Comparative analyses of small molecule and antibody inhibition on glycoprotein-mediated entry of Měnglà virus with other filoviruses

Laura Cooper | Jazmin Galvan Achi | Lijun Rong

Abstract
The ability of viruses in the Filoviridae family (Ebola virus [EBOV] and Marburg virus [MARV]) to cause severe human disease and their pandemic potential makes all emerging filoviral pathogens a concern to humanity. Měnglà virus (MLAV) belonging to the new genus *Dianlovirus* was recently discovered in the liver of bats from Měnglà County, Yunnan Province, China. The capacity of MLAV to utilize NPC1 as an endosomal receptor, to transduce mammalian cells, and suppress IFN response suggests that this potential pathogen could cause human illness. Despite great effort by researchers, only the viral genome has been recovered and isolation of live MLAV had been unsuccessful. Here using a pseudovirus model baring the MLAV glycoprotein (GP), we studied the protease dependence of the MLAV-GP, and the ability of small molecules and antibodies to inhibit MLAV viral entry. Like EBOV and MARV, the MLAV-GP requires proteolytic processing but like MARV it does not depend on cathepsin B activity for viral entry. Furthermore, previously discovered small-molecule inhibitors and antibodies are MLAV inhibitors and show the possibility of developing these inhibitors as broad-spectrum filovirus antivirals. Overall, the findings in the study confirmed that MLAV viral entry is biologically distinct but has similarities to MARV.

**KEYWORDS**
Ebola virus, emerging pathogens, filovirus, Marburg virus, Měnglà virus

1 | INTRODUCTION

In 2019 a new filovirus was discovered in the Rousettus bat in the Yunnan Province of China; this filovirus would come to be known as Měnglà virus (MLAV). Rousettus bats are the natural reservoir for Marburg virus (MARV) and the suspected reservoir for Ebola virus (EBOV).\(^1\) While most of the filovirus outbreaks have originated in Africa, filoviruses have been found in other parts of the world. For example, Lloviu virus, which has been assigned the genus *Cuevavirus*, was found in bats from Hungary, while Reston virus (RESTV) was found in the Philippines.\(^2,3\) Furthermore, there has been previous evidence of *Ebolavirus* in bats from China before the discovery of MLAV.\(^4\) These findings suggest that filoviruses may be hiding in animal reservoirs outside of Africa and have the potential to start an epidemic in highly populated countries such as China, or even a global pandemic.
MLAV was identified from the liver samples collected from bats in Mènglà Country, Yunnan Province, China. The whole-genome sequence was determined from the liver samples of bat-9447 using next-generation sequencing (NGS) and was found to be related to the Filoviridae family. To date, only the viral genome has been recovered since researchers have been unable to isolate infectious MLAV. Based on analysis of the L protein, MLAV is genetically more closely related to that of MARV with a nucleotide homology of 54% compared to EBOV, with a homology of 41%. The genome organization is like that of other filoviruses with seven potential open reading frames (ORFs). Given the low homology to the other filoviruses, MLAV is classified into its own genus taxon, Dianlovirus.

Overall, the MLAV-GP has an amino acid homology of 27% with the EBOV-GP and 40% with the MARV-GP, respectively. Sequence analysis of the MLAV-GP gene showed conservation in the receptor-binding domain (RBD) of the GP suggesting that MLAV, like EBOV and MARV, uses NPC1 as an endosomal receptor. Yang et al. used a vesicular stomatitis virus (VSV)-based pseudovirus assay and confirmed that NPC1 is a critical host factor and MLAV has a broad species tropism with the ability to transduce the cells from multiple mammalian species. In addition, it has been shown that the MLAV is able to suppress the type I interferon (IFN) response in human cells and the MLAV VP35 and VP40 are able to block the human IFN response. This is consistent with what has been seen for EBOV and MARV, suggesting that MLAV has the potential to cause human infection and interspecies transmission.

