A biaryl sulfonamide derivative as a novel inhibitor of filovirus infection

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Abstract

Ebolaviruses and marburgviruses, members of the family *Filoviridae*, are known to cause fatal diseases often associated with hemorrhagic fever. Recent outbreaks of Ebola virus disease in West African countries and the Democratic Republic of the Congo have made clear the urgent need for the development of therapeutics and vaccines against filoviruses. Using replication-incompetent vesicular stomatitis virus (VSV) pseudotyped with the Ebola virus (EBOV) envelope glycoprotein (GP), we screened a chemical compound library to obtain new drug candidates that inhibit filoviral entry into target cells. We descovered a biaryl sulfonamide derivative that suppressed *in vitro* infection mediated by GPs derived from all known human-pathogenic filoviruses. To determine the inhibitory mechanism of the compound, we monitored each entry step (attachment, internalization, and membrane fusion) using lipophilic tracer-labelled ebolavirus-like particles and found that the compound efficiently blocked fusion between the viral envelope and the endosomal membrane during cellular entry. However, the compound did not block the interaction of GP with the Niemann-Pick C1 protein, which is believed to be the receptor of filoviruses. Using replication-competent VSVs pseudotyped with EBOV GP, we selected escape mutants and identified two EBOV GP amino acid residues (positions 47 and 66) important for the interaction with this compound. Interestingly, these amino acid residues were located at the base region of the GP trimer, suggesting that the compound might interfere with the GP conformational change required for membrane fusion. These results suggest that this biaryl sulfonamide derivative is a novel fusion inhibitor and a possible drug candidate for the development of a pan-filovirus therapeutic.

Keywords

Ebolavirus, Marburgvirus, Glycoprotein, Compound, Entry inhibitor, Membrane fusion

1. Introduction

Ebola- and marburgviruses are non-segmented negative-stranded RNA viruses that belong to the family *Filoviridae*, which includes among others the genus *Marburgvirus* with a single species, *Marburg marburgvirus*, including Marburg virus (MARV) and Ravn virus, and the genus *Ebolavirus* with five species, *Zaire ebolavirus*, *Sudan ebolavirus*, *Tai Forest ebolavirus*, *Bundibugyo ebolavirus*, and *Reston ebolavirus*, represented by Ebola virus (EBOV), Sudan virus (SUDV), Taï Forest virus (TAFV), Bundibugyo virus (BDBV), and Reston virus (RESTV), respectively (Sahul Hameed et al., 2019). An additional ebolavirus species, *Bombali ebolavirus*, represented by Bombali virus (BOMV), has been proposed recently (Goldstein et al., 2018). These filoviruses, with the exception of RESTV and BOMV, are known to cause severe hemorrhagic fever in humans (Emanuel et al., 2018; Rollin et al., 1999). The case fatality rate is up to about 90% but varies among virus species and even variants (Baseler et al., 2017).

EBOV caused the largest outbreak of Ebola virus disease (EVD) in 2013-2016 in West Africa, with over 28,000 cases and 11,000 deaths. In the second largest EVD outbreak in the Democratic Republic of the Congo (DRC), declared by the Ministry of Health of the DRC on 1st August 2018, a total of 3,481 EVD confirmed and probable cases and 2,299 deaths have been reported (as of July 3, 2020) (Mbala-Kingebeni et al., 2019; Tariq et al., 2019; WHO, 2019). Clinical trials of antibody therapies (i.e., ZMapp, REGN-EB3, and mAb114) were conducted during the outbreaks in West Africa and the DRC (The PREVAIL II Writing Group, 2016; WHO, 2018). Several small compound

candidates for treatment of EVD such as T-705 (favipiravir), and GS-5734 (remdesivir), all of which are nucleotide analogues, have also been tested in animal models and clinical cases (Cardile et al., 2016; Nakkazi, 2018; Sissoko et al., 2016). In particular, remdesivir is currently under clinical trials in the DRC (NIH, 2019). However, there are no approved antiviral therapeutics against EVD.

