1 **Screening and Identification of Lassa Virus Endonuclease-targeting Inhibitors from a**

2 **Fragment-based Drug Development Library**

3 Xiaohao Lan,^{a, b, c} Yueli Zhang,^{a, b, c} Yang Liu,^a Jiao Guo,^{a, b} Xiaoying Jia,^{a, b} Mengmeng Zhang,

4 ^{a, b, c} Junyuan Cao,^{a, b} Gengfu Xiao,^{a, b} Yu Guo,^c Wei Wang,^{a, b #}

5 State Key Laboratory of Virology, Wuhan Institute of Virology, Center for Biosafety

6 Mega-Science, Chinese Academy of Sciences, Wuhan 430071, China ^a

7 University of the Chinese Academy of Sciences, Beijing 100049, China ^b

8 College of Pharmacy and State Key Laboratory of Medicinal Chemical Biology, Nankai

9 University, Tianjin, 300450 China ^c

10

11 **Running Title:** FBDD screen for LASV EN inhibitor

12 **Key Words:** Lassa virus (LASV), cap-snatching, endonuclease (EN), fragment-based drug

13 development (FBDD), Lymphocytic choriomeningitis virus (LCMV), severe fever with

14 thrombocytopenia syndrome virus (SFTSV), benzotriazole compounds

15 **Word count:** abstract = 198, text = 2771

16 #Address correspondence to Wei Wang, wangwei@wh.iov.cn

17

18

19 **Abstract**

20 Lassa virus (LASV) belongs to the Old World genus *Mammarenavirus*, family *Arenaviridae*,
 21 and order *Bunyavirales*. Arenavirus contains a segmented negative-sense RNA genome,
 22 which is in line with the bunyavirus and orthomyxoviruses. The segmented negative-sense
 23 RNA viruses utilize a cap-snatching strategy to provide primers cleaved from the host
 24 capped mRNA for viral mRNA transcription. As a similar strategy and the conformational
 25 conservation shared with these viruses, the endonuclease (EN) would serve as an attractive
 26 target for developing broad-spectrum inhibitors. Using the LASV minigenome (MG) system,
 27 we screened a fragment-based drug development library and found three candidates (F1204,
 28 F1781, and F1597) inhibited MG activity. All three candidates also inhibited the prototype
 29 arenavirus Lymphocytic choriomeningitis virus (LCMV) MG activity. Furthermore, the
 30 investigation revealed that two benzotriazole compounds (F1204 and F1781) effectively
 31 inhibited authentic LCMV and severe fever with thrombocytopenia syndrome virus (SFTSV)
 32 infections. The combination of either compound with an arenavirus entry inhibitor had
 33 significant synergistic antiviral effects. Moreover, both F1204 and F1781 were found to exert
 34 the binding ability of LASV EN with binding affinity at the micromolar level. These findings
 35 provide a basis for developing benzotriazole compounds as potential candidates for the
 36 treatment of segmented negative-sense RNA virus infections.

37

38 **Importance**

39 Cap-snatching is the mRNA transcription strategy shared by all the segmented, negative-sense
 40 RNA viruses. Using a fragment-based drug development (FBDD) library, we tried to screen

out the backbone compound to inhibit the endonuclease activity and thus block this kind of virus infection. Two benzotriazole compounds, F1204 and F1781, were identified to inhibit the Lassa virus (LASV) minigenome activity by targeting the LASV EN.

Introduction

Lassa virus (LASV) is an enveloped, negative-sense, bi-segmented RNA virus that belongs to the genus *Mammarenavirus* (family *Arenaviridae*) (1). LASV causes the hemorrhagic disease Lassa fever (LF), an annual epidemic in West Africa and peaks during the dry season. In 2020, Nigeria faced a large outbreak of LF, with 1,189 confirmed cases and 244 deaths, according to the Nigeria Centre for Disease Control. As LASV is associated with a high mortality rate in humans, it is listed as a biosafety level 4 (BSL-4) agent. Most pathogenic mammarenaviruses, including the Junín virus (JUNV), Machupo virus (MACV), Guanarito virus (GTOV), Chapare virus (CHAPV), Sabiá virus (SBAV), and Lujo viruses (LUJV), are known to cause severe hemorrhagic fever and are also listed as BSL-4 agents. However, the prototypes of *Mammarenavirus*, Lymphocytic choriomeningitis virus (LCMV), a close relative of LASV, is usually asymptomatic and is categorized as a BSL-2 agent (2).

Mammarenavirus RNA genomes contain the S segment, encoding glycoprotein complex (GPC) and nucleoprotein (NP), and the L segment, encoding matrix protein (Z) and viral polymerase (L). In line with other segmented, negative-sense RNA viruses, such as influenza A virus (IAV), which contains eight segments, and bunyaviruses (such as severe fever with thrombocytopenia syndrome virus (SFTSV)), which contains a tri-segment, *Mammarenavirus* utilizes the cap-snatching mechanism to start viral mRNA transcription (3-6). Cap-snatching

63 involves recognizing capped cellular mRNAs followed by the cleavage of 10–14 nucleotides
 64 downstream by the polymerase's endonuclease (EN) to provide a primer for viral mRNA
 65 transcription (7-10). The N-terminal 173-aa region of the LASV L protein has been identified
 66 as the EN domain, and its structure is highly homologous to other known viral endonucleases
 67 (10).

