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Arbidol and other Low Molecular Weight Drugs That Inhibit Lassa and Ebola Viruses

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Abstract

Antiviral therapies that impede virus entry are attractive because they act on the first phase of the infectious cycle. Drugs that target pathways common to multiple viruses are particularly desirable when laboratory-based viral identification may be challenging, e.g. in an outbreak setting. We are interested in identifying drugs that block both Ebola virus (EBOV) and Lassa virus (LASV), two unrelated but highly pathogenic hemorrhagic fever viruses that have caused outbreaks in similar regions in Africa and share features of virus entry: use of cell surface attachment factors, macropinocytosis, endosomal receptors and low pH to trigger fusion in late endosomes. Towards this goal, we directly compared the potency of eight drugs known to block EBOV entry with their potency as inhibitors of LASV entry. Five drugs (amodiaquine, apilimod, arbidol, niclosamide, and zoniporide) showed roughly equivalent inhibition of LASV and EBOV glycoprotein (GP)-bearing pseudoviruses; three (clomiphene, sertraline and toremifene) were more potent against EBOV. We then focused on arbidol, which is licensed abroad as an anti-influenza drug and exhibits activity against a diverse array of clinically relevant viruses. We found that arbidol inhibits infection by authentic LASV, inhibits LASV GP-mediated cell-cell fusion and virus-cell fusion and, reminiscent of its activity on influenza hemagglutinin, stabilizes LASV GP to low pH exposure. Our findings suggest that arbidol inhibits LASV fusion, which may partly involve blocking conformational changes in LASV GP. We discuss our findings in terms of the potential to develop a drug cocktail that could inhibit both LASV and EBOV.

Importance

Lassa and Ebola viruses continue to cause severe outbreaks in humans, yet there are only limited therapeutic options to treat the deadly hemorrhagic fever diseases they cause. Because of overlapping geographic occurrences and similarities in mode of entry into cells, we seek a

practical drug or drug cocktail that could be used to treat infections by both viruses. Towards this goal, we directly compared eight drugs, approved or in clinical testing, for their ability to block entry mediated by the glycoproteins of both viruses. We identified five drugs with approximately equal potency against both. Among these we investigated the modes of action of arbidol, a drug licensed abroad to treat influenza infections. We found, as shown for influenza, that arbidol blocks fusion mediated by the Lassa virus glycoprotein. Our findings encourage the development of a combination of approved drugs to treat both Lassa and Ebola virus diseases.

Introduction

Lassa virus (LASV) is an enveloped ambisense RNA virus belonging to the Arenaviridae. As the most clinically significant member of this large family, LASV is a major pathogen in West Africa, where it infects an estimated 300,000 people each year. LASV has also been responsible for a number of imported cases of Lassa hemorrhagic fever (LHF) in Europe and North America in recent years (1). The 2018 outbreak of LHF in Nigeria was particularly severe, with over 430 confirmed positive cases and a case fatality rate of ~25% (2). Classic symptoms of acute LHF include malaise, headache, fever, vomiting, respiratory distress, facial edema, and hemorrhaging of mucosal surfaces (3). Even in fatal cases, however, patients may not present with redolent hemorrhagic fever symptoms, complicating diagnosis (4).

The only antiviral treatment option for LHF is the guanosine analogue, ribavirin. There are a substantial number of contraindications and adverse effects associated with ribavirin, and its efficacy in clinical trial settings remains controversial and under-evaluated. Furthermore, while ribavirin is effective against other hemorrhagic fever arenaviruses, it has limited efficacy against filoviruses. Thus, current guidelines recommend ribavirin only after high-risk exposures

to LASV (5). Given the partial geographic overlap between EBOV and LASV in West Africa and similar clinical presentation in early infection stages, it would be advantageous to have a common therapeutic effective against both viruses (6, 7).

Promising new compounds against LASV have been identified (6, 8-17), but the limited geographical endemicity of LASV, its inefficient person-to-person transmission, and low re-infection rates make the prospect of collecting adequate clinical trial data on new drugs challenging. Thus, a practical approach to more expeditiously grow the arsenal of drugs against these highly pathogenic viruses is to screen approved drugs for antiviral activity. When this strategy was employed, many FDA approved compounds with repurposing potential were identified that showed inhibitory effects against EBOV (18-24). Many of these are thought to act upon the entry stages of EBOV infection. A similar recent screen revealed FDA-approved drugs with potential activity against LASV (8).

Viral entry inhibitors are valuable as therapeutics since blocking infection early in the lifecycle will reduce cellular and tissue damage associated with the replication of incoming viruses and the production of viral progeny. LASV employs several key features in common with EBOV for its entry: 1) it is internalized into the endocytic pathway by a macropinocytotic-like process after initial contact with surface receptors/attachment factors, 2) low pH is needed to trigger fusion, and 3) an endosomal, cholesterol binding, receptor promotes endosomal escape (Lamp1 for LASV and NPC1 for EBOV) (12, 25-32). Hence for this study, we selected eight low molecular weight drugs shown to inhibit EBOV entry and directly compared their inhibitory activity against LASV and EBOV. Five of these drugs have FDA approval (amodiaquine, clomiphene, niclosamide, sertraline, and toremifene), one is licensed abroad (arbidol), and two have been evaluated in clinical trials (apilimod and zoniporide).