The ebolaviruses contain at least seven structural proteins: nucleoprotein (NP), viral protein (VP) 35, VP40, glycoprotein (GP), VP30, VP24, and RNA-dependent RNA polymerase (L). GP is the only viral surface protein that forms spikes on the virion surface: It is responsible for receptor binding and membrane fusion (Hunt et al., 2012; Takada et al., 1997) and thus is thought to be an important target of antivirals. The entry of ebolaviruses into host cells is initiated by attachment of GP to cell surface attachment receptors such as C-type lectins and TIM-1 (Alvarez et al., 2002; Kondratowicz et al., 2011; Takada et al., 2004). The virus particle is then internalized into endosomes by macropinocytosis (Nanbo et al., 2010; Saeed et al., 2010). In endosomes, GP is proteolytically processed by cathepsins B and L in a low pH environment (Schornberg et al., 2006). The processed GP interacts with the endosomal fusion receptor, Niemann-Pick C1 (NPC1), and subsequently fusion between the viral envelope and the endosomal membrane occurs, leading to the release of the viral ribonucleoprotein (RNP) complex into cytoplasm (Carette et al., 2011; Côté et al., 2011). The RNP complex is formed by the genomic RNA, NP, VP24, VP35, VP30, and L. EBOV VP35 and VP24 are known interferon antagonists (Basler et al., 2000; Zhang et al., 2012). VP40, the matrix protein, is the most abundant protein in the virus particle and plays a key role in the budding from infected cells (Jasenosky and Kawaoka, 2004; Noda et al., 2002).

We discovered a novel compound, HUP2976, that specifically inhibits the entry

of filoviruses into target cells. We determined the inhibitory mechanism of this drug candidate by monitoring each step of the viral entry process (attachment, internalization, and membrane fusion) using virus-like particles (VLPs) consisting of EBOV NP, VP40, and GP. HUP2976 efficiently blocked the membrane fusion in cellular endosomes, though it did not inhibit VLP binding to the NPC1 receptor, suggesting that the mechanism of action of HUP2976 was fusion inhibition independent from the GP-NPC1 engagement.

2. **Materials and Methods**

2.1. Cells and cellular assays

Human embryonic kidney (HEK) 293T, African green monkey kidney Vero E6, NPC1-knockout Vero E6 (Vero E6/NPC1-KO) (Kondoh et al., 2018), eGFP-Rab7-expressing Vero E6 (Vero E6/eGFP-Rab7) kindly provided by Dr. A. Nanbo, Nagasaki University, Japan, and human hepatoma Huh7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma) supplemented with 10% fetal calf serum (FCS) (Cell Culture Bioscience), 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Gibco). Cell viabilities were measured with the alamar blue assay (Biosource International) according to the manufacturer's instructions. Endosomal pH in Vero E6/eGFP-Rab7 was tested using LysoTracker Red DND-99 (Life Technologies).

2.2. Vesicular stomatitis virus (VSV) pseudotyped with filovirus GPs

The expression plasmids for filovirus GPs were constructed as described previously (Takada et al., 1997). Mutant GP genes were constructed by site-directed mutagenesis with KOD-Plus Neo (Toyobo). Using vesicular stomatitis virus (VSV) containing the green fluorescent protein (GFP) gene instead of the VSV glycoprotein (G)

gene (VSVΔG*-G), GP-expressing plasmids, and HEK293T cells, replication-incompetent VSVs pseudotyped with ebolavirus and marburgvirus GPs were generated as described previously (Furuyama et al., 2017; Takada et al., 1997). VSVs pseudotyped with GPs of EBOV (Mayinga), BDBV (Butalya), TAFV (Pauléoula), SUDV (Boniface), RESTV (Pennsylvania), and MARV (Angola) were preincubated with an anti-VSV G monoclonal antibody, VSV-G [N] 1-9, to abolish the background infectivity of parental VSVΔG*-G (Nakayama et al., 2011). For virus titration, 10-fold serial dilutions of pseudotyped VSVs were inoculated into confluent monolayers of Vero E6 cells cultured on 96-well plates, and infectious units (IUs) were determined 18 hr later by counting the number of GFP-expressing cells with IN Cell Analyzer (GE Healthcare).