68 To date, no vaccines or specific antiviral agents against LASV have been developed.
 69 Therapeutic strategies are limited to ribavirin administration in the early stages of the illness.
 70 We have focused on developing entry inhibitors against LASV (11-13). Fragment-based drug
 71 discovery (FBDD) can identify early lead candidates for treatment, provide a backbone for
 72 drug optimization, and facilitate elucidation of the mechanism underlying the infection (14).
 73 As the EN domain might be an attractive target for replication inhibitors, shedding light on
 74 other negatively segmented RNA viruses, we used the LASV minigenome (MG) system to
 75 screen a library of 1,015 fragment-based drugs. After two rounds of screening, two
 76 benzotriazole compounds (F1204: 2-Amino-6-ethoxybenzothiazole, F1781:
 77 2-Amino-6-chlorobenzothiazole) and 4,4'-dihydroxybiphenyl (F1597) were found to inhibit
 78 LASV MG activity. Furthermore, both benzotriazole compounds were capable of inhibiting
 79 other authentic negative segmented RNA viruses, such as LCMV and SFTSV.

80

81 **Results**

82 **Screening of an FBDD library for inhibitors against LASV MG activities.**

83 To construct a high-throughput system to study viral replication in BSL-2 containment and
 84 facilitate high-throughput screening (HTS), LASV MG system based on the ambisense LASV

85 S segment genome, in which the NP and GPC coding sequences were replaced with ZsGreen
 86 (ZsG) and *Gaussia princeps* luciferase (gLuc) reporter genes, co-transfected with the
 87 supportive pCAGGS-NP and pCAGGS-L plasmids, was constructed as previously reported
 88 (14). A ratio of 3:1:5 for S (ZsG/gLuc), pCAGGS-L, and pCAGGS-NP yielded an optimal
 89 signal-to-noise ratio >200 (Fig. 1A). Using ribavirin as the positive control, the Z' factor was
 90 determined to be 0.87, indicating that the assay is robust and suitable for HTS.

91 A flowchart of the HTS is depicted in Figure 1B. Inhibitors were defined as prime candidates,
 92 with LASV MG inhibition >80% and no apparent cytotoxicity at a concentration of 100 μ M.
 93 Of the 1,015 tested compounds, six (5.91%) were identified as prime candidates (Fig. 1C).
 94 The confirmed screen was then carried out over a broader concentration range from 9.6 nM to
 95 150 μ M. Three compounds (2.96%) were identified as candidates based on their
 96 dose-dependent inhibition and a selective index (SI) >10. Among the three candidates, both
 97 F1204 and F1781 were benzotriazole compounds, with IC₅₀ values of 5.384 and 2.100 μ M,
 98 respectively. As both compounds showed little or mild cytotoxicity, the SI values of both
 99 compounds were >70. The third candidate, F1597 (4,4'-dihydroxybiphenyl), also showed
 100 dose-dependent inhibition against LASV MG with an IC₅₀ of 9.696 μ M (Fig. 1D to 1G).

101 Both F1204 and F1781 inhibited LCMV replication.

102 To investigate the inhibitory effects of the candidates on an authentic *Mammarenavirus*,
 103 BSL-2 compatible LCMV was utilized. First, the inhibition of LCMV MG activity was
 104 verified using LCMV MG on 293T cells. As shown in Figures 2A to 2C, although the
 105 inhibition on LCMV MG was slightly lower than that on LASV MG, all the candidates could
 106 effectively inhibit the LCMV MG activities in a dose-dependent manner. Notably, as F1597

107 showed cytotoxicity in 293T cells with a $CC_{50} < 100 \mu M$, it was eliminated for further research.

108 The antiviral effects of the remaining two candidates were validated using LCMV strain C113.

109 As shown in Figures 2D and 2E, both F1204 and F1781 inhibited the authentic LCMV

110 infection with IC_{50} values of 13.43% and 27.12%, respectively, suggesting that both

111 compounds inhibited *Mammarenavirus* infection by blocking the replication step.

112 **F1204 and F1781 had little effects on *Mammarenavirus* entry and budding.**

113 To further assess the role of both F1204 and F1781 on other steps in the life cycle of

114 *Mammarenavirus*, the pseudotype virus was used to evaluate the effect on the virus entry step.

115 In contrast, the Z protein-expressing plasmid was used to investigate viral budding. Figures

116 3A and 3B showed neither F1204 nor F1781 blocked LASVpv infection as the percentage of

117 inhibition barely reached 50%, even at the highest tested concentration (200 μM). Similarly,

118 both compounds could hardly block LCMVpv infection (Fig. 3C and 3D), suggesting that

119 neither compound had any effect on *Mammarenavirus* entry. Furthermore, we investigated the

120 effects of the hit compounds on the budding ability of the matrix Z protein by detecting

121 virus-like particles (VLPs) with a Z self-budding assay (15-17). As shown in Figures 3E-3H,

122 even when treated with either F1204 or F1781 at 100 μM , LASV Z could efficiently produce

123 VLP, relative to the vehicle group, suggesting that neither F1204 nor F1781 impaired

124 *Mammarenavirus* Z budding ability. Collectively, these results demonstrated that both F1204

125 and F1781 exerted antiviral effects on the replication of mammarenaviruses.