The compound we investigated in most detail was the anti-influenza drug arbidol (Umifenovir), which was developed and is currently used as an antiviral. Arbidol has been reported to have inhibitory effects on a diverse array of viruses, including DNA and RNA viruses as well as capsid- and membrane-enclosed viruses (33-36). Studies aimed at determining the mechanism of action of arbidol implicate a number of possible antiviral effects, including several steps of entry as well as later phases of the infectious cycle (36). The principle inhibitory effect of arbidol on influenza virus, for which it has been a licensed treatment in China and Russia for many years, appears to be during a late stage of entry, when influenza fuses with an endosomal membrane. While arbidol can bind directly to influenza HA and inhibit its ability to transition to an activated conformation (37-39), it is not yet clear whether this is its sole or primary mechanism of anti-influenza activity, or if arbidol also impairs fusion by intercalation into the viral or target membrane thereby rendering the membrane less yielding for fusion (35).

MATERIALS AND METHODS

Chemicals and cell culture. Dulbecco's modified Eagle's medium (DMEM), phenol-red free DMEM, Opti-MEM (OMEM), sodium pyruvate, antibiotic/antimycotic, trypsin-EDTA 0.05%, phenol-red free trypsin-EDTA 0.5 % and neutral red were from ThermoFisher Scientific. Phosphate-buffered saline (PBS) was from Corning. Cosmic Calf Serum (CCS), Fetal Bovine Serum (FBS) and Supplemented Calf Serum (SCS) were from HyClone. Fibronectin was from Millipore. Polyethylenimine (PEI) and Non-Enzymatic Cell Dissociation Solution were from Sigma. Lipofectamine 2000 was from Invitrogen. Toremifene citrate was from Selleck Chemicals. Zoniporide, amodiaquine, niclosamide and clomiphene citrate were from Sigma.

Apilimod was from Axon MedChem. Sertraline HCl was from Toronto Research Chemicals. Arbidol was synthesized commercially, and the purity and structure of the product were confirmed as described previously (34).

HEK293T/17 and BSC-1 cells were from the ATCC, Lamp1 KO HEK293T/17 cells (clone 1D4) were described in Hulseberg et al., 2018. COS7 cells were from the ATCC and a kind gift from Douglas DeSimone at the University of Virginia. BHK-21 cells were from the ATCC and a kind gift from James Casanova at the University of Virginia. Vero76 cells were from the United States Army Medical Research Institute of Infectious Diseases (USAMRIID). HEK293T/17 and BSC-1 cells were maintained in DMEM containing 10% CCS. BHK21 cells were maintained in DMEM containing 10% SCS. COS7 and Lamp1 KO HEK293T/17 cells were maintained in DMEM containing 10% FBS, 1% sodium pyruvate, and1% antibiotic/antimycotic. Vero76 cells were maintained in Corning DMEM with 10% Gibco FBS, 1% penicillin/streptomycin, 1% L-Glutamine, and 1% sodium-pyruvate.

Plasmids and Virus. The pCMV-LASV-GPC Josiah strain plasmid was from F.L. Cosset (Université de Lyon, France) via Gregory Melikian (Emory University), the LASV-GPC-Flag pCC421 Josiah strain was from Jason Botten (University of Vermont). VSV-G plasmid was from Michael Whitt (University of Tennessee); pDisplay-EBOV-GPΔ Mayinga strain was from Erica Saphire (Scripps Research Institute). TG-Luc plasmid was from Jean Dubuisson (Centre National de la Recherche Scientifique, Lille, France) via Gary Whittaker (Cornell University), pCMV-Gag-Pol plasmid was from Jean Millet and Gary Whittaker (Cornell University) and Jean Dubuisson; Gag-βlaM plasmid was made by James Simmons (University of Virginia). The pcDNA3-luciferase (Firefly) plasmid was from Addgene. The DSP_{1-7} and DSP_{8-11} plasmids were

from Naoyuki Kondo (Kansai Medical University, Japan). The WSN HA and NA plasmids were from Gary Whittaker (Cornell University). Plasmids encoding LCMV GP and Junin GP were from Jack Nunberg (University Montana).

The stock of LASV (Josiah strain) used was generated by infecting Vero E6 cells in complete EMEM (VWR) containing 10% FBS (HyClone) and 2% L-Glutamine (ThermoFisher Scientific). Infected cells were incubated at 37°C/5% CO_2.Virus-containing cell culture supernatant was harvested 3 days post-inoculation, clarified at 10,000 x g at 4°C for 10 min, and frozen at -80°C. All work with native LASV was conducted in a BSL4 containment suite with personnel in positive pressure encapsulating suits following appropriate institutional SOPs.

Antibodies and immunoprecipitation reagents. For western blotting, the mouse-anti-LASV-GP L52-134-23A was from USAMRIID and anti-mouse-IR680RD was from Licor. For LASV GP bead capture, α-Flag®M2 magnetic beads were from Sigma.

Pseudovirus production. To produce VSV pseudoviruses, 1 x 10^6 BHK-21 cells were seeded in each of multiple 10 cm^2 dishes. Cells in each dish were transfected with 12 µg of plasmid encoding LASV-GPC using PEI. The following day, cells were infected with 40µl (per dish) VSV-ΔG helper virus (from pre-titered plaque eluate) encoding *Renilla* luciferase (diluted in serum-free media) for 1 hr at 37°C. After infection, cells were washed extensively with cold PBS and incubated overnight in complete DMEM. Supernatants containing pseudoviruses were collected, clarified and pelleted through a 20% sucrose-HM (20 mM HEPES, 20 mM MES, 130 mM NaCl, pH 7.4) cushion. The pellet was re-suspended in 10% sucrose-HM. VSV-ΔG helper

179 virus was produced following the same procedure, by infecting VSV-G transfected cells with
180 eluate from VSV-ΔG plaques.