Replication-competent recombinant VSV (rVSV-EBOV) was generated as described previously (Takada et al., 2003) and its titer was determined by a conventional plaque assay. Briefly, Vero E6 cells were seeded on 12-well plates (3.0×10^5 cells/well) and incubated in a 5% CO_2 incubator at 37˚C for 18 hr. After aspirating the medium, serial dilutions (10-fold) of rVSV-EBOV were inoculated (100 µl/well) onto the cells. After incubation in a 5% CO_2 incubator at 37˚C for 1 hr with rocking every 15 min, the inoculum was removed and cells were washed with FCS free-DMEM. Following this, they were overlaid with Eagle's minimal essential medium (MEM) (Invitrogen) containing 1.0% Bacto Agar (BD), 0.3% bovine serum albumin (BSA)(Sigma), 100 U/ml penicillin, and 0.1 mg/ml streptomycin, and then incubated in 5% CO_2 incubator at 37˚C for 48 hr. Numbers of plaques at appropriate dilutions were counted to determine plaque forming units (PFU).

2.3. Recombinant EBOV

Recombinant EBOV encoding GFP (EBOV-GFP) was generated as described previously (Ebihara et al., 2007). EBOV-GFP was inoculated onto confluent monolayers of Vero E6 cells and incubated for 1 hr. Then the inoculum was removed and a 1.2% carboxymethyl cellulose (CMC)/MEM solution was added. Following incubation for 2-3 days at 37˚C, images were captured by fluorescent microscopy. Numbers of GFP-expressing foci were counted under a microscope. All infectious work with EBOV-GFP was performed in the biosafety level-4 laboratory at the Integrated Research Facility of the Rocky Mountain Laboratories, Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, Montana, USA. All standard operating procedures were approved by the Institutional Biosafety Committee.

2.4. Screening of the compounds

A chemical compound library consisting of 9,600 compounds was provided by the Drug Development Initiative (DDI), University of Tokyo. Vero E6 cells were seeded on 96-well plates (3.0×10^4 cells/well) and incubated for 18 hr. Then equal volumes of VSVs pseudotyped with filovirus GPs (1000 IUs/ml) diluted in DMEM with 2% FCS and P/S and chemical compounds (10 μM) were mixed, and added to theVero E6 cells (100 μl/well). After 18 hr incubation in a 5% CO_2 incubator at 37˚C, GFP-expressing cells were counted using IN Cell Analyzer (GE Healthcare).

2.5. DiI assay

VLPs containing EBOV GP, VP40, and NP were purified as described previously (Furuyama et al., 2016; Nanbo et al., 2010). VLPs were labeled with a lipophilic tracer,

1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) (Invitrogen) at room temperature with gentle rocking in the dark for 1 hr. Labeled VLPs were diluted with PBS to 1 μg/ml. Vero E6/eGFP-Rab7 cells (1.5×10^5 cells/well) were cultured in 8-well chamber slides (Merck). Then they were washed with 250 μl of FCS-free DMEM and incubated with DiI-labelled VLPs on ice for 30 min. The cells were washed with FCS-free DMEM to remove unbound VLPs and incubated with 250 μl of DMEM containing HUP2976 (25 μM) for 0, 2, and 6 hr at 37°C to analyze attachment, internalization, and membrane fusion, respectively. In this assay, the fluorescent signal is enhanced once the DiI-labelled VLP envelope fuses with the endosomal membrane (Nanbo et al., 2010). To count the number of DiI-labelled VLPs, the cells were fixed with 4% paraformaldehyde for 15 min at room temperature. Then nuclei were stained with 1 μg/ml 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI) for 10 min at room temperature. Images were captured with a 63× oil objective lens using a Zeiss LSM780 inverted microscope and a ZEN 2010 software. Images of 4-20 optical sections were acquired in 0.5-1.0 μm steps to analyze the number of DiI-labeled VLPs (0 hr), the percentage of DiI-labeled VLPs that colocalized with eGFP-Rab7 (2 hr), and relative sizes and intensities of DiI dots (6 hr). Quantitative analyses were conducted with Image J software (NIH, USA).