126 **Binding ability of both hits to LASV EN.**

127 To investigate whether the endonuclease was the target of both hit compounds, we further

128 analyzed the binding affinity of the hits to LASV EN using isothermal titration calorimetry

129 (ITC) in the presence of divalent metal ions (Mg^{2+} and Mn^{2+}). As shown in Figure 4, F1204
130 and F1781 bound to LASV EN with the equilibrium dissociation constant (K_D) of 35.3 and
131 12.8 μM , respectively, suggesting that LASV EN might serve as the target of both F1204 and
132 F1781.

133 **Combinatorial effects of hit compounds and *Mammarenavirus* entry inhibitor.**

134 As both F1204 and F1781 targeted the endonuclease and thus inhibited *Mammarenavirus*
135 replication, we hypothesized that these replication inhibitors might synergize with the entry
136 inhibitor. To address this, we utilized casticin, a botanical drug demonstrated to inhibit
137 *Mammarenavirus* entry by blocking GPC-mediated membrane fusion (13), to study the
138 combined therapeutic effects. The prototype *Mammarenavirus*, LCMV, was used again
139 because each step of the authentic virus life cycle could be studied in the BSL-2 lab. As
140 expected, both the combinations of F1204 with casticin and F1781 with casticin had
141 significant synergistic antiviral effects (18, 19) with different volumes of 194.54 and 148.45
142 nM^2 %, respectively (Fig. 5) (20).

143 **F1204 and F1781 exerted an antiviral effect against SFTSV.**

144 To test whether both hit compounds could extend the antiviral spectrum to other negative
145 segmented RNA viruses, we investigated the inhibitory effect of both compounds against
146 SFTSV, a tick-borne virus belonging to the genus *Banyangvirus* (family Phenuiviridae) (21).
147 As shown in Figure 6, both F1204 and F1781 inhibited SFTSV infection in a dose-dependent
148 manner. However, the inhibitory effect was less pronounced than that of LCMV. F1204 and
149 F1781 (200 μM) robustly inhibited LCMV infection to ~90% (Fig. 2), while the inhibition of
150 SFTSV was only ~60% (Fig. 6), which was similar to a previous report that the approved IAV

151 EN inhibitor, L-742001, which inhibited SFTSV only at high concentrations ($>0 \mu\text{M}$) (5).
 152 This might be due to the subtle structural difference in the EN domain, which resulted in the
 153 difference in the antiviral effects (6).

154

155 **Discussion**

156 Based on the 2020 International Committee on Taxonomy of Viruses (ICTV)-taxonomic
 157 update reports, the genus *Mammarenavirus*, family *Arenaviridae*, is added to the order
 158 Bunyavirales, which belongs to the class Ellioviricetes, subphylum Polyplvircotina.
 159 Polyplvircotina contains a negative-sense RNA virus that encodes the L protein without
 160 mRNA capping activity. The other class in Polyplvircotina is Insthoviricetes, which
 161 includes the family Orthomyxoviridae, which contains IAV and IBV (1). The cap-snatching
 162 mechanism in the initial phase of mRNA transcription employed in all negative-sense,
 163 segmented RNA viruses is a potential target for developing broad-spectrum antiviral drugs.
 164 Notably, the endonuclease inhibitor baloxavir marboxil (BXM) was approved in 2018 to treat
 165 IAV and IBV infections (22). The active metabolite (baloxavir acid, BXA) of BXM targets the
 166 IAV EN with a metal chelating mechanism (23-25), similar to the first-generation
 167 endonuclease inhibitors, such as L-742,001 (26, 27). It has been recently reported that BXA
 168 exhibited a micromolar level inhibition on SFTSV EN activity and SFTSV plaque formation
 169 (5).

170 As cap-snatching is the replication strategy shared by all segmented negative-sense RNA
 171 viruses, and these viruses have a similar EN structure, EN is an attractive target for small
 172 molecule inhibitors. In this study, we utilized the FBDD library to screen for inhibitors

173 targeting LASV EN. Among the three identified candidates, F1204
 174 (2-Amino-6-ethoxybenzothiazole) and F1781 (2-Amino-6-chlorobenzothiazole) are
 175 benzotriazole compounds. To this end, we first reviewed all the nine benzotriazole
 176 compounds included in the current library. We found that three additional compounds, F1145,
 177 F1406, and F1486, showed inhibition levels of 50%–79% on the primary screen, while the
 178 other four compounds showed cytotoxicity or little inhibition (Table 1). Based on these results,
 179 we hypothesized that benzotriazole might serve as a backbone for the development of hit
 180 drugs. We selected two approved drugs, riluzole: 2-Amino-6-(trifluoromethoxy)
 181 benzothiazole, and Frentizole: 1-(6-Methoxy-2-benzothiazolyl)-3-phenylurea with the
 182 benzotriazole backbone and tested its effects on LASV MG. Unfortunately, neither showed
 183 inhibition of LASV MG activity. These results, however, were instrumental in highlighting
 184 the structure-activity relationship optimization of lead compounds.

