause viral hemorrhagic fevers (VHFs) associated with substantial mortality and morbidity (Geisbert and Jahrling, 2004). LASV (family *Arenaviridae*) is endemic in West Africa, with an estimated 100,000–300,000 annual cases and 5,000 deaths (McCormick and Fisher-Hoch, 2002). EBOV (family *Filoviridae*) is responsible for recurring epidemics in Africa, including the 2013–2016 outbreak in West Africa that resulted in over 28,000 cases (World Health Organisation, 2016). No vaccines or therapeutics are currently approved for the prevention or treatment of either infection, leaving supportive care or “off-label” use of existing drugs the only options for health care workers (Geisbert and Jahrling, 2004; Jacobs et al., 2015).

The requirement to handle these viruses at biosafety level 4 (BSL-4) limits research to only a few institutions worldwide. In lieu of using live virus at BSL-4, many groups have used virus-like particles containing the LASV or EBOV envelope glycoproteins, or pseudotyped lentivirus or vesicular stomatitis virus particles to study viral entry or egress and to screen inhibitors of these processes (Anantpadma et al., 2016; Han et al., 2014; Kouznetsova et al., 2014; Maisa et al., 2009; Yonezawa et al., 2005). One largely unexplored option is the identification of antivirals targeting viral replication and transcription; using nucleoside analogs to inhibit these processes has proven highly successful for other viral infections, such as HIV and hepatitis C virus (HCV) (Cihlar and Ray, 2010; Gane et al., 2013). Minigenome assays emulate viral replication and transcription, and may be used to identify inhibitors of these processes (McCarthy et al., 2016; Uebelhoer et al., 2014). However, while the signal generated by a minigenome is dependent on its replication by viral proteins, the expression of these proteins, usually from co-transfected plasmids, requires cellular factors like RNA polymerase II (PolII). Thus, the activity of compounds identified during screening using a minigenome system must be confirmed in additional assays using infectious virus.

Here we describe the development of a LASV minigenome assay that can be performed at BSL-2. We also generated a novel recombinant LASV expressing the fluorescent reporter protein ZsGreen (ZsG) to facilitate high-throughput screening (HTS) compound testing at BSL-4. We used these novel LASV minigenome and reporter virus assays alongside the existing EBOV assays (Albariño et al., 2016; Albariño et al., 2015) to test the ability of 27 selected commercially available nucleoside analogs to inhibit LASV and EBOV minigenome function. We verified the specificity of these compounds using reporter and authentic wild-type viruses.

2. Materials and Methods

2.1 Biosafety

65 Work with infectious wild-type and recombinant LASV and EBOV was performed under BSL-4
66 containment. Experiments involving cDNA encoding viral sequences were performed in accordance
67 with approved Institutional Biosafety Committee (IBC) protocols. Minigenome experiments were
68 conducted under BSL-2 conditions.

69

70 2.2 Cell culture, viruses, and compounds

71 Huh7 cells (Apath), Vero-E6 cells, and BSR-T7/5 cells were cultured in Dulbecco's modified Eagle's
72 medium (DMEM) supplemented with 5% (v/v) fetal calf serum (FCS), 100 U/mL penicillin, and 100
73 µg/mL streptomycin. Huh7 cells were further supplemented with 1× non-essential amino acids (NEAA).
74 BSR-T7/5 cells were selected with 1 mg/mL G418 (Invitrogen) added every other passage. The viruses
75 used in generating the recombinant strains were LASV (Strain Josiah, Genbank HQ688673.1,
76 HQ688675.1) and EBOV (Zaire ebolavirus strain Ebola virus/H.sapiens-rec/LBR/2014/Makona-L2014,
77 Genbank KR781608). Nucleoside analogs were obtained from Sigma-Aldrich, Selleckchem, U.S.
78 Pharmacopeia, Carbosynth, and Emory Institute of Drug Development (EIDD).