2.6. GP-NPC1 binding assay

Vero E6/NPC1-KO cells and Vero E6/NPC1-KO cells expressing HA-tagged NPC1 (Kondoh et al., 2018) were seeded in T-75 flasks. After harvesting with trypsin, the cells were sedimented at 1,000 rpm for 5 min. CHAPS-NTE buffer (0.5% wt/vol CHAPS, 140 mM NaC1, 10 mM Tris-HC1, 1 mM EDTA; pH 7.5) (Miller et al., 2012) was added to cells to a final concentration of 10^7 cells/ml. Then, EDTA-free Complete Protease

Inhibitor Cocktail (Roche) was added. The cells were sedimented at 10,000 × g for 10 min at 4˚C and the supernatant was harvested. VLPs (1 mg/ml in PBS) were treated with thermolysin (Sigma) at 37˚C for 30 min. The VLP solution was diluted at 1:10 with 0.05 M carbonate buffer (pH 9.6). ELISA plates (Nunc, Maxisorp) were coated with the diluted VLPs, and incubated at 4˚C overnight. The VLPs were removed and the plates were blocked with BSA (10 mg/ml in PBS) and incubated at room temperature for 2 hrs. Recombinant monoclonal antibody mAb114, which blocks binding of NPC1 to GP via interaction with the glycan cap and the inner chalice of GP, was generated as a positive control based on the sequence described previously using γ1HC, and κLC vectors (Cagigi et al., 2018; Saito et al., 2019; Tiller et al., 2008). Serially diluted HUP2976 and mAb114 (in PBS) were mixed with cell lysates (diluted at 1:20 with CHAPS-NTE buffer) and incubated at room temperature for 10 min. After washing the plates with 0.05% Tween 20 in PBS (PBST), the mixture was added to each well and incubated at 4˚C overnight. After removal of the mixture, the plates were washed with PBST 3 times, and rat anti-HA antibody 3F10 (Sigma) diluted with PBST containing BSA (5 mg/ml) was added, followed by incubation at room temperature for 1 hr. After washing 3 times with PBST, horseradish peroxidase (HRP)-conjugated anti-rat IgG (H+L) (Jackson ImmunoResearch) was added to each well. After incubation at room temperature for 1 hr, the plates were extensively washed and a 3,3',5,5'-tetramethyl-benzidine (TMB) substrate (Sigma) was added, followed by incubation in the dark at room temperature for 30-60 min. The optical density (OD) value at 450 nm was measured after stopping the reaction with 1M phosphoric acid.

2.7. Selection of HUP2976 escape mutants

Tenfold serial dilutions of rVSV-EBOV (approximately 2.0×10^2 to 2.0×10^6 PFU/0.1ml in FCS free-DMEM) were mixed with equal volumes of HUP2976 (20 μM in FCS free-DMEM) and incubated at room temperature for 1 hr, then inoculated onto confluent Vero E6 cells grown in 6-well tissue culture plates. After adsorption for 1 hr, the cells were overlaid with MEM (Invitrogen) containing 1.0% Bacto Agar (BD) and 20 μM HUP2976, and then incubated in a 5% CO_2 incubator at 37˚C for 48 hrs. Mutant viruses growing in the presence of HUP2976 were purified from single isolated plaques and propagated on Vero E6 cells. Viral RNAs were extracted from the supernatant, the nucleotide sequences of the GP genes of the parent viruses and the escape mutants were determined and the amino acid sequences were compared among the viruses.

3. Results

3.1. Inhibitory effect of HUP2976 against VSVs pseudotyped with filovirus GPs

Using VSV pseudotyped with EBOV GP, a total of 9,600 compounds from the DDI of the University of Tokyo were screened for antiviral effects (i.e., entry inhibitors), and we obtained approximatey 100 compounds that inhibited the virus entry into target cells. Based on the structural simplicity, potential toxicity data, and antiviral efficacy, we selected a biaryl sulfonamide derivative. The structure-activity correlation of derivatives of this lead compound enabled us to improve the inhibitory activity and the cytotoxicity (data not shown) and, thus, we obtained a viable drug candidate, HUP2976 (Fig. 1A).