181 For MLV pseudoviruses, HEK293T/17 cells were seeded in 10 cm^2 dishes. The following
182 day, cells were transfected with 6 μg of total DNA using a 2:1:1:1 ratio of pTG-Luc:pCMV Gag-
183 Pol:Gag-βlam:glycoprotein. At 48 hr post-transfection, virus-containing medium was harvested,
184 clarified, pelleted through a 20% sucrose-HM cushion, resuspended in 10% sucrose-HM, and
185 stored at -80°C in single use aliquots.

186

187 **LASV plaque reduction assay.** Vero76 cells were seeded on 6-well plates. At full confluency,
188 cells in duplicate wells were pretreated with the indicated concentration of arbidol, 25 mM
189 NH_4Cl, or 10% ethanol vehicle for 1 hr at 37°C. Cells were then infected with LASV at a
190 multiplicity of infection (MOI) of 0.01 for 1 hr in the presence of the indicated concentration of
191 arbidol or vehicle. Cells were washed twice to remove unbound virus and incubated for 24 hr at
192 37°C in drug-containing medium. Supernatants were harvested and 10-fold serial dilutions were
193 made to infect ~90% confluent monolayers of Vero76 cells in 6-well plates for 1 hr at 37°C, with
194 rocking every 15 min. A primary overlay consisting of a 1:1 mixture of 1.6% SeaKem agarose
195 (Lonza) and 2x EBME (ThermoFisher Scientific) supplemented with 20% FBS and 8%
196 Glutamax (ThermoFisher Scientific) was then added on top of the infected cells and allowed to
197 solidify. Cells were incubated for 4 days at 37°C, followed by addition of a secondary overlay
198 consisting of a 1:1 mixture identical to the above but with the addition of 8% Neutral Red (final
199 NR concentration was 4%). Plaques were counted the following day. Plaque counts were
200 averaged from duplicate wells and then multiplied by dilution factor to establish starting titer of
201 input supernatants.

Biosafety. All manipulations involving live LASV were performed in a biosafety level 4 containment suite at USAMRIID with personnel wearing positive-pressure protective suits fitted with HEPA filters and umbilical fed air. USAMRIID is registered with the Centers for Disease Control Select Agent Program for the possession and use of biological select agents and toxins and has implemented a biological surety program in accordance with U.S. Army regulation AR 50-1 "Biological Surety."

Pseudovirus Infection assay. BSC-1 cells were seeded on white 96-well plates (1.5×10^4 cells/well). The following day, cells were pretreated with drugs (or mock) for 1 hr in OMEM and then, while maintaining the presence of drug, were infected with an input of EBOV GP- and LASV GP-pseudoviruses that had been pre-titered to achieve roughly equivalent RLU signals in the mock-treated samples. After 24 hr at 37°C/5% CO_2, the cells were lysed with Britelite reagent (PerkinElmer) and luminescence was measured. IC_{50} concentrations and statistical analysis of all data were performed using GraphPad Prism 7 (GraphPad Software, Inc.): Log(Agonist) vs. response-variable slope (four parameters) constrained to bottom=0.

Cell-cell fusion (CCF) assay. Effector (HEK293T/17) cells were seeded on 6-well plates (6.75×10^5 cells/well). Target (HEK293T/17) cells were seeded on fibronectin-coated opaque white 96-well plates (3.5×10^4 cells/well). Effector cells were transfected with 1 µg/well of GP plasmid and 1 µg/well of DSP_{1-7} plasmid. Target cells were co-transfected with 33 ng/well pmLamp1 and 33 ng/well of DSP_{8-11} plasmid. Cells were transfected using Lipofectamine 2000, according to the manufacturer's instructions. Twenty-four hours post-transfections, effector cells were loaded

225 with EnduRen™ luciferase substrate (Promega) (60 µM in complete DMEM) for 2 hr at 37°C.
226 Effector cells were then rinsed with PBS and lifted with Non-Enzymatic Cell-Dissociation
227 Solution. Effector cells were re-suspended in complete DMEM and 1 x 10^5 effector cells were
228 overlaid onto each well of target cells (96-well plate). Cells were co-cultured for 3 hr. At this
229 time, a low pH pulse was applied with fusion buffer (100mM NaCl, 15 mM HEPES, 15 mM
230 succinate, 15 mM MES, 2 mg/mL glucose) adjusted to pH 5.0, for 5 min at 37°C. The pH was re-
231 neutralized by replacing the fusion buffer with complete DMEM, and the cells were returned to
232 37°C for 1 hr before measuring luciferase activity.
233
234 **Forced fusion at the plasma membrane (FFPM) assay.** COS7 cells were seeded in 6-well
235 plates (4 x 10^5 cells/well). ~24 hr post seeding, the cells were transfected with 1 µg of plasmid
236 encoding firefly luciferase using Lipofectamine 2000 according to the manufacturer's
237 instructions. ~24 hr post transfection, the cells were washed, lifted, and reseeded at 1.5 x 10^4
238 cells/well on fibronectin-coated opaque white 96-well plates. The day after re-seeding, cells were
239 chilled on ice for 15 min and LASV-GP VSV-luciferase (*Renilla*) pseudoviruses, which had been
240 titered to reach a target signal of at least 1 x 10^6 RLUs in a standard infection assay, were added
241 to cells in quintuplicate in serum-free DMEM. Pseudoviruses were bound to the cells by
242 centrifugation (250 x g, 1 hr, 4°C). Cells were returned to ice and washed once with cold PBS.
243 Fusion was triggered by applying a pulse of pre-warmed low pH fusion buffer (as in CCF assay)
244 for 5 min at 37°C adjusted to the indicated pH values. Cells were returned to ice and the fusion
245 buffer was replaced with complete DMEM containing 40 mM NH_4Cl (to block virus entry via
246 the normal endocytic route) was added. Sixteen hr later, luciferase activity was measured using
247 the Dual-Glo® Luciferase Assay System (Promega) according to the manufacturer's instructions