79

80 2.3 Rescue of rLASV/ZsG reporter virus

81 To rescue recombinant LASV expressing ZsG (rLASV/ZsG), BSR-T7/5 cells were transfected with
82 plasmids containing full-length, antigenomic-sense LASV L and recombinant S genome segments
83 under transcriptional control of a T7 promoter element. The recombinant S segment contained the
84 inserted ZsG-P2A coding sequence immediately 5' to the nucleoprotein (NP) coding sequence. The
85 P2A sequence causes self-cleavage of the nascent peptide chain during translation, resulting in the
86 expression of the 2 separate proteins, ZsG and NP (Kim et al., 2011). Cells were transfected with 1 µg
87 of each plasmid, and 5 days post transfection the supernatant was removed and used to infect Vero-E6
88 cells as previously described (Albariño et al., 2015). rLASV/ZsG tissue culture infective dose 50
89 (TCID₅₀) titers were determined in Vero-E6 cells by immunofluorescence assays using anti-LASV
90 polyclonal antibodies.

91

92 2.4 Minigenome and reporter virus assay screening of nucleoside analogs

93 For minigenome screening, Huh7 cells were seeded 2×10^4 cells/well in a 96-well plate 1 day prior to
94 the assay. The plasmids used in the assay were pLASV-S (ZsG/gLuc), which expresses the
95 minigenome segment, and the support plasmids pC-LASV-L and pC-LASV-NP, which express LASV

polymerase (L) and NP respectively. The minigenome is based on the LASV S segment, in which the NP and GPC coding sequences have been replaced with those of ZsG and *Gaussia princeps* luciferase (gLuc), respectively, leaving the authentic untranslated regions and intergenic regions unchanged. The support plasmids express the viral proteins required for replication and transcription. 50 ng of pLASV-S (ZsG/gLuc), 10 ng of pC-LASV-L, and 30 ng of pC-LASV-NP were transfected per well using 0.27 μ L TransIT-LT-1. One hour post transfection (hpt), nucleoside analogs diluted in DMSO were added to the cells (final DMSO concentration 0.5%) and incubated for 48 h before gLuc expression was quantified using Renilla Luciferase Assay System (Promega). Luminescence was measured using a microplate reader (BioTek Synergy H1MD), with levels recorded at a height of 6 mm after a 2 s delay, 10 ms integration, and 75 gain/sensitivity for LASV minigenome and 50 gain/sensitivity for EBOV minigenome.

For reporter virus assays, Huh7 cells were seeded 1×10^4 cells/well of a 96-well plate in Fluorobrite cell culture media (Thermo Fisher) 1 day prior to the assay. Nucleoside analogs were added to the cells 1 h before infection with reporter virus (MOI = 0.1). Cells were incubated for 72 h before ZsG fluorescence levels were determined using a BioTek Synergy reader (height 6 mm; 120 gain/sensitivity). Cell viability was determined concurrently for both minigenome and reporter virus assays on compound-treated, mock transfected/infected cells using CellTiter-Glo (Promega), which measures cellular ATP content. All experiments were conducted in quadruplicate, and all values normalized to mock transfected/treated cells (i.e., DMSO only) for analysis.

2.5 Virus titer reduction assay

Huh7 cells were seeded 1×10^4 cells/well of a 96-well plate 1 day prior to the assay. Cells were treated with the compound for 1 h prior to infection at MOI = 0.1; 3 days later, cell culture supernatants were harvested and virus titrations were performed in Vero-E6 cells. Three days after infection, the cells were fixed, permeabilized, and stained with virus-specific polyclonal antibodies to visualize viral proteins. The TCID₅₀ of each sample was calculated using the Reed-Muench method (Reed and Muench, 1938).

2.6 Data analysis

Normalized values of both gLuc and ZsG expression were used to fit a 4-parameter equation to semilog plots of the concentration-response data, and to interpolate the nucleoside analog concentration that inhibited 50% of the gLuc or ZsG expression (EC₅₀) in transfected or infected cells, respectively. Cell viability data were similarly used to derive the 50% cytotoxicity concentration of each compound (CC₅₀).

128 The selectivity index (SI) was calculated by dividing the CC_{50} by the EC_{50} . All analyses were performed
129 using GraphPad Prism (GraphPad Software). Z prime (Z') score, a measure of statistical effect size,
130 was calculated for each assay as a measure of suitability for HTS, with anything between 0.5 and 1.0
131 being considered acceptable (Zhang et al., 1999).