We found that HUP2976 efficiently inhibited infection by VSVs pseudotyped with filovirus GPs, but not VSV G in Vero E6 and Huh7 cells, suggesting the potential of HUP2976 to specifically inhibit the entry of filoviruses (Fig. 1B). Notably, VSV pseudotyped with EBOV or BDBV GPs was almost completely neutralized at 0.31 μM

in both Vero E6 and Huh7 cells. Among ebolaviruses, HUP2976 showed the weakest effect on RESTV GP-mediated infection. Although HUP2976 showed less efficacy against VSV pseudotyped with MARV GP , it reduced the infectivity at 5 μM in both Vero E6 and Huh7 cells (Fig. 1B). The 50% inhibitory concentrations (IC_{50}) of HUP2976 for pseudotyped VSVs using Vero E6 cells were 0.086 μM (EBOV GP), 0.270 μM (SUDV GP), 0.307 μM (TAFV GP), 0.050 μM (BDBV GP), 1.117 μM (RESTV GP), and 4.79 μM (MARV GP). Regarding Huh7 cells, IC_{50} of HUP2976 for pseudotyped VSVs were 0.010 μM (EBOV GP), 0.020 μM (SUDV GP), 0.151 μM (TAFV GP), 0.010 μM (BDBV GP), 0.280 μM (RESTV GP), and 0.570 μM (MARV GP). Significant cytotoxicity was not observed in both cell lines except the highest concentration (80 μM) for Huh7 cells. Dose-dependent inhibitory effects of HUP2976 on EBOV entry were confirmed using infectious EBOV-GFP (Fig. 2). We found that sizes of virus-infected cell foci formed in the presence of HUP2976 were markedly smaller than in control cells treated with 0.5% DMSO, and that HUP2976 significantly reduced the number of visible foci formed by EBOV-GFP (Fig. 2). The IC50 value for EBOV-GFP was around 0.1 μM, which was similar to that for VSV pseudotyped with EBOV GP in Vero E6 cells.

3.2. Inhibition of fusion between virus and endosomal membrane by HUP2976

We then investigated the inhibitory effects of HUP2976 on viral attachment (0 hr), internalization (2 hr), and membrane fusion (6 hr) using DiI-labelled VLPs consisting of EBOV NP, VP40, and GP (Fig. 3). The number of VLPs attached to the surface of Vero E6 cells in the presence of HUP2976 was not significantly different from that of untreated (i.e., DMSO-treated) cells, indicating that HUP2976 did not interfere with VLP attachment (Fig. 3A, B). Likewise, the number of VLPs colocalizing with eGFP-Rab7, a

late endosome marker, was similar for HUP2976-treated and untreated cells, suggesting that HUP2976 did not affect subsequent uptake of VLPs into cellular endosomes (Fig. 3C, D). Finally, we analyzed membrane fusion efficiency by detecting dequenched DiI fluorescence signals (Kuroda et al., 2015; Nanbo et al., 2010). In this assay, when DiI-labeled VLP envelopes fuse with the endosomal membrane, enhanced fluorescent signals are observed. We observed significantly enhanced DiI signals colocalizing with Rab7 in untreated cells, indicating that membrane fusion occurred efficiently in endosomes (Fig. 3E, F). In contrast, dequenched DiI signals were significantly reduced in the presence of HUP2976, indicating that HUP2976 prevented GP-mediated membrane fusion in endosomes (Fig. 3E, F). Using a LysoTracker marker, we examined endosomal acidification in the presence or absence of HUP2976 and found that this compound did not significantly raise the endosomal pH (Fig. 3G).

3.3. Effects of HUP2976 and mAb114 on EBOV GP-NPC1 binding

Since EBOV GP is known to bind the endosomal fusion receptor NPC1 to mediate membrane fusion, we conducted EBOV GP-NPC1 binding assays to examine whether the interaction between EBOV GP and NPC1 was inhibited by HUP2976. Consistent with a previous study (Misasi et al., 2016), the positive control antibody mAb114, which binds to the NPC1 binding region of EBOV GP, inhibited the interaction of these molecules in a dose-dependent manner (Fig. 4). In contrast, HUP2976 did not reduce the binding activity of EBOV GP to NPC1 even at the highest concentration tested (Fig. 4). These results indicated that HUP2976 did not affect the interaction between EBOV GP and NPC1.