using a Promega GloMax® luminometer. The ratio of *Renilla* luciferase activity (an indicator for pseudovirus infection) over firefly luciferase activity (to account for the number of cells) was calculated to assess viral GP-mediated fusion with the plasma membrane.

GP1 dissociation assay. Lamp1 KO HEK293T/17 cells were seeded in 6-well plates (6.25 x 10^5 cells/well). The following day, cells were transfected with 1 µg of LASV-GPC-Flag using PEI. At 48 hr post-transfection, the cells were lysed with NETI buffer (150 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl, 0.5% Igepal) at pH 8. After clearing cell debris (centrifugation for 15 min at 21,000 x g), the lysate was incubated with α-Flag® M2 Magnetic Beads (that were prewashed twice in NETI buffer pH 8) for 1 hr at 4°C. Arbidol was then added to the bead plus lysate mixture as indicated, and the samples were incubated for an additional hour at 4°C. For pH-dependent dissociation experiments, beads with captured LASV GP and pretreated +/- arbidol were then pulled over on a magnetic stand and quickly washed with cold NETI buffer (without arbidol) at the indicated pH. The cold NETI buffer was then replaced with pre-warmed NETI buffer at the same pH +/- arbidol, as indicated. Samples were incubated at 37°C for 1 min. For time-dependent dissociation experiments, beads with captured LASV GP and pretreated +/- arbidol as above were quickly washed with cold NETI buffer at pH 6.5. The cold pH 6.5 buffer was then replaced with pre-warmed NETI buffer at pH 6.5 +/- arbidol. Samples were then incubated at 37°C for 0.5, 1.0, 2.5 or 5.0 min. The "0 min" samples were treated with pre-warmed buffer, and then placed immediately on ice post buffer addition. At the end of the indicated incubation period, the samples were immediately placed on the magnetic rack and supernatants collected. Proteins were then eluted from the residual beads using 100 mM glycine pH 3.5 for 15 min at 25°C with constant vortexing, and the eluted samples were neutralized by

the addition of 1M Tris-HCl, pH 8.5. Supernatant and bead samples were then analyzed by SDS-PAGE and western blotting, with a primary antibody against LASV GP1. The signal intensity of the GP1 bands in the supernatant and corresponding bead samples was measured using ImageJ. Percent GP1 dissociation was calculated as the signal intensity of the GP1 band in the supernatant divided by the summed signal intensity of the GP1 bands in the supernatant and bead samples.

RESULTS

Comparison of the potency of small molecule inhibitors against LASV GP- and EBOV GP-mediated entry. Enveloped viruses that are endocytosed rely on their glycoproteins (GPs) to mediate the entire entry process, from attachment to the cell surface to fusion within endosomal membranes. Here we directly compared the effects of eight drugs that block EBOV entry for their effects on LASV GP-mediated entry. To do this, we used murine leukemia viruses (MLV) carrying a luciferase reporter and pseudotyped with either LASV or EBOV GP. Drug dosing ranges were determined by establishing the concentration of each drug needed to elicit a near-total inhibition of infection. The remaining doses in each set were 2-fold serial dilutions. A mock (vehicle-only) treatment was included as an anchor point in each series to assess the extent of inhibition in treated cells.

Representative direct comparative dose response curves for LASV and EBOV for each of the eight drugs are presented in Fig. 1. Each drug was tested in parallel against LASV GP- and EBOV GP-MLV pseudoviruses in three to five independent experiments. Table 1 reports the

294 average ratio of the IC_{50} value against LASV GP divided by that for EBOV GP, analyzed in
295 parallel, for each of the eight drugs tested. These ratios indicated that the IC_{50} values against
296 LASV GP-MLV pseudoviruses for five drugs (zoniporide, amodiaquine, niclosamide, apilimod,
297 and arbidol) were either approximately the same or lower than the corresponding values for
298 EBOV GP-MLV pseudoviruses, indicating similar or enhanced potency against LASV GP-
299 mediated infection. For three drugs, clomiphene, sertraline and toremifene, the IC_{50} values for
300 LASV are ~3-6-fold greater than those for EBOV, indicating that these drugs are more potent
301 against EBOV. It is noteworthy that the latter three drugs are cationic amphiphilic drugs (CADs),
302 which may be especially active against EBOV (19, 20, 23, 40).

303 For the remainder of the study we focused on arbidol, for reasons outlined in the
304 introduction. Pécheur and colleagues reported an EC_{50} of 5.8 μM in Vero cells for arbidol against
305 the New World arenavirus, Tacaribe virus; to the best of our knowledge the latter is the only
306 published evaluation of the efficacy of arbidol against an arenavirus. Using MLV pseudoviruses
307 we found that in addition to inhibiting entry mediated by LASV GP (Fig. 1), arbidol inhibited
308 entry mediated by the GPs of two other arenaviruses, those of LCMV and Junin (Fig. 2A). We
309 also found, using MLV pseudoviruses, that arbidol is somewhat more potent against LASV GP-
310 mediated infection than against infection mediated by influenza HA from the WSN (H1N1)
311 strain (Fig. 2B).