185 The FBDD library contains compounds that are small in size and low in molecular weight
 186 (~200 Da), and FBDD library screens usually utilize biophysical methods such as X-ray
 187 crystallography, nuclear magnetic resonance, and mass spectrometry to identify the hits (28,
 188 29). The binding affinity of the hits to the targeting protein is usually weak because the
 189 fragments could only occupy part of the binding pocket. Sometimes, the hit fragments showed
 190 no activity in the functional assays (30). We screened the FBDD library using the enzyme
 191 activity-based high concentration screening strategy, which has been reported to be an
 192 effective and rapid approach for identifying fragments (31, 32). Both the hit compounds,
 193 F1204 and F1781, showed the micromolar binding ability to LASV EN affinity. Furthermore,
 194 both hit compounds could effectively inhibit authentic LCMV and SFTSV infection,

195 suggesting the potential to develop hit compounds to broad-spectrum antiviral inhibitors. The
196 synergistic effects of the combination of the hit compounds with the entry inhibitors shed
197 light on developing a successful treatment for pathogenic segmented, negative-sense RNA
198 virus infectious diseases.

199

200 **Materials and Methods**

201 **Cells and viruses.**

202 Vero, 293T, BSR-T7, and BHK-21 cells were cultured in Dulbecco's modified Eagle's
203 medium (DMEM; HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum
204 (Gibco, Grand Island, NY, USA).

205 LCMV clone 13 was rescued using genome RNA L (DQ361066) and S (DQ361065)
206 segments, as previously reported (13, 33). Briefly, BSR-T7 cells were transfected with a
207 mixture of plasmids containing pCAGGS-LCMVNP (300 ng), pCAGGS-LCMV-L (600 ng),
208 pT7-LCMV-L (600 ng), and pT7-LCMV-S (300 ng). The supernatant was collected 72 h later
209 and inoculated into BHK-21 cells for amplification. The titer of LCMV was determined to be
210 1×10^7 /ml by plaque assay. SFTSV (WCH/97/HN_Henan_2011) was provided by the
211 Microorganisms and Viruses Culture Collection Center, Wuhan Institute of Virology, Chinese
212 Academy of Sciences. The titer of SFTSV was determined to be 1×10^5 /ml by the TCID₅₀
213 assay. LASVpv and LCMVpv were generated using the VSV-based pseudotype virus system
214 (34, 35). Briefly, 293T cells transfected with pCAGGSGPC were infected with pseudotype
215 VSV, in which the G gene was replaced with a luciferase gene. The culture supernatants were
216 harvested 24 h later, and the viral titers of LASVpv and LCMVpv were determined to be $3 \times$

217 10^7 /ml and 1×10^6 /ml, respectively (11, 13).

218 **MG assay.**

219 For the LASV MG assay, Vero cells were seeded at 1.5×10^4 cells per well in a 96-well plate.

220 After overnight incubation, cells were transfected with 90 ng of mixed plasmids with a 3:1:5

221 ratio of pLASV-S (ZsG/gLuc), pCAGGS-L, and pCAGGS-NP. The minigenome is based on

222 the LASV S segment, as previously reported (14). The NP and GPC were replaced with ZsG

223 and gLuc, respectively, and the authentic untranslated regions intergenic regions were

224 unchanged (Josiah strain; GenBank number HQ688673.1). The cells were lysed 24 h later,

225 and luciferase activity was measured using the Rluc assay system (Promega, Madison, WI).

226 For the LCMV MG assay, 293T cells were used.

227 **HTS assay of an FBDD library.**

228 A library of 1,015 fragment-based drugs was purchased from Selleck Chemicals (Cat: L1600;

229 Houston, TX, USA). Compounds were stored in 10 mM stock solution in DMSO at -80 °C

230 until use. The first-round HTS was performed, as shown in Figure 1B. Briefly, cells

231 transfected with MG mixed plasmids were treated in duplicate with the compounds (100 μ M);

232 24 h later, cells were lysed to measure luciferase activity. Prime candidates were identified

233 using criteria of no apparent cytotoxicity and an average >80% inhibition in duplicate wells

234 and subsequently confirmed by serial dilution in triplicate plates to evaluate the IC₅₀

235 (GraphPad Prism 6). Using the criteria of dose-dependent inhibition and SI > 10, three

236 compounds were selected. Cytotoxicity was determined using the

237 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay.

238 **Antiviral assay.**

239 Vero cells were seeded at a density of 1×10^4 cells per well in a 96-well plate. After
240 incubation overnight, cells were incubated with compounds at the indicated concentrations for
241 1 h. LCMV and SFTSV (MOI: 0.1) were added to the cells and incubated for 1 h. The cells
242 were then incubated with the compounds for an additional 24 h. For the anti-LCMV assay, the
243 cell lysates were subjected to RT-qPCR using the primers LCMV-f:
244 AGAATCCAGGTGGTTATTGCC and LCMV-r: GTTGTAGTCAATTAGTCGCAGC and
245 GAPDH-f: 5'-TCCTTGGAGGCCATGTGGGCCAT-3' and GAPDH-r:
246 5'-TGATGACATCAAGAAGGTGGTGAAG-3'. In the anti-SFTSV assay, the cells were
247 subjected to an immunofluorescence assay using rabbit anti-NP serum (kindly provided by
248 Prof. Fei Deng at Wuhan Institute of Virology, CAS).