3.4. Amino acid substitutions in the EBOV GP escape mutants

To identify amino acid residues that could potentially interact with HUP2976, escape mutants were selected using replication-competent rVSV-EBOV. We isolated 6 escape mutants, and amino acid substitutions in the GP genes were analyzed (Fig. 5A). All the GP mutants had amino acid substitutions at positions 47 (Asp-to-Gly [1/6] or Asp-to-Glu [1/6]) or 66 (Val-to-Phe [4/6]), both of which are located in the base region (amino acid positions 33-70) of the GP molecule (Fig. 5B) (King et al., 2018).

After cloning these mutant GP genes into expression vectors, VSVs pseudotyped with mutant GPs were prepared to analyze whether these amino acid substitutions affected the inhibitory efficacy of HUP2976 (Fig. 5C). We found that single substitutions at position 47 and 66 similarly reduced the sensitivity of the virus to HUP2976 as indicated by approximately 60- to 100-fold increased IC_{50} values. There was no significant difference in the extent of the resistance among the mutants with single and double substitutions, indicating that either of the mutations at position 47 or 66 was sufficient to escape from the inhibitory effect of HUP2976. However, it is worth noting that neither single nor multiple mutations in GP made the virus fully resistant to this compound. The amino acid residues at positions 47 and 66 mapped on the GP trimeric structure (PDB code: 5JQ3) revealed that the distance between Cα atoms of these two residues was 19.9 Å (in a GP monomer) in the base region of the chalice-like GP structure (Fig. 6). The distances from positions 47 to 47, from 66 to 66, and from 47 to 66 between the GP monomers were 37.8 Å, 33.3 Å, and 28.0 Å, respectively. Interestingly, D47 and V66 were located in the same cavity formed by 2 neighboring GP monomers (Fig. 6B).

4. Discussion

During the epidemic of EVD in West Africa, favipiravir (T-705), a viral RNA polymerase inhibitor approved for conditional use to treat influenza virus infections in Japan, was investigated for treatment of EVD patients (Bai et al., 2016; Sissoko et al., 2016). However, its therapeutic potency remains questionable due to the limited efficacy for patients with high EBOV titers. Other experimental drugs such as remdesivir have also been used in the latest outbreak in the DRC. All these chemical compound-based drug candidates are nucleotide analogues expected to interfere with the function of the viral RNA polymerase L. HUP2976 inhibits ebolavirus infection by blocking the entry of these viruses into cells. We found that HUP2976 had the potential to inhibit GP-mediated infection with VSV pseudotypes carrying GPs of representative members of all known human-pathogenic filovirus species. Notably, the inhibitory effects on EBOV GP- and BDBV GP-mediated infection were particularly strong. Our data suggest that HUP2976 may be a pan-filovirus therapeutic.

We found that HUP2976 inhibited fusion between the endosomal membrane and viral envelope. It has been shown that low endosomal pH leads to proteolytic processing of filovirus GPs during the transport of filovirus particles to late endosomes, and the exposed receptor binding site of the proteolytically processed GP is thought to interact with NPC1, followed by membrane fusion (Carette et al., 2011; Côté et al., 2011). Since the endosomal pH was not significantly changed in HUP2976-treated cells, it is unlikely that altered acidification conditions affected the viral infectivity. The fact that HUP2976 did not reduce the infectivity of VSVΔG*-G supports this notion. Thus, we first hypothesized that HUP2976 inhibited the binding between GP and NPC1. However, contrary to our expectation, the binding of these molecules was not blocked by HUP2976. By analyzing the GP sequences of escape mutants, we found that amino acid residues

D47 and V66 might be important for the antiviral effect of HUP2976. These amino acid residues are well conserved among ebolaviruses and located in the base region (Fig. 5D), and seem to be important to form or stabilize the trimeric structure of GP.