132

133 3. Results

134 3.1 Design and optimization of a LASV minigenome for HTS of nucleoside analogs

135 A LASV minigenome pC-LASV-S (ZsG/gLuc) was designed using a modified S segment in which the
136 NP and GPC coding sequences were replaced with those for ZsG and gLuc, respectively (Figure 1a).
137 Using 2 reporters allows for rapid qualification of minigenome activity by visualizing the cells under
138 fluorescent microscopy, but further permits a more sensitive quantification of activity via measurement
139 of expressed gLuc in the media. The HTS assays described here used the gLuc signal because of its
140 higher sensitivity. A ratio of 3:1:5 of pC-LASV-S (ZsG/gLuc), pC-LASV-L, and pC-LASV-NP gave the
141 optimal signal-to-noise ratio of ~200:1 at 48 hpt (Supplementary Figure 1). GLuc expression was
142 detectable in the absence of LASV polymerase (~60-fold over mock-transfected cells at 48 hpt; data
143 not shown), and was likely the result of cryptic cellular RNA PolIII promoters in the minigenome plasmid
144 (Boehmann et al., 2005; Jasenosky et al., 2010; Muhlberger et al., 1999; Uebelhoer et al., 2014). Here,
145 we used gLuc expression in the absence of polymerase (or, in the case of the EBOV minigenome, with
146 an inactive form of EBOV polymerase [Albariño et al., 2016]) to evaluate the ability of compounds to
147 inhibit cellular transcription mechanisms. Titrations of ribavirin, a guanosine analog with known activity
148 against RNA virus replication (McCormick et al., 1986; Uebelhoer et al., 2014), were used to evaluate
149 both minigenome assays in a 96-well format (Figures 1b and 1c). Two measures of selectivity were
150 used to assess the inhibitory activity of ribavirin. First, the SI value (CC_{50}/EC_{50}) was calculated to obtain
151 a ratio between the antiviral activity of a compound and its cytotoxicity. Second, we also calculated the
152 ratio of the background EC_{50} value (defined as the concentration at which gLuc expression in the
153 minigenome assay without an active polymerase was reduced by 50%) to the EC_{50} value of the
154 minigenome with an active polymerase; this was termed the SI_{pol} value. SI_{pol} was used to determine if
155 minigenome inhibition was specific to the viral polymerase; if the inhibitory activity of a nucleoside was
156 mediated through the inhibition of cellular RNA PolIII, then the inhibition of the background signal would
157 also be observed, giving an SI_{pol} value of approximately 1. Ribavirin concentration-dependent inhibition
158 was observed in both LASV and EBOV minigenomes, with EC_{50} values of 2.51 and 5.04 μ M,
159 respectively (Figure 1d). For both assays, the SI_{pol} value was >20, indicating specific inhibition of the
160 viral polymerase. The EC_{50} values were lower than those previously reported (Hass et al., 2004;
161 Oestereich et al., 2016; Uebelhoer et al., 2014), although this discrepancy may be due to differences

between the minigenome designs and cell lines used. Using 50 μ M ribavirin as the positive control and DMSO vehicle-only signal as the negative control, the Z' scores were calculated to be 0.86 for LASV minigenome and 0.60 for EBOV minigenome assays. These scores indicated the suitability of both minigenomes to be used in HTS assays.

Next, we tested the ability of 27 selected nucleoside analogs to inhibit the LASV and EBOV minigenomes, using 3 concentrations of each compound (5000, 500, and 50 nM; Supplementary Table 1). Using a cut-off of <15% minigenome activity compared to the mock-treated control, we selected for further evaluation 6 compounds that inhibited activity of LASV minigenome and 3 that inhibited activity of the EBOV minigenome. Titrations were then performed for these compounds using the respective minigenomes (Figure 2). Any inhibitory effects of 8-azidoadenosine on LASV minigenome activity and toyocamycin on EBOV minigenome activity were difficult to distinguish from the associated cytotoxicity. Toyocamycin, 5-fluorouridine and 8-azaadenosine reduced the LASV minigenome signal in a concentration-dependent manner without cytotoxicity, but also concomitantly inhibited background gLuc expression in the absence of viral L polymerase. The SI_{pol} values of these compounds ranged from 1-2 indicating an indirect inhibitory mechanism.