249 **VLP assay.**

250 The VLP assay was conducted as described previously (15-17). Briefly, 293T cells were
251 seeded at 8×10^5 cells per well in a 6-well plate. After overnight incubation, the cells were
252 transfected with pCAGGS-LASV-Z and pCAGGS-LCMV-Z. Four hours later, 100 μ M hit
253 compounds were added to the cells for 48 h. The supernatant was centrifuged at $15,000 \times g$
254 for 10 min at 4 ° C. The supernatant was then treated with 8% PEG8000 and 0.5 M sodium
255 chloride, followed by centrifugation at $15,000 \times g$ for 15 min. The samples collected from the
256 supernatant and cell lysate were subjected to WB assay using anti-LASV Z antibody
257 (GeneTex, cat: GTX134874, CA, USA) and anti-HA antibody (Proteintech, cat: 66006-2-Ig,
258 IL, USA) to detect the LASV and LCMV VLPs, respectively.

259 **LASV EN protein expression and purification.**

260 *E. coli* codon-optimized coding sequences were synthesized (Sanger) for residues 1-173

(LASV EN) of LASV L protein (7). LASV EN was cloned into pET-9a (Novagen) with an N-terminal His-tag, and a tobacco etch virus (TEV) cleavage site (MGHHHHHHHDYDIPTTENLYFQG-). The protein was expressed in *E. coli* strain BL21 (DE3) at 16 °C in LB media for 16 h after induction with (IPTG 0.2 mM). The protein was purified using a Ni-NTA column followed by removing the His-tag by TEV protease as previously described (6).

ITC assay.

ITC assays were performed at 25 °C using a MicroCal PEAQ-ITC (MicroCal, Inc.). The experiments comprised 19 injections of 2 µl of 0.9 mM compound into the sample cell containing 200 µl of 45 µM Lassa EN. The heat produced by the compound dilution in the buffer was subtracted from the heat obtained in the presence of the protein. Binding isotherms were fitted to one-site binding using MicroCal PEAQ-ITC Analysis software.

ACKNOWLEDGMENTS

We thank Dr. César G. Albariño for general advice on the creation of the LASV and LCMV minigenome system. We thank Dr. Liangdan Fei from Wuhan University for providing assistance in the ITC assay. We thank the Center for Instrumental Analysis and Metrology and Core Facility and Technical Support, Wuhan Institute of Virology, for providing technical assistance.

This work was supported by the National Key Research and Development Program of China (2018YFA0507204), the National Natural Sciences Foundation of China (31670165), Wuhan National Biosafety Laboratory, Chinese Academy of Sciences Advanced Customer