To date, only a couple of studies have been reported on MLAV. Yang et al. were the first to report and characterize MLAV, and Williams et al. more recently investigated the role of the MLAV viral proteins on host innate immune pathways. Except for the demonstrated role of NPC1 in MLAV entry, little is known about the entry mechanism of this virus. In this study, using an HIV-based pseudovirus as a surrogate entry assay, we examined (1) the inhibition profiles of several small-molecule inhibitors on MLAV entry in comparison with that of EBOV and MARV, (2) the protease dependence of the MLAV-GP, and (3) the effectiveness of EBOV and MARV monoclonal antibodies on neutralizing MLAV entry.

2 | MATERIALS AND METHODS

2.1 | Cell culture

Human A549 lung epithelial cells (ATCC# CCL185) and 293T embryonic kidney cells (ATCC# CRL-1573) were cultured in DMEM supplemented with 10% (v/v) fetal bovine serum (Gibco), 100 units of penicillin, and 100 µg/ml streptomycin (Invitrogen), at 37°C and 5% CO₂.

2.2 | Pseudoviron production

Pseudoviruses for initial IC₅₀ drug screening were created using the following plasmids: Marburg virus Musoke glycoprotein, Ebola virus Zaire Mayinga glycoprotein, Mènglà virus glycoprotein, and the HIV-1 pro-viral vector pNL4-3-LucR’E’, which was obtained through the NIH AIDS Research and Reference Reagent Program. All pseudoviruses were produced by transient cotransfection of 293T cells using a polyethyleneimine (PEI)-based transfection protocol. Five hours after transfection, cells were washed with phosphate-buffered saline (PBS), and 20 ml of fresh media was added to each 150 mm plate. Twenty-four hours post-transfection, the supernatant was collected and filtered through a 0.45 µm pore size filter and stored at 4°C before use.

2.3 | Measuring IC₅₀ and CC₅₀ against pseudovirus

Low passage A549 cells were seeded in 96-well plates at the density of 5000 cell/well and incubated at 37°C and 5% CO₂ for 24 h before infection. In the presence of a range of drug concentrations, A549 cells were infected with pseudovirions containing a luciferase reporter gene. All drugs were dissolved in dimethyl sulfoxide (DMSO) and final treatment DMSO concentrations never exceeded 1%. For antibody neutralization, the antibodies were incubated with the pseudovirus for 1 h before being added to the A549 cells. Plates were incubated at 37°C and 5% CO₂ for 48 h and viral infection was determined by luminescence using the neolite reporter gene assay system (PerkinElmer). Virus with 1% DMSO was used as a negative control and data were normalized to the negative control. Drug cytotoxicity was assessed using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega) in the A549 cells treated the same way as for the antiviral screen. IC₅₀ and CC₅₀ values were determined by fitting dose–response curves with four-parameter logistic regression in Graphpad.

3 | RESULTS

3.1 | Small molecule inhibition of MLAV entry

The glycoprotein (GP) of MLAV, like all filoviruses mediates viral entry. Sequence comparison of MLAV-GP with that of other filoviruses generated the same phylogenetic tree as that of the L proteins (Figure 1). We have established an MLAV-GP-based pseudovirus platform using a pNL4-3-LucR’E’ lentiviral system, which gave a very high signal-to-noise ratio (~2500) in A549 cells (Figure 2A). This system has been used successfully to model the GP-mediated entry of EBOV and MARV. Using this system, we analyzed MLAV-GP-mediated entry and examined small-molecule inhibition characteristics. We evaluated eight previously identified FDA-approved inhibitors of EBOV and MARV for their ability to inhibit MLAV. All eight inhibitors are chemically diverse and have been FDA approved for different indications.