In contrast, 6-azauridine and 2'-deoxy-2'-fluorocytidine (2'-dFC) reduced minigenome signal without affecting background gLuc levels. 6-azauridine, which was previously shown to block EBOV and Marburg virus minigenome activity in BSR-T7/5 cells (Uebelhoer et al., 2014), inhibited both LASV and EBOV minigenomes with EC_{50} values of 4.76 and 3.88 μ M, respectively. 2'-dFC inhibited the LASV minigenome with an EC_{50} value of 1.35 μ M. Neither drug was cytotoxic at the highest concentration tested. SI_{pol} values >10 for both compounds indicated that they acted specifically on viral proteins, and not indirectly by inhibiting cellular polymerases such as RNA PolII.

3.2 Generation of rLASV/ZsG reporter virus

To further evaluate the efficacy of the compounds against authentic viral replication and transcription, we used recombinant LASV and EBOV, both expressing ZsG. The development of the reporter EBOV has been described previously (Albariño et al., 2015). Generating recombinant arenaviruses expressing foreign proteins, however, is complicated by the compact nature of the genome, which lacks non-essential elements that can be replaced with a reporter gene. One previous attempt to generate a reporter arenavirus lymphocytic choriomeningitis virus (LCMV) incorporated an additional third genome segment containing a reporter protein coding sequence (Emonet et al., 2009). A similarly designed recombinant LASV was rescued, but grew poorly and lost the reporter segment after 2–3 passages (unpublished data). However, fusing a fluorescent reporter protein, such as ZsG, to the N-terminus of a

viral protein (with the P2A peptide linkage sequence from porcine teschovirus-1) resulted in successful rescue of the reporter EBOV and also a reporter LCMV expressing GFP (Ngo et al., 2015). Incorporating P2A results in self-cleavage and the expression of 2 proteins from a single promoter (Kim et al., 2011). Using this approach, we generated the recombinant rLASV/ZsG virus, in which the ZsG-P2A coding sequence was inserted at the N terminus of LASV NP (Figure 3a). The recombinant viruses were successfully rescued in BSR-T7/5 cells, with ZsG expression detected in Huh7 cells at 72 hpi using MOI of 0.1 of both viruses (Figure 3b). Characterization of the growth kinetics of rLASV/ZsG versus the wildtype Josiah strain showed modest attenuation of the recombinant virus, with titers reduced by approximately 1 log at 72 hpi; however, ZsG expression was sufficient to quantify using a fluorescence plate reader, allowing its use in HTS (Figure 3c). To evaluate the suitability of rLASV/ZsG and rEBOV/ZsG for HTS, a dose-response curve was performed for both viruses using ribavirin. Concentration-dependent reduction of ZsG fluorescence was observed in cells infected with both rLASV/ZsG and rEBOV/ZsG, with EC_{50} values of 2.03 and 5.34 μ M, respectively, and no associated loss in cell viability (Figures 3c and 3d). The signal-to-noise ratios for rLASV/ZsG and rEBOV/ZsG were 32:1 and 87:1, respectively. Using 50 μ M ribavirin as the positive control and DMSO vehicle-only signal as the negative control, the Z' scores were 0.84 for rLASV/ZsG and 0.75 for rEBOV/ZsG, indicating the suitability of both reporter virus assays to be used for HTS of compounds.

3.3 Evaluating antiviral characteristics of selected nucleoside analogs

As 6-azauridine and 2'-dFC had SI_{pol} values >10, they were selected for further testing using the reporter virus assays. Dose-response assays were performed with the nucleoside analogs in Huh7 cells infected with MOI = 0.1 of either rLASV/ZsG or rEBOV/ZsG. Inhibition was quantified by measuring ZsG fluorescence at 3 dpi. A dose-dependent reduction in fluorescence was observed in 6-azauridine treated cells for rLASV/ZsG and rEBOV/ZsG (EC_{50} : 10.95 μ M for rLASV/ZsG, 11.32 μ M for rEBOV/ZsG; Figure 4a). Similarly, a reduction in fluorescence was observed in 2'-dFC treated cells infected with rLASV/ZsG, with an EC_{50} of 226 nM (Figure 4b). To confirm that antiviral activity of the nucleoside analogs was not specific to the ZsG-expressing viruses, the compounds were tested against wild-type LASV and EBOV. Both reduced wild-type virus titers in a concentration-dependent manner (Figure 4c): at the highest concentrations used, both compounds reduced the titers of their respective viruses by approximately 2 logs. The EC_{50} values of both the compounds tested against wild-type virus were lower than those determined when using the corresponding reporter virus. The chemical structures of 2'-dFC and 6-azauridine are shown in Figure 4d.