283 Cultivation Project (2019ACCP-MS03), and the Open Research Fund Program of the State

284 Key Laboratory of Virology of China (2018IOV001).

285

286 **References**

- 287 1. Kuhn JH, Adkins S, Alioto D, Alkhovsky SV, Amarasinghe GK, Anthony SJ, Avsic-Zupanc T,
288 Ayllon MA, Bahl J, Balkema-Buschmann A, Ballinger MJ, Bartonicka T, Basler C, Bavari S, Beer
289 M, Bente DA, Bergeron E, Bird BH, Blair C, Blasdel KR, Bradfute SB, Breyta R, Briesse T, Brown
290 PA, Buchholz UJ, Buchmeier MJ, Bukreyev A, Burt F, Buzkan N, Calisher CH, Cao M, Casas I,
291 Chamberlain J, Chandran K, Charrel RN, Chen B, Chiumenti M, Choi IR, Clegg JCS, Crozier I, da
292 Graca JV, Dal Bo E, Davila AMR, de la Torre JC, de Lamballerie X, de Swart RL, Di Bello PL, Di
293 Paola N, Di Serio F, Dietzgen RG, et al. 2020. 2020 taxonomic update for phylum
294 Negarnaviricota (Riboviria: Orthornavirae), including the large orders Bunyavirales and
295 Mononegavirales. *Arch Virol* 165:3023-3072.
- 296 2. Buchmeier MJ, de la Torre JC, Peters CJ. 2007. Arenaviridae: the viruses and their replication,
297 p 1791–1828. *In* Knipe DM HP, Griffin DE, Lamb RA, Martin MA, Roizman B, Straus SE (ed),
298 Fields virology, 5 ed. Lippincott-Raven, Philadelphia, PA.
- 299 3. Plotch SJ, Bouloy M, Ulmanen I, Krug RM. 1981. A unique cap(m7GpppXm)-dependent
300 influenza virion endonuclease cleaves capped RNAs to generate the primers that initiate viral
301 RNA transcription. *Cell* 23:847-58.
- 302 4. Garcin D, Lezzi M, Dobbs M, Elliott RM, Schmaljohn C, Kang CY, Kolakofsky D. 1995. The 5'
303 ends of Hantaan virus (Bunyaviridae) RNAs suggest a prime-and-realign mechanism for the
304 initiation of RNA synthesis. *J Virol* 69:5754-62.
- 305 5. Wang W, Shin WJ, Zhang B, Choi Y, Yoo JS, Zimmerman MI, Frederick TE, Bowman GR, Gross
306 ML, Leung DW, Jung JU, Amarasinghe GK. 2020. The Cap-Snatching SFTSV Endonuclease
307 Domain Is an Antiviral Target. *Cell Rep* 30:153-163 e5.
- 308 6. Reguera J, Gerlach P, Rosenthal M, Gaudon S, Coscia F, Gunther S, Cusack S. 2016.
309 Comparative Structural and Functional Analysis of Bunyavirus and Arenavirus Cap-Snatching
310 Endonucleases. *PLoS Pathog* 12:e1005636.
- 311 7. Saez-Ayala M, Yekwa EL, Carcelli M, Canard B, Alvarez K, Ferron F. 2018. Crystal structures of
312 Lymphocytic choriomeningitis virus endonuclease domain complexed with diketo-acid ligands.
313 *IUCrJ* 5:223-235.
- 314 8. Reich S, Guigligay D, Pflug A, Malet H, Berger I, Crepin T, Hart D, Lunardi T, Nanao M, Ruigrok
315 RW, Cusack S. 2014. Structural insight into cap-snatching and RNA synthesis by influenza
316 polymerase. *Nature* 516:361-6.
- 317 9. Morin B, Coutard B, Lelke M, Ferron F, Kerber R, Jamal S, Frangeul A, Baronti C, Charrel R, de
318 Lamballerie X, Vonnrhein C, Lescar J, Bricogne G, Gunther S, Canard B. 2010. The N-terminal
319 domain of the arenavirus L protein is an RNA endonuclease essential in mRNA transcription.
320 *PLoS Pathog* 6:e1001038.
- 321 10. Wallat GD, Huang Q, Wang W, Dong H, Ly H, Liang Y, Dong C. 2014. High-resolution structure
322 of the N-terminal endonuclease domain of the Lassa virus L polymerase in complex with
323 magnesium ions. *PLoS One* 9:e87577.
- 324 11. Wang P, Liu Y, Zhang G, Wang S, Guo J, Cao J, Jia X, Zhang L, Xiao G, Wang W. 2018. Screening
325 and Identification of Lassa Virus Entry Inhibitors from an FDA-Approved Drugs Library. *J Virol*
326 92:e00954-18.
- 327 12. Zhang G, Cao J, Cai Y, Liu Y, Li Y, Wang P, Guo J, Jia X, Zhang M, Xiao G, Guo Y, Wang W. 2019.
328 Structure-activity relationship optimization for lassa virus fusion inhibitors targeting the

transmembrane domain of GP2. *Protein Cell* 10:137-142.

13. Liu Y, Guo J, Cao J, Zhang G, Jia X, Wang P, Xiao G, Wang W. 2021. Screening of Botanical Drugs against Lassa Virus Entry. *J Virol* doi:10.1128/JVI.02429-20.

14. Welch SR, Guerrero LW, Chakrabarti AK, McMullan LK, Flint M, Bluemling GR, Painter GR, Nichol ST, Spiropoulou CF, Albarino CG. 2016. Lassa and Ebola virus inhibitors identified using minigenome and recombinant virus reporter systems. *Antiviral Res* 136:9-18.

15. Fehling SK, Lennartz F, Strecker T. 2012. Multifunctional nature of the arenavirus RING finger protein Z. *Viruses* 4:2973-3011.

16. Shao J, Liang Y, Ly H. 2018. Roles of Arenavirus Z Protein in Mediating Virion Budding, Viral Transcription-Inhibition and Interferon-Beta Suppression. *Methods Mol Biol* 1604:217-227.

17. Mpingabo PI, Urata S, Yasuda J. 2020. Analysis of the Cell Type-Dependence on the Arenavirus Z-Mediated Virus-Like Particle Production. *Front Microbiol* 11:562814.

18. Prichard MN, Shipman C, Jr. 1990. A three-dimensional model to analyze drug-drug interactions. *Antiviral Res* 14:181-205.

19. Prichard MN, Prichard LE, Baguley WA, Nassiri MR, Shipman C, Jr. 1991. Three-dimensional analysis of the synergistic cytotoxicity of ganciclovir and zidovudine. *Antimicrob Agents Chemother* 35:1060-5.

20. Jones GS, Yu F, Zeynalzadegan A, Hesselgesser J, Chen X, Chen J, Jin H, Kim CU, Wright M, Geleziunas R, Tsiang M. 2009. Preclinical evaluation of GS-9160, a novel inhibitor of human immunodeficiency virus type 1 integrase. *Antimicrob Agents Chemother* 53:1194-203.

21. Yu XJ, Liang MF, Zhang SY, Liu Y, Li JD, Sun YL, Zhang L, Zhang QF, Popov VL, Li C, Qu J, Li Q, Zhang YP, Hai R, Wu W, Wang Q, Zhan FX, Wang XJ, Kan B, Wang SW, Wan KL, Jing HQ, Lu JX, Yin WW, Zhou H, Guan XH, Liu JF, Bi ZQ, Liu GH, Ren J, Wang H, Zhao Z, Song JD, He JR, Wan T, Zhang JS, Fu XP, Sun LN, Dong XP, Feng ZJ, Yang WZ, Hong T, Zhang Y, Walker DH, Wang Y, Li DX. 2011. Fever with thrombocytopenia associated with a novel bunyavirus in China. *N Engl J Med* 364:1523-32.