Recently, selective estrogen receptor modulators (SERM), painkillers, antianginals antidepressants, and antipsychotics have been reported to have potential as fusion inhibitors against ebolaviruses (Johansen et al., 2015; Shaikh et al., 2019; Zhao et al., 2018, 2016). While clomiphene, a SERM, blocks filovirus entry indirectly by affecting the function of NPC1 (Shoemaker et al., 2013), toremifene is thought to bind the GP molecule directly, to decrease its stability, and thus to prevent the fusion between the viral envelope and endosomal membrane. Interestingly, drugs such as toremifene, bepridil, paroxetine and sertraline seem to interact with V66 in the cavity on the GP surface (Fig. 6) (Ren et al., 2018; Zhao et al., 2016). It is conceivable that the interactions of inhibitors with the GP base involving V66 may affect the structural flexibility or stability of the GP molecule, resulting in reduced membrane fusion activity (Ren et al., 2018; Shaikh et al., 2019). However other possible mechanisms caused by HUP2976 (e.g., cholesterol accumulation in endosomes and inhibitory effects on proteolytic cleavage of GP) are not ruled out.

Although direct evidence needs to be provided, escape mutations of amino acids D47 and V66 suggest that HUP2976 may interact with this cavity like other previously found fusion inhibitors such as toremifene, bepridil, paroxetine and sertraline. However, the amino acid comparison among filovirus GPs (Fig. 5D) suggests that D47 and V66 are not the only amino acids that are involved in the interaction between HUP2976 and GP. EBOV, BDBV, and TAFV GPs are identical at these positions but EBOV and BDBV GPs are more sensitive to HUP2976 than TAFV GP, and RESTV and SUDV GPs having E47

and V66 displayed differential sensitivities to HUP2976. Detailed molecular mechanisms of HUP2976-mediated fusion inhibition, including identification of the amino acids responsible for the HUP2976-GP interaction, need to be clarified in future studies.

Although many entry inhibitors against EBOV such as ion channel blockers (e.g., amiodarone and bepridil), antimicrobials (e.g., amodiaquine and teicoplanin), psychoactive drugs (e.g., benztropine and imipramine), and protein kinase inhibitors (e.g., erlotinib and sunitinib) have been reported, none of them have been approved by the the United States Food and Drug Administration for the treatment of EVD (Salata et al., 2019). It has been reported that most of these drug candidates inhibit ebolavirus infection by affecting host intracellular factors such as NPC1, host protease cathepsin, calcium signaling required for endosomal fusion, and so on. It should be considered that such compounds that affect cellular functions may have the potential to cause detrimental effects in clinical use. Although further studies are required, candidate compounds that interfere with the GP function by direct interactions with the GP molecule may be promising candidates for the development of EVD drugs in the future.

Other viral proteins are also useful targets to develop EVD therapeutics. For example, NP, VP35, and VP24 play key roles in the life cycle of ebolaviruses by mediating nucleocapsid transport (Takamatsu et al., 2018). Inhibition of the functions of these proteins may reduce the replication efficiency of filoviruses. Indeed, it was reported that siRNA therapeutics targeting VP24 and VP35 might be one of the options as indicated by their efficacy in nonhuman primate models (Geisbert et al., 2010; Warren et al., 2010). Interactions between VP40 and other host proteins may also be an interesting target. Further studies on drug design focusing on therapeutic agents that directly inhibit viral protein functions and/or interfere with viral-host protein interaction are needed.

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Figure legends

Fig. 1. HUP2976 and its inhibitory activity against VSVs pseudotyped with filovirus GPs. (A) Chemical structure of HUP2976 (MW: 470.6). (B) Pseudotyped VSVs (1,000 IU/ml) were mixed with equal volumes of HUP2976 and inoculated onto Vero E6 and Huh7 cells in 96-well plates. The cells were incubated at 37˚C for 18 hr and the numbers of GFP-expressing cells were counted using IN Cell Analyzer. One experiment performed in triplicate is shown; averages and standard deviations are presented. (C) Vero E6 and Huh7 cells were incubated with the indicated concentrations of HUP2976 or 0.8% DMSO. Cell viabilities were measured after 24 hour-incubation.