3.4 Evaluating inhibitory properties of anti-HIV nucleoside analogs against LASV and EBOV

During the 2013–2016 EBOV disease epidemic in West Africa, reports emerged suggesting that the anti-HIV nucleoside analog lamivudine was effective in treating EBOV infection. Furthermore, a recent study reported that lamivudine and 3 other anti-HIV nucleoside analogs - stavudine, zidovudine, and tenofovir - inhibited an EBOV minigenome (McCarthy et al., 2016). Other studies, however, reported no inhibition of EBOV by lamivudine (Hensley et al., 2015). We assessed the antiviral activity of each above-mentioned anti-HIV compound using dose-response assays with both minigenomes and reporter viruses. All 4 compounds exhibited no cytotoxicity, but also showed little specific antiviral activity (Figure 5). Tenofovir inhibited both LASV and EBOV minigenomes, but this effect was non-specific, as gLuc expression in the absence of viral polymerase was similarly inhibited. Tenofovir also did not significantly inhibit either reporter virus even at the highest concentration used (50 μ M). Lamivudine showed no significant inhibition in any assay at the highest tested concentration (50 μ M).

4. Discussion

A significant problem hindering the study of hemorrhagic fever viruses is the need to conduct experiments under high containment. The ability to deconstruct the viral lifecycle into discrete functions is a useful tool, proving especially invaluable in the study of BSL-4 pathogens by allowing experiments to be performed at lower biosafety containment levels. Here we used LASV and EBOV minigenome assays to evaluate their potential as BSL-2 surrogates for both BSL-4 reporter virus and wild-type virus in HTS of antiviral compounds. To confirm any potential hits, we also developed a novel recombinant LASV expressing the ZsG reporter protein to complement the existing reporter EBOV. The activity of both the minigenome and the ZsG reporter virus can be quantified rapidly, in a non-destructive manner, and using the same plate reader, thus allowing large numbers of compounds to be screened simultaneously for activity against LASV and EBOV. During the recent EBOV outbreak the challenges of VHF outbreak management in a region with two hemorrhagic fever viruses circulating became apparent (Goba et al., 2016). Given that both infections can occur in overlapping geographic areas and present with similar initial clinical signs, obtaining an exact diagnosis is generally dependent upon molecular diagnostic assays. High-throughput screening of inhibitors targeting multiple VHF pathogens simultaneous will hopefully identify compounds active against both LASV and EBOV, thus allowing treatment to begin immediately upon suspicion of a VHF infection in known LASV/EBOV regions without having to wait for a definite diagnosis.

260 Several compounds were identified by our screens as potentially having anti-EBOV and anti-LASV
261 activity. A measure of selectivity, SI_{pol} , was included to assess whether the inhibition of minigenome
262 signal expression occurred through inhibition of the viral polymerases, or whether the putative antiviral
263 compounds also blocked background luciferase signals in the absence of viral polymerase. 5-
264 fluorouridine, 8-azaadenosine, 8-azidoadenosine, toyocamycin, and tenofovir inhibited minigenome
265 signals, but exhibited low SI_{pol} values (<2), suggesting non-specific activity. This was consistent with
266 their inhibition of cellular factors required for the function of the assay, possibly the cellular RNA
267 polymerases required for transcription and expression from the minigenome plasmids. These findings
268 highlight a potential issue with using minigenome systems alone for antiviral screening, as inhibitors of
269 the system itself, rather than of viral polymerase-dependent processes, may be identified as hits. The
270 SI_{pol} value may therefore be used to assess the specificity of inhibition and prioritize compounds for
271 antiviral HTS in the more expensive and technically challenging BSL-4 containment.