312

313 **Arbidol blocks authentic LASV infection**. To evaluate the efficacy of arbidol against authentic
314 LASV, we performed LASV (Josiah) plaque reduction assays under BSL4 conditions, testing the
315 effects of concentrations of arbidol up to 40 μM. Cells were pretreated with arbidol for 1 hr and
316 then infected with LASV (Josiah) in the continued presence of arbidol for 24 hr. In the first of

317 three experiments the IC_{50} was ~ 5-10 μM and the maximum inhibition was 98% (Fig. 3A); in
318 the second, the IC_{50} was ~20 μM and the maximum inhibition was 100% (Fig. 3B). In a third
319 experiment, testing only 20 μM arbidol, the percent inhibition was 74% (Fig. 3C). The average
320 percent inhibition caused by 20 μM arbidol from the three experiments was 72.5% (Fig. 3D). By
321 visual inspection 40 μM arbidol had no effect on Vero cell monolayers for up to five days (data
322 not shown). We note that the apparent IC_{50} for arbidol vs. authentic LASV (Fig. 3) is higher than
323 that seen with MLV pseudoviruses bearing LASV GP (Figs. 1 and 2).
324
325 **Arbidol blocks LASV GP-mediated fusion**. We next asked if arbidol impairs LASV GP-
326 mediated fusion, as it does for other viruses (33, 35, 38, 39, 41). Given that optimal LASV fusion
327 requires the endosomal protein Lamp1 (26, 31, 42), we used cells expressing Lamp1 at the
328 plasma membrane (pmLamp) as fusion targets. Cell-cell fusion (CCF) was then induced between
329 co-cultured effector cells (expressing LASV GP at their surface) and target cells (expressing
330 Lamp1 at their surface) by briefly exposing the cells to low pH, as described previously (31). To
331 assess the effects of arbidol, effector cells (expressing LASV GP) were pretreated for 1 hr with
332 the indicated concentration of arbidol, co-cultured with pmLamp1-expressing target cells, and
333 then triggered to fuse by brief exposure to pH 5 (all in the continued presence of arbidol). The
334 efficiency of CCF was then determined by measuring the activity of the luciferase reporter that is
335 functionally restored upon cytoplasmic mixing of fused cells (43). As seen in Fig. 4A, CCF by
336 LASV GP (at pH 5.0) was suppressed by 20 μM and 40 μM arbidol. Based on findings in
337 parallel experiments (Fig. 4B), arbidol appeared more potent at impeding LASV-GP than
338 influenza HA-mediated CCF, consistent with its somewhat stronger effect on LASV GP-
339 compared to influenza HA-MLV pseudovirus infection (Fig. 2B).

As a complement to the CCF study (Fig. 4), we employed a forced fusion at the plasma membrane (FFPM) assay and assessed fusion of LASV GP-VSV pseudoviruses with the surface of cells expressing pmLamp1 (i.e., with Lamp1 at the cell surface), as previously described (31). As seen in Fig. 5A, arbidol suppressed LASV-GP-mediated FFPM with strong and complete inhibition seen with 20 and 40 μM doses, respectively. The experiment shown in Fig. 4A was conducted with a low pH pulse of pH 5.0. As seen in Fig. 5B, 40 μM arbidol strongly inhibited LASV GP-mediated FFPM at both pH 5.0 and pH 5.5.

Effects of arbidol on LASV GP1 dissociation. In the case of influenza, arbidol stabilizes HA (the fusion protein) such that the pH dependence for its fusion-inducing conformation change is shifted by 0.2-0.3 units in the more acidic direction (37, 39). Stabilization of HA is considered part of the mechanism of arbidol against influenza virus (38, 39, 44, 45). Since two independent assays (CCF and FFPM) showed that arbidol impairs the fusion activity of LASV GP, we next asked whether it impairs a conformational change in GP1 required for fusion activation. Upon exposure to low pH, LASV GP undergoes structural rearrangements, one of the earliest being dissociation of GP1, the receptor binding subunit, from GP2, the fusion subunit. This early change is thought to license subsequent changes that allow the fusion loop (in GP2) to access the target membrane and then to permit GP2 to fold back into a trimer-of-hairpins, which brings the viral and endosomal membranes into intimate contact leading to their fusion (46-49). Experiments using isolated LASV GP1/GP2 captured on beads showed that in this system, dissociation of the 44 kDa GP1 subunit occurs optimally at 37°C and half maximally at pH ~6.4 at 37°C following a 1 min low pH pulse (data not shown). We therefore treated LASV GP1/GP2 immobilized on beads with either 0 or 40 μM arbidol and then exposed the beads to buffers of

different pH values for 1 min at 37°C. As seen in Fig. 6A, the presence of 40 μM arbidol shifted the pH dependence for GP1 dissociation by ~0.5 units in the more acidic direction, suggesting that, as for influenza HA, arbidol can stabilize LASV GP. If arbidol stabilizes LASV GP, then it might delay GP1 dissociation in this system. To test this idea, we again captured LASV GP1/GP2 on beads, pre-treated the samples with 0 or 40 μM arbidol, treated the beads at pH 6.5 and 37°C in the presence of arbidol, and then took samples from 0-5 min and assayed them for GP1 dissociation. As seen in Fig. 6B, arbidol appeared to introduce an ~30 second lag, thereby slowing GP1 dissociation.