Fig. 2. Inhibitory activity of HUP2976 against EBOV-GFP. EBOV-GFP was diluted to infect Vero E6 cells at high (0.5-1.0) and low (0.05-0.1) multiplicities of infection. Following infection, the cells were incubated with the indicated concentrations of HUP2976 for 2 days and fluorescent images were captured (A). EBOV-GFP was diluted (200 focus forming units/well) and inoculated to Vero E6 cells on 96-well plates. Then, the cells were incubated as described in Materials and Methods. The numbers of GFP-expressing foci were counted from triplicate wells (low moi samples) and averages and standard deviations are shown (B). Statistical analysis was performed using Student's *t*-test (*$p < 0.05$).

Fig. 3. Membrane fusion inhibition by HUP2976. DiI-labelled VLPs (red) were inoculated into confluent Vero E6 cells expressing eGFP-Rab7 (green) and incubated for 30 min on ice. After adsorption, the cells were incubated for 0 (A,B), 2 (C,D), or 6 hrs

(E,F) at 37˚C in the presence of HUP2976 (25 μM) or 0.25% DMSO. The cells were fixed with 4% paraformaldehyde and nuclei were stained with DAPI (blue). DiI signals on the cell surface and in the cytoplasm were monitored by confocal laser scanning microscopy (A,C,E). Scale bars represent 10 μm. (B,D,F) Three microscopic fields were acquired randomly, and the number of DiI-labeled virions was measured in approximately 50 individual cells (B,D). Percentage of colocalization (D) and size (B,F) and fluorescence intensity (F) of DiI dots were measured in approximately 50 individual cells and quantified using Image J software. Averages and standard deviations of three independent experiments are shown (B,D,F). Statistical analysis was performed using Student's *t*-test (*$p < 0.05$). (G) Vero E6/eGFP-Rab7 cells were incubated with HUP2976 (25 μM), NH_4Cl (25 mM), or DMSO (0.25%) and stained with LysoTracker Red DND-99. Acidic endosomes are viualized in red.

Fig. 4. Effect of HUP2976 on the GP-NPC1 interaction. ELISA plates were coated with thermolysin-treated VLPs, followed by incubation with HUP2976 or mAb114, HA-tagged NPC1 or mock cell lysates, a rat anti-HA antibody, and HRP-conjugated anti-rat IgG (H+L). The reaction was visualized with the TMB substrate. The OD values of mock cell lysates were subtracted from those of HUP2976- or mAb114-treated lysates at each concentration. The experiment was performed in triplicate and averages and standard deviations are shown .

Fig. 5. Identification of amino acid substitutions allowing escape in EBOV GP. (A) The primary structure of GP and amino acid sequences at positions 45-70 are shown. The primary GP structure contains the base, glycan cap, mucin-like domain (MLD), internal

fusion loop (IFL), and transmembrane region (TM) and cytoplasmic tail (CT). Amino acid substitutions found in the EBOV GP escape mutants selected under HUP2976 pressure are shown in red. (B) The trimeric structure of EBOV GP (PDB code: 6G95) was constructed using PyMOL 1.2r3pre (Schrödinger) and the colored corresponding sequence map above. (C) VSVs pseudotyped with wildtype and mutant EBOV GPs were diluted to 1,000 IU/ml, mixed with equal volumes of HUP2976, and inoculated onto Vero E6 cells. The cells were incubated at 37˚C for 18 hr, and GFP-expressing cells were counted with IN Cell Analyzer. Averages and standard deviations from 3 independent experiments are shown. (D) Amino acid sequences at positions 45-70 (EBOV numbering) of ebolavirus and marburgvirus GPs. Amino acid residues conserved among all of the filoviruses and those conserved among all of the ebolaviruses but not marburgvirus are highlighted in light blue and pink, respectively.

Fig. 6. Substituted amino acid residues mapped on the GP trimeric structure. The amino acid residues at positions 47 ad 66 are mapped on a ribbon model (A) and surface model (B) of the EBOV GP trimeric structure constructed using PyMOL 1.2r3pre (Schrödinger) based on the crystal structure (PDB code: 6G95). A close-up of the EBOV GP inhibitor-binding pocket in a surface representation in the solid black square. GP1 and GP2 are shown in black and orange in a GP monomer and in gray and yellow in another monomer, respectively. Green and red spheres represent D47 and V66 residues, respectively.