We demonstrated that all these drugs inhibited MLAV entry. Sertraline, a drug FDA approved to treat depression, was one of the most potent inhibitors tested against MLAV with an IC₅₀ value of 1.40 ± 0.54 µM and a favorable selectivity index (SI) of 11. In addition to sertraline, two other drugs, imipramine and benzotropine, had SI values greater than 10 with MLAV suggesting that they have
potential as drug leads (Figure 2B and Table 1). Sertraline and benzotropine were also potent inhibitors of EBOV (IC$_{50}$ values = 0.52 ± 0.21 and 0.40 ± 0.52 µM, respectively) and MARV (IC$_{50}$ values = 1.18 ± 0.10 and 2.54 ± 0.527 µM, respectively). Paroxetine was the third most potent inhibitor of MLAV, MARV, and EBOV with IC$_{50}$ values of 4.88 ± 1.70, 1.83 ± 0.66, and 0.36 ± 0.40 µM, respectively. However, due to increased cytotoxicity with paroxetine this drug had a less desirable SI value of 5 with MLAV. In contrast, imipramine was less potent in MLAV with an IC$_{50}$ value of 7.91 ± 0.81 µM but it had a favorable SI value of 10 due to decreased cytotoxicity. However, it should be noted that all inhibitors tested were less potent against MLAV compared to MARV and EBOV.
Protease dependence of MLAV entry

It is known that all filoviruses require host proteolytic processing of their GP for viral entry. Host proteases remove the glycan cap and heavily glycosylated mucin domain, a process that is required for viral entry. It has been shown that all *Ebolavirus* species are activated and depend on the cysteine proteases cathepsins B and L; in contrast, MARV activation is only dependent on cathepsin L and independent of cathepsin B. Here we tested if MLAV requires host proteases during the entry process. We selected small-molecule inhibitors that have been previously characterized to have various activities against cathepsin B and cathepsin L (Figure 3A). Using the same MLAV/HIV pseudovirus model, we evaluated these inhibitors for their ability to inhibit MLAV pseudovirus entry. These results were compared with EBOV and MARV, as well as VSV, a pseudovirus that does not require host proteases as a control.

As expected, MLAV, EBOV, and MARV were sensitive to the broad-spectrum protease inhibitor leupeptin, which is a naturally occurring covalent inhibitor of cysteine, serine, and threonine peptidases. It efficiently blocked viral entry of all three filoviruses but was ineffective against the control virus VSV. Consistent with Figure 2, leupeptin was a more potent inhibitor of EBOV and MARV (IC\(_{50}\) values = 0.41 ± 0.04 and 0.92 ± 0.27 µM, respectively) than MLAV (IC\(_{50}\) value = 1.24 ± 0.29 µM, Figure 3B). Like MARV, MLAV was resistant to the Cathepsin B-specific inhibitors, whereas leupeptin inhibited EBOV and MARV with comparable potency. These results suggest that MLAV entry is not dependent on cathepsin B.

### Table 1

| Compounds       | IC\(_{50}\) HIV/EBOV (µM) | IC\(_{50}\) HIV/MARV (µM) | IC\(_{50}\) HIV/MLAV (µM) | CC\(_{50}\) (µM) |
|-----------------|---------------------------|---------------------------|---------------------------|-----------------|
| Toremifene      | 0.06 ± 0.05               | 1.75 ± 0.86               | 3.63 ± 0.72               | 25.3 ± 1.6      |
| Imipramine      | 2.00 ± 0.65               | 5.55 ± 1.12               | 7.91 ± 0.81               | 76.3 ± 7.4      |
| Paroxetine      | 0.36 ± 0.40               | 1.83 ± 0.66               | 4.88 ± 1.70               | 23.6 ± 2.5      |
| Bepridil        | 3.27 ± 0.40               | 7.37 ± 0.90               | 9.74 ± 0.94               | 34.7 ± 4.6      |
| Dibucaine       | 2.93 ± 1.04               | 5.82 ± 0.37               | 10.80 ± 1.68              | 51.3 ± 1.2      |
| Orphenadrine    | 1.45 ± 0.56               | 10.13 ± 3.58              | 23.02 ± 4.71              | >100            |
| Benztropine     | 0.40 ± 0.52               | 2.54 ± 0.57               | 4.93 ± 0.24               | 54.5 ± 2.0      |
| Sertraline      | 0.54 ± 0.21               | 1.18 ± 0.10               | 1.40 ± 0.54               | 15.0 ± 0.9      |
| Fluoxetine      | 3.00 ± 0.54               | 2.44 ± 0.58               | 7.84 ± 0.54               | 28.7 ± 0.1      |

Note: All error bars represent SD from three independent experiments.