272 Two compounds, 2'-dFC and 6-azauridine, had SI_{pol} values >10 , indicating that their mechanism of
273 action may be virus-specific. 6-azauridine was previously shown to inhibit EBOV and Marburg virus
274 (Uebelhoer et al., 2014). Here we demonstrated that 6-azauridine has anti-LASV activity, and confirmed
275 its anti-EBOV activity in the human Huh7 cell line. Previous assays used golden hamster BSR-T7/5
276 cells, but human cells are preferred, especially for testing nucleoside analogues which, if acting through
277 incorporation into viral RNA, need cellular factors to be phosphorylated to an active form. 6-azauridine
278 is known to inhibit multiple viruses by blocking cellular pyrimidine biosynthesis (Chen and Jones, 1979;
279 Flint et al., 2014; Lo et al., 2014; Uebelhoer et al., 2014). Its SI_{pol} value suggests that the LASV and
280 EBOV polymerases may be more susceptible to this mechanism of inhibition than cellular RNA
281 polymerases. 2'-dFC is an analog of PSI-6130, a potent inhibitor of HCV (Murakami et al., 2007;
282 Stuyver et al., 2006), differing from PSI-6130 by the absence of a methyl group at the 2' position
283 (Figure 4d and Supplementary Figure 2). The addition of the methyl group lowered the EC_{90} value
284 against an HCV replicon from 6.5 μ M with 2'-dFC to 4.6 μ M with PSI-6130. However, though PSI-6130
285 was included in our initial screen of analogs, it did not inhibit LASV minigenome activity, suggesting that
286 this 2'-methyl group negatively affects compound effectiveness against LASV. Both our minigenome
287 and reporter virus assays proved useful in examining recent reports concerning the effectiveness of the
288 anti-HIV nucleoside analogs lamivudine, stavudine, zidovudine, and tenofovir against EBOV. Our data
289 contradict previous reports by McCarthy *et al.* 2016, which showed that these compounds inhibited an
290 EBOV minigenome. Other studies, however, showed no inhibition of EBOV by lamivudine (Hensley et
291 al., 2015). Similarly, in our studies, none of these compounds showed specific anti-EBOV or anti-LASV
292 activity.

293 In conclusion, we have described the development of LASV and EBOV minigenome and reporter virus
294 assays adaptable to HTS of antiviral compounds. Furthermore, the use of identical reporter proteins

295 and HTS methodologies described here for both of these pathogens should greatly facilitate compound
296 testing under BSL-4 containment. We have shown that the anti-EBOV activity of 6-azauridine reported
297 in BSRT-7 cells is also observed in cells of human origin, and that this compound also has anti-LASV
298 activity. We have further shown that 2'-dFC potently inhibits LASV, much more so than ribavirin, which
299 is currently the only available treatment option for LASV infection. Further experimentation in animal
300 models will be needed to confirm the antiviral activity *in vivo*. We believe the findings from our study will
301 aid the development of badly needed antiviral agents against EBOV and LASV.

302

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310

Figure 1: Design and validation of the Lassa virus and Ebola virus minigenomes. **a.** Genome schematics of the Lassa virus (LASV) and Ebola virus (EBOV) minigenome (MG) segments. The LASV minigenome contains the antigenome 5' and 3' UTRs of LASV S segment (Strain Josiah, Genbank HQ688673.1) in which the nucleocapsid protein (NP) and glycoprotein precursor (GPC) coding sequences have been replaced with those for ZsGreen (ZsG) and *Gaussia princeps* luciferase (gLuc), respectively. Support plasmids encode LASV polymerase (L) and NP. The EBOV minigenome contains the antigenome 5' and 3' UTRs of Ebola (Zaire ebolavirus strain Ebola virus/H.sapiens-rec/LBR/2014/Makona-L2014, Genbank KR781608) flanking the gLuc coding sequence. Support plasmids encode EBOV L and NP, and transcriptional accessory proteins VP30 and VP35. **b.** The optimized LASV and **c.** EBOV minigenome assays were evaluated with the antiviral compound ribavirin. Reductions in gLuc expression in cells transfected with the minigenome and helper plasmids including viral polymerase (blue) or without an active viral polymerase (red) were measured, with gLuc values normalized to mock-treated cells (DMSO only). Cell viability was assessed at each ribavirin concentration by measuring ATP content (green), and values were normalized to mock-treated cells. Each point represents the mean of quadruplicate wells, with error bars indicating standard deviation; graphs are representative of 2 independent experiments. **d.** EC₅₀ values for each compound, including EC₅₀ for inhibition of background gLuc expression in the assays not containing an active polymerase (EC₅₀ BG), along with CC₅₀ and SI values. SI_{pol} is the ratio of background EC₅₀ to EC₅₀ values.