DISCUSSION

Drugs that block LASV- and EBOV GP-mediated entry with similar potency. We began this study by comparing the ability of eight drugs to inhibit LASV and EBOV GP-mediated infection. All eight are orally available, room temperature stable small molecules that block EBOV entry (18-20, 22, 23, 40, 50) and target entry processes also used by LASV (20-23, 51-53). Six are approved for clinical use and two are in advanced clinical testing. These collective features offer practical advantages (e.g., net costs and ease of transport and delivery) compared to novel drugs, many of which are designed in a 'one drug-one bug' approach (54).

Five drugs showed similar potency against LASV- and EBOV GP-mediated entry (Table 1). Zoniporide, an inhibitor of the plasma membrane Na^+/H^+ exchanger, blocks infection by the arenavirus LCMV by thwarting macropinocytotic uptake of viral particles (52). As both LASV (30) and EBOV (55, 56) are taken into cells by macropinocytosis, we expected and found zoniporide to have similar activity against both. Two drugs that impair endosome acidification, needed for the entry of both viruses (25, 49)---amodiaquine (an antimalarial) and niclosamide (an

386 anthelminthic)---also showed similar potency against both viral GPs in our direct comparative
387 tests. Both have been shown, albeit not in direct comparative studies, to inhibit many pathogens
388 that enter cells by endocytosis (21-23, 57-60). The fourth drug with similar activity against both
389 viruses is apilimod, which inhibits PIKfyve, an enzyme required for late endosome maturation
390 (51, 53, 61) and EBOV entry (27). Apilimod blocks EBOV trafficking to late endosomes (51,
391 53), which serve as portals for both EBOV and LASV (25, 26, 62-64). The fifth drug with
392 similar potency against LASV GP- and EBOV GP- mediated entry is arbidol (Umifenovir), a
393 synthetic antiviral approved and used in Russia and China to combat influenza, and shown to
394 have broad-spectrum antiviral activity (33, 34, 36, 38, 39).

395 The other three drugs---clomiphene, toremifene, and sertraline---showed ~3-6-fold
396 greater activity against EBOV GP than LASV GP-mediated entry. They are CADs that block
397 EBOV infections (19, 20, 22, 23, 40) and cause cholesterol accumulation in late endosomes,
398 mimicking effects of dysfunctional NPC1, the EBOV receptor (27, 28, 65). Since LASV also
399 enters cells through late endosomes (31), CADs may interfere with LASV entry due to general
400 impairments of late endosome function. The enhanced activity of toremifene and sertraline
401 against EBOV (Table 1), may be because they can bind to both EBOV GP, as shown by thermal
402 stability assays and X-ray crystallography (66, 67), as well as to the viral and/or endosomal
403 membrane (35, 39, 45, 66).

404

405 **Mechanisms by which arbidol may block LASV entry and infection.** Arbidol showed
406 roughly equivalent potency against LASV and EBOV GP-mediated pseudovirus infection (Fig.
407 1, Table 1), blocked infection by authentic LASV (Fig. 3), and was somewhat more effective at
408 blocking LASV GP- vs. influenza HA-mediated fusion and entry (Figs. 2B and 4B). Three

409 mechanisms have been proposed for the action of arbidol against influenza: direct binding to HA
410 (K_d 47 μM to PR8 influenza HA) (38, 44) and, as proposed for other viruses, binding to the viral
411 and/or target membrane so as to reduce fusion fitness (34, 35). By binding to and stabilizing
412 HA, arbidol shifts the pH threshold for fusion-inducing conformational changes by ~0.2 to 0.3
413 units in the more acidic direction (37, 38). This is noteworthy as even such small changes in
414 fusion pH can significantly impact influenza infectivity (67). Similarly, we found that arbidol
415 shifts the pH dependence for LASV GP1 dissociation from GP2 by ~0.5 units (in the more acidic
416 direction). This event is thought to unclamp the LASV fusion subunit (GP2), analogous to the
417 unclamping of influenza HA2 and HIV gp41. Hence, in addition to its likely general fusion-
418 impairing effects caused by interclation into the viral and/or endosomal membrane (35, 45, 66),
419 it is plausible that arbidol additionally affects the stability of LASV GP in a manner similar to its
420 effects on influenza HA (37-39). And, as reviewed elsewhere (36), arbidol may also affect steps
421 upstream or downstream of fusion.
422
423 **Therapeutic potential of drugs with similar potency against LASV and EBOV.** As reasoned
424 in the Introduction, a long-term goal is to identify a drug cocktail that inhibits both LASV and
425 EBOV. Our focus is on orally available, room temperature stable drugs that target processes used
426 in common for LASV and EBOV entry into cells. Here we identified five drugs that target
427 discrete steps of entry and show approximately equal potency against LASV and EBOV GP-
428 mediated entry: the macropinocytosis inhibitor, zoniporide; the endosome acidification inhibitors
429 amodiaquine and niclosamide; the trafficking inhibitor, apilimod; and the fusion inhibitor,
430 arbidol.