Abbreviations: EBOV, Ebola virus; MARV, Marburg virus; MLAV, Mènglà virus.
inhibitor CA-074. However, CA-074 selectively inhibited EBOV with an IC₅₀ of 28.43 ± 6.56 µM. Along with VSV, both MARV and MLAV were resistant to CA-074 treatment at 100 µM (Figure 3C).

Cathepsin L inhibitor III is a selective inhibitor of cathepsin L and cathepsin B with a higher affinity for cathepsin L. Consistent with previous findings, EBOV showed an increased sensitivity to cathepsin L inhibitor III with an IC₅₀ value of 0.75 ± 0.19 µM. MARV and MLAV also were inhibited with IC₅₀ values of 2.38 ± 0.40 and 7.14 ± 1.50 µM, respectively. The viral entry inhibition by cathepsin L inhibitor III was filovirus specific with VSV showing no inhibition at 100 µM. The increased inhibition seen with EBOV could be from its increased susceptibility to cathepsin B inhibition (Figure 3D).

MLD-28170 is a calpain inhibitor that also inhibits cathepsins B and L and can block EBOV, MARV, and MLAV in a dose-dependent manner (Figure 3E). The results of MLD-28170 further validate that MLAV can be inhibited by cathepsin L. AEBSF is an irreversible serine protease inhibitor that has no appreciable activity against cathepsins B and L. Consistent with previous reports there was limited inhibition of EBOV or MARV viral entry by AEBSF, most inhibition seen can be attributed to cytotoxicity. Like EBOV and MARV, AEBSF was unable to effectively inhibit MLAV entry (Figure 3F and Table 2). These results suggest that MLAV is dependent on the proteolytic process of its GP by host proteases for entry and that like MARV, MLAV does not depend on cathepsins B activity for entry into host cells.

### 3.3 Analysis of monoclonal antibodies against MLAV-GP

For enveloped viruses, such as EBOV, neutralizing antibodies can bind to the surface GP and interfere with the ability of the virus to enter the host cell. However, some antibodies can inhibit egress by binding to the glycoprotein on the surface of the host cell and prevent new virus from budding off the cell. This activity prevents progenesis from infecting other cells and inhibits already formed virions from entering new target cells. Therefore, neutralizing antibodies have potential as therapeutics. In fact, Ebanga (ansuvimab), a single monoclonal antibody, and Inmazeb (atoltivimab/maftivimab/odesivimab), a triple monoclonal antibody therapy, have been approved by the FDA for the treatment of Ebola virus disease.¹³,¹⁴

The heavily glycosylated mucin-like domains (MLDs) mask the conserved NPC1 RBD of EBOV-GP but not the MARV-GP. In the MARV-GP, the MLD is bound to the GP1 and GP2 subunit, but the EBOV-GP MLD is only bound to GP1. MR-78 and MR-191 are MARV-neutralizing antibodies that bind to MARV-GP with and without cleavage and EBOV-GP after cleavage. The differing positions of the MLD give rise to the selectivity of the MR-series of antibodies that bind to the highly conserved NPC1 RBD. Using these antibodies, we were able to probe the possible location of the MLD for MLAV.