22. Hayden FG, Sugaya N, Hirotsu N, Lee N, de Jong MD, Hurt AC, Ishida T, Sekino H, Yamada K, Portsmouth S, Kawaguchi K, Shishido T, Arai M, Tsuchiya K, Uehara T, Watanabe A, Baloxavir Marboxil Investigators G. 2018. Baloxavir Marboxil for Uncomplicated Influenza in Adults and Adolescents. *N Engl J Med* 379:913-923.

23. Kowalinski E, Zubieta C, Wolkerstorfer A, Szolar OH, Ruigrok RW, Cusack S. 2012. Structural analysis of specific metal chelating inhibitor binding to the endonuclease domain of influenza pH1N1 (2009) polymerase. *PLoS Pathog* 8:e1002831.

24. Omoto S, Speranzini V, Hashimoto T, Noshi T, Yamaguchi H, Kawai M, Kawaguchi K, Uehara T, Shishido T, Naito A, Cusack S. 2018. Characterization of influenza virus variants induced by treatment with the endonuclease inhibitor baloxavir marboxil. *Sci Rep* 8:9633.

25. Jones JC, Pascua PNQ, Fabrizio TP, Marathe BM, Seiler P, Barman S, Webby RJ, Webster RG, Govorkova EA. 2020. Influenza A and B viruses with reduced baloxavir susceptibility display attenuated in vitro fitness but retain ferret transmissibility. *Proc Natl Acad Sci U S A* 117:8593-8601.

26. Stevaert A, Dallochio R, Dessi A, Pala N, Rogolino D, Sechi M, Naesens L. 2013. Mutational analysis of the binding pockets of the diketo acid inhibitor L-742,001 in the influenza virus PA endonuclease. *J Virol* 87:10524-38.

27. Tomassini J, Selnick H, Davies ME, Armstrong ME, Baldwin J, Bourgeois M, Hastings J, Hazuda

373 D, Lewis J, McClements W, et al. 1994. Inhibition of cap (m7GpppXm)-dependent
374 endonuclease of influenza virus by 4-substituted 2,4-dioxobutanoic acid compounds.
375 Antimicrob Agents Chemother 38:2827-37.

376 28. Erlanson DA. 2012. Introduction to fragment-based drug discovery. Top Curr Chem 317:1-32.

377 29. Troelsen NS, Clausen MH. 2020. Library Design Strategies To Accelerate Fragment-Based Drug
378 Discovery. Chemistry 26:11391-11403.

379 30. Erlanson DA, Fesik SW, Hubbard RE, Jahnke W, Jhoti H. 2016. Twenty years on: the impact of
380 fragments on drug discovery. Nat Rev Drug Discov 15:605-619.

381 31. Godemann R, Madden J, Kramer J, Smith M, Fritz U, Hestekamp T, Barker J, Hoppner S,
382 Hallett D, Cesura A, Ebnet A, Kemp J. 2009. Fragment-based discovery of BACE1 inhibitors
383 using functional assays. Biochemistry 48:10743-51.

384 32. Artis DR, Lin JJ, Zhang C, Wang W, Mehra U, Perreault M, Erbe D, Krupka HI, England BP,
385 Arnold J, Plotnikov AN, Marimuthu A, Nguyen H, Will S, Signaevsky M, Kral J, Cantwell J,
386 Settachatgull C, Yan DS, Fong D, Oh A, Shi S, Womack P, Powell B, Habets G, West BL, Zhang KY,
387 Milburn MV, Vlasuk GP, Hirth KP, Nolop K, Bollag G, Ibrahim PN, Tobin JF. 2009. Scaffold-based
388 discovery of indeglitazar, a PPAR pan-active anti-diabetic agent. Proc Natl Acad Sci U S A
389 106:262-7.

390 33. Sanchez AB, de la Torre JC. 2006. Rescue of the prototypic Arenavirus LCMV entirely from
391 plasmid. Virology 350:370-80.

392 34. Whitt MA. 2010. Generation of VSV pseudotypes using recombinant DeltaG-VSV for studies
393 on virus entry, identification of entry inhibitors, and immune responses to vaccines. J Virol
394 Methods 169:365-74.

395 35. Tani H, Shiokawa M, Kaname Y, Kambara H, Mori Y, Abe T, Moriishi K, Matsuura Y. 2010.
396 Involvement of ceramide in the propagation of Japanese encephalitis virus. J Virol
397 84:2798-807.