While zoniporide is not approved, it has advanced to Phase II clinical trials for treatment of cardiovascular diseases. As an alternate, the FDA-approved drug aripiprazole (trade name Abilify) may have utility. Aripiprazole blocks EBOV infection and synergizes with other entry inhibitors (20, 23). Preliminary data suggest that it blocks EBOV particle internalization as well as LASV GP-mediated infection (White Lab, unpublished data). The endosome acidification inhibitors amodiaquine and niclosamide are orally available and FDA-approved to treat malaria and helminthic diseases, respectively. For both, the C_{max} is within range of the IC_{50} for anti-EBOV/LASV activity (20-23, 57, 68, 69). Newer amodiaquine derivatives (59) or synergistic drug pairs containing amodiaquine or niclosamide and another agent could lower the needed doses (23). And while the trafficking inhibitor apilimod is well tolerated and a potent antagonist of EBOV and LASV (51, 53, 70) (and this study), in a first test, it did not protect mice from lethal EBOV challenge (see (23)), likely due to its inhibition of IL12/23 production ((61) and Rogers et al, manuscript submitted).

Among the fusion inhibitors tested, arbidol emerges as a candidate for future consideration, as it shows similar activity against LASV and EBOV and appears, in our assays, somewhat more potent against LASV- and EBOV GP- vs. influenza HA-mediated fusion and entry. Arbidol is approved and used in China and Russia against influenza and has shown strikingly broad anti-viral activity (33, 36, 71). A single standard human dose (200 mg) of arbidol yields a C_{max} lower (33, 72-74) than our preliminary indication (Fig. 3) of its IC_{50} against authentic LASV. We note, however, that the estimated IC_{50}s for arbidol against authentic LASV (Fig. 3) and influenza (39, 45, 75) are similar (roughly ~10 μM; variable for different influenza strains). As standard dosing of arbidol for influenza is 200 mgs three (72) or four (75) times per day, and as arbidol has a long half-life (36), the net (cumulative) C_{max} with multiple daily dosing

is expected to be significantly higher (S. Polyak and M. Paine, unpublished calculation). Hence, as standard dosing of arbidol is clinically beneficial against influenza (see for example, (75)), it may have utility against LASV and EBOV. However, the only modest reduction in titer seen (Fig. 3) coupled with experience with another hemorrhagic fever virus (76) argue against proposing arbidol as a stand-alone therapeutic. A tolerated higher dose of arbidol (74), a new arbidol derivative (39, 44), or a combination of arbidol with another drug (23, 77, 78) might lower the dose needed to be in line with attainable anti-viral efficacy. With respect to a potential drug cocktail including arbidol, it is interesting that amantadine, rimantadine, ribavirin, and ribamidil have been reported to enhance the activity of arbidol against influenza (79).

We envision that an orally-available, room temperature stable, approved drug or cocktail of approved drugs could be rapidly deployed for treatment or prophylaxis against (suspected) cases of LASV and EBOV, especially in regions around the globe that are challenged in terms of resources, infrastructure and accessibility. Such a drug, or drug cocktail, might be valuable before a definitive diagnosis has been made, concurrent with vaccination (e.g., of healthcare workers and/or first responders to outbreaks), and/or during the set-up of ring vaccination. Here we have identified several drugs with these attributes that show similar inhibition of LASV and EBOV entry. Moreover, three of them---amodiaquine, niclosamide, and arbidol---inhibit multiple enveloped viruses (33, 36, 57, 60, 71, 80-83). Hence, a drug or drug cocktail containing these drugs could inhibit multiple enveloped viruses that enter cells through the endocytic pathway (49).

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Drug	Step Blocked	n	Average Ratio (+/- SEM) IC_{50} LASV/ IC_{50} EBOV
Zoniporide	Internalization	3	0.72 +/- 0.14
Amodiaquine	Acidification	4	0.41 +/- 0.14
Niclosamide	Acidification	5	0.54 +/- 0.13
Apilimod	Traffic	3	1.50 +/- 0.16
Arbidol	Fusion	3	0.59 +/- 0.12
Clomiphene	Fusion	4	4.72 +/- 2.41
Sertraline	Fusion	3	4.99 +/- 2.37
Toremifene	Fusion	3	6.71 +/- 3.09

Table 1: Comparative ability of drugs to block infections by LASV- and EBOV GP-MLV pseudoviruses. In each experiment (n=3-5 as indicated) cells were pretreated with 8 doses of the indicated drugs and their IC_{50}s for blocking infections by LASV and EBOV GP MLV pseudoviruses were determined in triplicate samples as described in the Materials and Methods section. For each experiment, the following ratio was then calculated: the IC_{50} for blocking LASV GP pseudovirus infection divided by the IC_{50} for blocking EBOV GP pseudovirus infection. Values in column 4 are the averages of those ratios +/- SEM. The average IC_{50} and maximal percent inhibition values across all experiments for LASV were: zoniporide (88 μM, 87%), amodiaquine (1.9 μM, 82%), niclosamide (0.12 μM, 100%), apilimod (0.04 μM, 80%), arbidol (1.7 μM , 95%), clomiphene (5.7 μM, 100%), sertraline (2.6 μM, 90%), toremifene (2.9 μM, 96%). The average IC_{50} and maximal percent inhibition values across all experiments for EBOV were: zoniporide (115 μM, 98%), amodiaquine (6.6 μM, 97%), niclosamide (0.24 μM, 100%), apilimod (0.03 μM, 100%), arbidol (2.8 μM, 100%), clomiphene (1.8 μM, 100%), sertraline (0.7 μM, 100%), toremifene (0.5 μM, 100%). Very similar ratios to those presented in column 4 above were obtained for the ratios of the average IC_{50} values across all experiments.