Figure 2: Screening of potential anti-viral nucleoside analogs using minigenome assays.

Concentration-response curves in Huh7 cells for **a.** LASV and **b.** EBOV minigenome (MG) transfected cells treated with nucleoside analogs. Reductions in gLuc expression in cells transfected with minigenome and helper plasmids including viral polymerase (blue) or without an active viral polymerase (red) were measured, with values normalized to mock-treated cells (DMSO only). Cell viability was assessed at each compound concentration by measuring ATP content (green), and values were normalized to mock-treated cells. Each point represents the mean of quadruplicate wells, with error bars indicating standard deviation; graphs are representative of 2 independent experiments. **c.** EC₅₀ values for each compound, including EC₅₀ values for inhibition of background gLuc expression in the assays not containing an active polymerase (EC₅₀ BG), are stated along with CC₅₀ and SI values. SI_{pol} is the ratio of EC₅₀ BG:EC₅₀ values. NT = not tested.

Figure 3: Design and evaluation of reporter viruses. **a.** Schematic representation of the genomes of recombinant EBOV and LASV expressing ZsGreen (ZsG) protein. Based on Zaire ebolavirus strain Ebola virus/H.sapiens-rec/LBR/2014/Makona-L2014 (Genbank KR781608), rEBOV/ZsG contains a

ZsG-P2A-VP40 coding sequence under transcriptional control of the parental VP40 promoter and terminator sequences. The P2A amino acid sequence self-cleaves, resulting in separate ZsG and VP40 proteins. Based on LASV strain Josiah (Genbank HQ688673.1, HQ688675.1), rLASV/ZsG contains a ZsG-P2A-NP coding sequence in the S segment. **b.** ZsG expression in Huh7 cells infected at MOI 0.1 with either rEBOV/ZsG or rLASV/ZsG 48 hpi. Cells imaged on an EVOS fluorescent microscope (Thermo Fisher), intensity 50%, $\times 4$ magnification. **c.** Characterization of growth kinetics of rLASV/ZsG versus wildtype LASV strain Josiah. Huh7 cells were infected at MOI 0.1 and titers determined at each time point by TCID₅₀. **d.** The optimized rLASV/ZsG and rEBOV/ZsG reporter virus assays were evaluated with the antiviral compound ribavirin. Reductions in ZsG fluorescence (blue) in infected cells were measured, with values normalized to mock-treated cells (DMSO only). Cell viability was assessed at each concentration by measuring ATP content (green), and values were normalized to mock-treated cells. Each point represents the mean of quadruplicate wells, with error bars indicating standard deviation; graphs are representative of 2 independent experiments. **e.** EC₅₀, CC₅₀, and SI values.

Figure 4: Antiviral activity of 6-azauridine and 2'-deoxy-2'-fluorocytidine. **a.** Representative concentration-response curves in Huh7 cells infected with either rLASV/ZsG or rEBOV/ZsG. Infected cells were treated with serial dilutions of either 6-azauridine or 2'-deoxy-2'-fluorocytidine (2'-dFC), and reduction in ZsG fluorescence (blue) was quantified; values were normalized to mock-infected cells. Cell viability was assessed at each concentration by measuring ATP content (green), and values were normalized to mock-treated cells. Each point represents the mean of quadruplicate wells, with error bars indicating standard deviation; graphs are representative of 2 independent experiments. NT = not tested. **b.** Reduction in ZsG fluorescence in 2'-dFC treated Huh7 cells infected at MOI 0.1 with rLASV/ZsG 48 hpi. Cells imaged on an EVOS fluorescent microscope (Thermo Fisher), intensity 50%, $\times 4$ magnification. **c.** Titer reduction assays using wild-type LASV (strain Josiah, Genbank AY628203.1) and EBOV (Zaire ebolavirus strain Ebola virus/H.sapiens-rec/LBR/2014/Makona-L2014, Genbank KR781608). Huh7 cells were treated with varying concentrations of compound 1 h prior to infection with either LASV or EBOV at MOI = 0.1. Three days post infection, cell culture supernatants were collected, and virus titers (TCID₅₀/mL) were determined in Vero-E6 cells by immunofluorescence assays. Mean titers are indicated, with errors bars representing standard deviation calculated from 3 independent infections. EC₅₀, CC₅₀, and SI values are stated for each compound. **d.** Chemical structure data for 2'-dFC and 6-azauridine.