398

399

400

401 **Figure legend**

402 **Fig. 1. High-throughput screening (HTS) for inhibitors against LASV MG replication**

403 **from a fragment-based drug library.** (A) Validation of the LASV MG. Varying ratios of the

404 LASV MG containing plasmids (total plasmid amount: 90 ng/well in 96-well plates) were

405 transfected into Vero cells. Ribavirin of 50 μ M, as a positive control, was added into the cells

406 5 h post-transfection. The cell lysates were subjected to test the gLuc activities 24 h later. (B)

407 HTS assay flowchart. (C) HTS of a library of 1,015 fragment compounds for prime

408 candidates inhibiting LASV MG activities. Each dot represents the percent inhibition

409 achieved with each compound at a concentration of 100 μ M. Six green dots represents the

410 prime candidates with inhibition >80% and no obvious cytotoxicity. (D-F) Dose-response

411 curves of F1204 (D), F1781 (E), and F1597 (F). Cells transfected with the LASV MG

412 containing plasmids were treated in duplicate with each compound at the indicated

413 concentrations; 24 h later, cells lysates were subjected to test the luciferase activities. Cell

414 viability was evaluated using MTT assay. (G) IC₅₀, CC₅₀, and SI values for the three

415 candidates. Data are presented as means $\pm$ standard deviations (SDs) for three to six

416 independent experiments.

417 **Fig. 2. Inhibitory effects of the candidates against LCMV MG and the authentic LCMV**

418 **CI13 strain.** (A-C) Inhibitory effects of F1204 (A), F1781 (B), and F1597 (C) against LCMV

419 MG. LCMV MG containing plasmids (LCMV-S: 30 ng; LCMV-NP: 50 ng; LCMV-L: 10 ng)

420 were transfected into 293T cells. Candidates with indicated concentration were added into the

421 cells 5 h post-transfection. The cell lysates were subjected to test the gLuc activities 24 h later.

422 The 293T cells viabilities were tested using MTT assay. (D-E) Dose-response curves of

423 F1204 (D) and F1781 (E) against authentic LCMV Cl13 strain. Vero cells were treated with
424 either compound; 1 h later, LCMV Cl13 of MOI: 0.1 was added to the cells; the supernatant
425 was removed 1 h postinfection, and the cells were incubated with either compound for
426 additional 23 h. The Vero cell lysates were assessed by qRT-PCR. Data are presented as
427 means $\pm$ standard deviations (SDs) for three independent experiments.

428 **Fig. 3. Effects of F1204 and F1781 on mammarenavirus entry and budding.** (A and B)

429 Effects of F1204 and F1781 on LASV entry. Vero cells were preincubated with F1204 (A)
430 and F1781 (B), respectively, for 1 h, followed by incubation with LASVrv (MOI, 0.1) in the
431 presence of compounds for 1 h. The cell lysates were assessed for luciferase activities 24 h
432 post-infection. (C and D) Effects of F1204 and F1781 on LCMV entry. (E) Effects of F1204
433 and F1781 on LASV VLP production. 293T cells were transfected with pCAGGS-LASV Z; 4
434 h later, compounds of 100 μ M were added and incubated for 48 h. The supernatant and cell
435 lysate were collected for WB assay. (F) Quantification results of WB assay were presented as
436 the mean $\pm$ SD from five independent experiments.

437 **Fig. 4. Binding of F1204 and F1781 to LASV endonuclease measured by ITC.** F1204 (A)

438 and F1781 (B) binding to 45 μ M LASV endonuclease protein were measured at 25 °C by ITC.
439 The upper plot showed the binding isotherm and the lower plot shows the integrated values.

440 **Fig. 5. Drug-drug interactions of F1204 and F1781 with casticin.** (A and B) Differential

441 surface plots at 95% confidence level (CI) were calculated and generated using MacSynergy
442 II for the drug-drug interactions for evaluating combinations of F1204 (A) and F1781 (B)
443 with casticin targeting LCMV.

444 **Fig. 6. Inhibitory effects of F1204 and F1781 against SFTSV.** (A) Inhibitory effect of

445 F1204 against SFTSV. Vero cells were treated with F1204; 1 h later, SFTSV of MOI: 0.1 was
 446 added to the cells; the supernatant was removed 1 h postinfection, and the cells were
 447 incubated with F1204 for additional 23 h. The percentage of inhibition was calculated using
 448 the Harmony 3.5 software in an Operetta high-content imaging system (PerkinElmer) (left),
 449 and the cells were imaged using the same system (right). (B) Inhibitory effect of F1781
 450 against SFTSV. Data are presented as means $\pm$ standard deviations (SDs) for three to four
 451 independent experiments.

452

LCMV-MG

LCMV CI13

A

B

Table 1. Benzotriazole compounds in FBDD library.

No.	name	CAS	structure	inhibition on LASV MG (%)*
F1204	2-Amino-6-ethoxy ybenzothiazole	94-45-1		97.81
F1781	2-Amino-6-chloro benzothiazole	95-24-9		85.89
F1145	2-Amino-6-methy lbenzothiazole	2536-91-6		78.88
F1406	2-Aminobenzothi azole	136-95-8		69.56
F1486	2-Mercaptobenzot hiazole	149-30-4		52.60
F1616	2-Chlorobenzothi azole	615-20-3		34.34
F1526	1-hydroxy-7-azab enzotriazole	39968-33-7		
F1654	Benzothiazole-6-c arboxylic acid	3622-35-3		-2.68
F2017	2-Amino-4-methy lbenzothiazole	1477-42-5		-13.77

F1137	2-Amino-6-fluoro benzothiazole	348-40-3		cytotoxic
-------	-----------------------------------	----------	--	-----------

* with concentration of 100 μ M.