When MR-78 and MR-191 were tested for MLAV pseudovirus neutralization only MR-191 showed neutralization but at a lower potency than that of MARV (23.10 ± 0.35 vs. 0.09 ± 0.03 µg/ml, respectively) (Figure 4A and Table 3). This is consistent with what has been previously published on the MR-series of antibodies; MR-191 also showed more potent binding of the cleaved EBOV-GP than MR-78. This suggests that the residues in the epitope region of MR-191 are more conserved than that of MR-78 but cleavage of the GP is still required for EBOV neutralization. The fact that MR-191 was able to neutralize the MLAV-GP to some degree without cleavage suggests that MLAV has a GP more similar to that of MARV-GP with the MR-series epitope not masked by the MLD (Figure 4C). The lack of neutralization of MR-78 is not surprising given that this antibody was only able to neutralize the authentic MARV species Musoke but not Uganda, Angola, or Ravn below 100 µg/ml.¹⁷

Further evidence of the location of the MLAV MLD comes from data obtained with EBOVneutralizing antibody ADI-15878. This antibody shows significant cross-reactivity across all Ebola virus species. ADI-15878 binds to an epitope region at the GP1/GP2 interface and is a competitive inhibitor of KS52 (an Ebola Zaire-specific neutralizing antibody). Despite amino acid sequence divergence in the epitope-binding region, all species of Ebola virus can be neutralized. Using escape mutagenesis, it has been shown that residue G528 in the EBOV-GP is key for ADI-15878 binding. This residue is conserved among all the filovirus species; however, ADI-15878 is unable to neutralize MARV. It is believed the lack of MARV neutralization is a result of a clash between the antibody and bulky residues in the epitope binding region in conjunction with the epitope being shielded by the location of the MARV MLD at the GP1/GP2 interface. As expected, we saw potent neutralization of the EBOV pseudovirus (EC₅₀ ≤ 0.01 µg/ml), and no neutralization of the MARV and MLAV pseudovirus (EC₅₀ > 100 µg/ml) (Figure 4A,B). This further confirms that MLAV is more MARV-like and may share a similar GP structure. Direct binding studies on cleaved and uncleaved MLAV should reveal more information on the location of the MLD.

### 4 DISCUSSION

The discovery of MLAV suggests there is a potential threat of novel filoviruses in parts of the world outside of Africa. This novel pathogen has been shown to be able to infect human cells and have broad tropism just like EBOV and MARV. However, little is known about the entry

| Compounds          | IC₅₀ HIV/EBOV (µM) | IC₅₀ HIV/MARV (µM) | IC₅₀ HIV/MLAV (µM) | CC₅₀ (µM)  |
|--------------------|--------------------|--------------------|--------------------|-----------|
| Leupeptin          | 0.41 ± 0.04        | 0.92 ± 0.27        | 1.24 ± 0.29        | >100      |
| CA-074             | 28.43 ± 6.56       | >100               | >100               | >100      |
| Cathepsin L        | 0.75 ± 0.19        | 2.38 ± 0.40        | 7.14 ± 1.50        | >100      |
| Inhibitor III      | MDL-28170          | 1.44 ± 0.20        | 1.69 ± 0.46        | 9.46 ± 1.69 | >100      |
| AEBSF              | 19.10 ± 0.58       | 29.00 ± 1.70       | 36.70 ± 10.40      | <100      |
mechanism of MLAV. In this study, using an HIV-based pseudovirus assay as a surrogate system, we compared the inhibition profiles of several small inhibitors, which were previously discovered as entry inhibitors of EBOV and MARV, on the MLAV entry. Not surprisingly, these inhibitors can all inhibit MLAV entry, albeit less effectively than that against EBOV or MARV.

In addition to the eight compounds tested here, we showed in Cooper et al. that toremifene and compounds 30 and 32 also inhibited MLAV. Compounds 30 and 32 had IC50 values of 1.40 and 1.84 µM making them two of the most potent MLAV entry inhibitors. Toremifene, sertraline, paroxetine, benztropine, bepridil, and imipramine have all been cocrystalized with the EBOV-GP and shown to bind directly to the EBOV fusion-loop-associated cavity. In Schafer et al., we confirmed that these compounds, in addition to dibucaine and orphenadrine, interact with the EBOV fusion-loop-associated cavity via mutational analysis of key residues in that binding pocket. We also identified the HR2 region as a possible site for small molecules to bind to EBOV-GP and MARV-GP. It is possible due to the high amino acid conservation of the HR2 region among the filoviruses (67% between MLAV & MARV and 58% MLAV & EBOV) that the HR2 region is one of the sites responsible for small molecule entry inhibition of MLAV. However, the exact mechanism for how these drugs inhibit MARV and MLAV viral entry needs to be further investigated. Overall, these results support the notion that it is possible to develop small-molecule pan-filovirus inhibitors.