Figure 5: Testing anti-HIV nucleoside analogs using minigenome assays and reporter viruses. **a.** Representative concentration-response curves for Huh7 cells transfected with LASV or EBOV

minigenomes. Reduction in gLuc expression in cells transfected with minigenome and helper plasmids including viral polymerase (blue) or without an viral active polymerase (red) was measured, with values normalized to mock-treated cells (DMSO only). Cell viability was assessed for each concentration of each compound by measuring ATP content (green); values were normalized to mock-treated cells. Each point represents the mean of quadruplicate wells, with error bars indicating standard deviation; graphs represent 2 independent experiments. EC_{50} values for each compound, including EC_{50} for inhibition of background gLuc expression in the assays not containing an active polymerase (EC_{50} BG), are stated, along with CC_{50} and SI values. SI_{pol} is the ratio of EC_{50} BG: EC_{50} values. **b.** Representative concentration-response curves in Huh7 cells infected with rLASV/ZsG or rEBOV/ZsG. Infected cells were treated with serial dilutions of compound, and reduction in ZsG fluorescence (green) was quantified; values were normalized to those from mock-infected cells. Each point represents the mean of quadruplicate wells, with error bars indicating standard deviation. EC_{50} , CC_{50} , and SI values are stated for each compound.

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a.

b.

c.

d.

Compound	LASV MG *					EBOV MG *			
	CC ₅₀	EC ₅₀	EC ₅₀ BG	SI	SI _{pol}	EC ₅₀	EC ₅₀ BG	SI	SI _{pol}
Ribavirin	>50	2.51	>50	>20	>20	5.04	>50	>20	>20

* All values in μM

a.

b.

c.

Compound	LASV MG *					EBOV MG *			
	CC ₅₀	EC ₅₀	EC ₅₀ BG	SI	SI _{pol}	EC ₅₀	EC ₅₀ BG	SI	SI _{pol}
2'-deoxy-2'-fluorocytidine	>50	1.35	>50	>37	>37	NT	NT	NT	NT
5-fluorouridine	>50	0.05	0.09	>1000	2	NT	NT	NT	NT
8-azaadenosine	>50	0.38	0.58	>132	2	0.68	0.77	>74	1
8-azidoadenosine	2.14	1.28	2.90	2	2	NT	NT	NT	NT
Toyocamycin	2.88	0.04	0.05	72	1	1.51	1.50	2	<1
6-azauridine	>50	4.76	>50	>11	>11	3.88	>50	>13	>13

* All values in μM

e.

Compound	CC ₅₀	rLASV/ZsG*		rEBOV/ZsG*	
		EC ₅₀	SI	EC ₅₀	SI
Ribavirin	>50	2.47	>20	5.34	>9

* All values in μM

a.

Compound	CC ₅₀	rLASV/ZsG*		rEBOV/ZsG*	
		EC ₅₀	SI	EC ₅₀	SI
2'-deoxy-2'-fluorocytidine	>50	0.23	>221	NT	NT
6-azauridine	>50	11	>5	12	>5

* All values in μM

b.

c.

d.

504

505

a.

Compound	LASV MG*					EBOV MG*			
	CC ₅₀	EC ₅₀	EC ₅₀ BG	SI	SI _{pol}	EC ₅₀ Active	EC ₅₀ BG	SI	SI _{pol}
Stavudine	>50	12	>50	>4	>4	>50	>50	>1	>1
Zidovudine	>50	>50	>50	>1	>1	>50	>50	>1	>1
Lamivudine	>50	>50	>50	>1	>1	>50	>50	>1	>1
Tenofovir	>50	3.92	1.27	13	<1	4.83	3.12	>11	<1

* All values in μM

b.

Compound	rLASV/ZsG*			rEBOV/ZsG*	
	CC ₅₀	EC ₅₀	SI	EC ₅₀	SI
Stavudine	>50	>50	>1	>50	>1
Zidovudine	>50	>50	>1	>50	>1
Lamivudine	>50	>50	>1	>50	>1
Tenofovir	>50	>50	>1	>50	>1

* All values in μM