FIGURE LEGENDS

FIG 1. Representative dose response curves for eight low molecular weight drugs against LASV GP- and EBOV GP-mediated MLV pseudovirus infection. BSC-1 cells were pretreated with the indicated dose of the indicated drug for 1 hr and then infected with MLV pseudoviruses encoding luciferase. Luciferase signals were measured 24 hr later and normalized to the maximal signal from a triplicate set of mock-treated cells. Data points indicate the average % inhibition from triplicate wells. Error bars represent the standard deviation (SD). The red horizontal dashed line indicates 50% inhibition. Each dose response comparison was conducted 3-5 times, with similar results.

FIG 2. Comparative effects of arbidol on infection by MLV pseudoviruses bearing LASV or other viral glycoproteins: (A) LASV, LCMV, and Junin GP; (B) LASV GP and influenza HA. MLV pseudoviruses bearing LASV GP, LCMV GP, Junin GP, or WSN influenza HA and NA were prepared as described in the Materials and Methods section. BSC-1 cells were pre-treated with the indicated concentrations of arbidol and then processed and analyzed for infection as described in the legend to Fig. 1. Data in **(A)** are the averages +/- SEM from three experiments, each performed with triplicate samples. Data in **(B)** represent the average +/- SD from triplicate samples from one experiment.

523 **FIG 3. Arbidol inhibits authentic LASV infection.** Duplicate wells of Vero76 cells were
524 pretreated with the indicated concentration of arbidol (or vehicle or 25 mM NH_4Cl) for 1 hr and
525 then infected with LASV (Josiah strain) virus at an MOI of 0.01. Following a 1 hr binding period
526 at 4°C, unadsorbed virus was removed and the cells were incubated for 24 hr in the presence of
527 drug in a 37°C CO_2 incubator. Culture supernatants were harvested, serially diluted 10-fold in
528 fresh medium, and then titered on Vero76 cells by a 96 hr plaque assay. Results in (**A-C)** are the
529 average titers from duplicate wells. The values in (**D)** indicate the average normalized infection
530 in samples treated with 20 μM arbidol (+/- SD) from the experiments shown in (**A-C**). The
531 asterisk indicates $^{**}p < 0.01$.
532
533 **FIG 4. Arbidol suppresses LASV GP-mediated cell-cell fusion (CCF).** Effector cells were
534 generated by transfecting HEK293T/17 cells with plasmids encoding DSP_{1-7} (the N-terminal
535 split luciferase plasmid) and either **(A)** LASV GP or **(B)** WSN influenza HA and NA. Target
536 cells were generated by transfecting HEK293T/17 cells with plasmids encoding DSP_{8-11} (the C-
537 terminal split luciferase plasmid) and pmLamp1. For the experiments, effector cells were
538 preloaded with a luciferase substrate and then pretreated for 1 hr with the indicated concentration
539 of arbidol or 10% EtOH (mock control). Effector cells were then co-cultured with target
540 HEK293T/17 cells (in the continued presence of arbidol or 10% EtOH) for 3 hr at 37°C. At this
541 time the cultures were pulsed with pH 5 buffer for 5 min at 37°C, reneutralized and then returned
542 to the 37°C CO_2 incubator for 1 hr, at which time the luminescent signal was measured. The data
543 represent the normalized luminescent signal (relative to mock-treated controls) from three
544 experiments, each performed with triplicate samples. Error bars indicate SD. Asterisks indicate:
545 $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

FIG 5: Arbidol inhibits LASV-GP-mediated forced fusion at the plasma membrane (FFPM). COS7 cells were transfected with plasmids encoding pmLamp1 and firefly luciferase (FLUC). Roughly 24 hr later, the cells were pretreated with the indicated dose of arbidol (or mock treated) for 1 hr. At this time, VSV pseudoviruses bearing LASV GP and encoding *Renilla* luciferase (RLUC) were allowed to bind at 4°C for 1 hr. The cells were then pulsed for 5 min at 37°C with prewarmed buffers at either **(A)** pH 5 or **(B)** the indicated pH and then the buffer was replaced with complete medium containing NH_4Cl to prevent infection via the normal endocytic route. After 12-18 hr at 37ºC, RLUC (an indicator of infection via FFPM) and FLUC (to standardize transfected cell numbers) were measured. The ratio of *Renilla* to firefly luciferase (RLUC/FLUC) was then **(A)** normalized to RLUC/FLUC ratio for the mock-treated cells or (**B**) directly plotted. Data are from a representative experiment performed with quintuplicate samples. Error bars indicate SD. ***$p < 0.001$, **** $p < 0.0001$. Each experiment was repeated one time with similar results.

FIG 6. Arbidol impairs LASV GP1 dissociation from GP2. (A) Flag-tagged LASV GP from cell lysates of Lamp1 KO HEK293T/17 cells was immobilized on anti-Flag (M2) magnetic beads, which were then treated with 0 or 40 µM arbidol for 1 hr at 4°C. The beads were then subjected to a pulse for 1 min at 37°C at the indicated pH in the presence or absence of arbidol. The extent of GP1 dissociation was then determined by Western blot analysis of GP1 in the supernatant and bead-bound fractions. Data are the averages from four experiments. Error bars represent SEM. **(B)** Flag-tagged LASV GP, prepared and immobilized as in panel **(A)**, was pretreated with 0 or 40 µM arbidol. The beads were then exposed to pH 6.5, at 37°C for the

indicated time, in the presence or absence of arbidol, and the extent of GP1 dissociation determined as in panel **(A)**. Data are the averages from five experiments. Error bars represent SEM.

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