In addition to sequence identity, MLAV shares many similarities with MARV. MLAV like MARV lacks RNA editing sites in its GP gene that results in the production of sGP and ssGP seen with the Ebolavirus/Cuevavirus species, has Rousettus bats as a natural reservoir, and possesses the ability to suppress IFN-induced signaling through VP40 as opposed to VP24. We found that MLAV requires host proteases for GP processing but, similarly to MARV, is not cathepsin B dependent. Interestingly, MARV-neutralizing antibody MR-191 shows some neutralization of MLAV in the absence of GP cleavage. This suggests that MLAV-GP MLD might be on the equatorial plane of the GP with an exposed RBD, like the structure of the MARV-GP.

Although MLAV shares many similarities with MARV, PASC analysis of the genome placed MLAV into its own distinct genus. Williams et al. further confirmed with functional evidence that MLAV is biologically distinctive from MARV, by showing an absence of MLAV VP24 Kelch-like ECH-associated protein 1 (Keap1)-binding motif preventing antioxidant response element (ARE) activation. The MARV VP24 has the ability to bind to Keap1 and disrupt the Keap1-nuclear factor erythroid 2-related factor 2 (Nrf2) interaction which causes the expression of genes possessing ARE. In MARV, this results

**FIGURE 4** Marburg monoclonal antibody MR-191 shows MLAV neutralization. (A) Dose–response curves for values in Table 3. (B) Amino acid sequence alignment of the epitope of MR-191. All error bars represent SD from three independent experiments. EBOV, Ebola virus; MARV, Marburg virus; MLAV, Mènglà virus; VSV, vesicular stomatitis virus.

**TABLE 3** Half maximal inhibitory concentration (IC50) for MARV and EBOV antibodies

| Antibody | IC50 HIV/EBOV (µg/ml) | IC50 HIV/MARV (µg/ml) | IC50 HIV/MLAV (µg/ml) | IC50 HIV/VSV (µg/ml) |
|----------|-----------------------|-----------------------|-----------------------|-----------------------|
| MR-191   | >100                  | 0.09 ± 0.03           | 23.10 ± 0.35          | >100                  |
| MR-78    | >100                  | 0.17 ± 0.05           | >100                  | >100                  |
| ADI-15878| <0.01                 | >100                  | >100                  | >100                  |

Note: All error bars represent SD from three independent experiments. Abbreviations: EBOV, Ebola virus; MARV, Marburg virus; MLAV, Mènglà virus; VSV, vesicular stomatitis virus.
in a cytoprotective state that extends the life of MARV-infected cells; therefore, MLAV fails to activate a cytoprotective response. The function of VP24 is not related to entry but confirms the biological diversity of MLAV within the Filoviridae family. Our data also support that MLAV is different from MARV based on the inhibition profile data with the tested small molecules and antibodies.

5 | CONCLUSION

In summary, our findings support that MLAV is more closely related to MARV than EBOV, yet distinctive from it, which is consistent with the previously published studies. Importantly, our work here indicates that the previously discovered small-molecule inhibitors are effective as entry inhibitors against MLAV and suggests the possibility of developing these inhibitors as broad-spectrum antivirals against different filoviruses.

AUTHOR CONTRIBUTIONS

Laura Cooper and Lijun Rong designed this study. Laura Cooper and Jazmin Galvan Achi gathered and verified the data. Laura Cooper carried out analysis and interpretation of data, and drafted the manuscript. Lijun Rong, Laura Cooper, and Jazmin Galvan Achi revised the manuscript. All authors read and approved the final manuscript.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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