Candidate medical countermeasures targeting Ebola virus cell entry

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Abstract

Medical countermeasures against virus infections ideally prevent the adsorption or entry of virions into target cells, thereby circumventing infection. Recent significant advances in elucidating the mechanism of Ebola virus (EBOV) host-cell penetration include the involvement of two-pore channels at the early stage of entry, and identification of cellular proteases for EBOV spike glycoprotein maturation and the intracellular EBOV receptor, NPC1. This improved understanding of the initial steps of EBOV infection is now increasingly applied to rapid development of candidate medical countermeasures, some of which have already entered the clinic. In this review, we summarize the currently known spectrum of EBOV cell entry inhibitors, describe their mechanism of action, and their potential for future development.

Background

Ebola virus (EBOV) is one of four members of the mononegaviral family *Filoviridae* that causes Ebola virus disease (EVD). EVD is a human viral hemorrhagic fever with an extremely high case-fatality rate (mean ≈42%) [1]. In the past, EVD outbreaks have been locally confined to small areas in Middle and Eastern Africa, encompassing maximally a few hundred cases (1,403 cases total since the discovery of EBOV in 1976 to 2013) [1]. Consequently, EBOV had the status of an "exotic" pathogen, and only limited resources were allocated to perform EBOV research. Such research requires performance in maximum containment (BSL-4) facilities.

From 2013 to 2016, EBOV caused an extraordinary EVD outbreak in Western Africa that involved 28,646 human infections and 11,323 deaths [2]. Developing medical countermeasures (MCMs) against EVD has since become high priority for the World Health Organization and large national medical institutions such as the National Institutes of Health in the US. Large emergency funds have been made available to maximum containment facilities and their collaborators to identify strategies to prevent and contain EVD [3, 4]. These strategies include the development of candidate vaccines (including post-exposure prophylactic vaccines) [5] and candidate antivirals, including EBOV-specific antibodies and small molecules [6]. Here, we focus on one aspect of candidate antiviral research, i.e., EBOV cell-entry inhibitors.

Ebola virus

EBOV has a linear, nonsegmented, monopartite, single-stranded RNA genome of negative polarity that encodes seven structural and at least three nonstructural proteins [7-10]. The structural proteins nucleoprotein (NP), phosphoprotein (VP35), matrix protein (VP40), surface glycoprotein ($GP_{1,2}$), transcriptional cofactor (VP30), secondary matrix protein (VP24), and

RNA-dependent RNA polymerase (L) assemble into enveloped particles and are necessary and sufficient for genome replication, gene transcription, and virion formation [11, 12]. The three nonstructural proteins sGP, ssGP, and Δ-peptide are secreted in high amounts from EBOV-infected cells [8-10], but their function in the EBOV lifecycle is still rather unclear.

The only viral protein protruding from the Ebola virion envelope is $GP_{1,2}$. Consequently, $GP_{1,2}$ is the primary target of host antibodies. Functionally, $GP_{1,2}$ alone mediates virion adsorption to host-cell surfaces, receptor binding, fusion of the virion envelope with host-cell membranes, and release of the viral ribonucleocapsid into the host-cell cytosol [13]. MCMs aiming at disrupting the EBOV cell-entry process therefore all target either $GP_{1,2}$ directly or its direct or indirect interactors.

$GP_{1,2}$ is produced via co-transcriptional editing of the mRNA derived from the fourth EBOV gene (*GP*) [8, 9]. The contiguous mRNA is translated into a typical preproprotein (pre-GP), which after signal peptide cleavage is proteolytically processed by furin into GP_1 and GP_2 subunits. GP_1 and GP_2 subunits remain attached to each other as disulfide-bridged heterodimers [14]. These dimers homotrimerize to form typical class I fusion machines that are transported to the cell membrane and from there are incorporated into budding EBOV particles. GP_1 contains the receptor-binding site [15, 16], whereas GP_2 contains a hydrophobic fusion peptide at the N-terminus followed by N- and C-terminal heptad repeats (NHR and CHR, respectively) and a transmembrane domain that anchors the $GP_{1,2}$ trimer to the membrane [16-19].

Ebola virion host-cell entry

The initiation of EBOV host-cell entry is incompletely understood. It is currently hypothesized that $GP_{1,2}$ interacts with host-cell surface attachment factors, such as integrins [20], lectins [21-

23], T-cell immunoglobulin mucin domain protein 1 and/or 4 (TIM-1/4) [24-27], or Tyro3 receptor tyrosine kinase family members such as Axl, DtK, and/or Mer (Figure 1) [28]. These interactions then initiate virion internalization into endosomes through a process that shares features with macropinocytosis [29-32]. As the hijacked endosome matures into the late endosome, its acidic environment triggers cellular cysteine proteases, cathepsin B and cathepsin L, to proteolytically process $GP_{1,2}$ [33, 34]. This processing results in the removal of a large portion of the GP_1 subunit (the protective "glycan cap"), resulting in a much smaller ("19-kD") $GP_{1,2}$ trimer with an exposed receptor-binding site [33, 35, 36]. This trimer subsequently interacts with Niemann-Pick disease, type C1 (NPC1), a multi-spanning membrane protein normally involved in cholesterol trafficking [37, 38]. The interaction between 19-kD $GP_{1,2}$ and NPC1 is necessary, but not sufficient, to trigger GP_2-mediated fusion of the virion envelope and the endosomal membrane, indicating the existence of an additional, unidentified cellular fusion trigger factor [37, 39-42]. Two-pore channels (TPCs) 1 and 2 have been implicated as cellular factors that are needed to initiate viral membrane fusion [43, 44]. TPCs play critical roles in endocytic trafficking [45], and therefore most likely inhibit virion delivery to late endosomes or endolysosomes.

By analogy to other class I viral membrane fusion machines, fusion triggering is proposed to involve release of the GP_2 fusion loops from their interactions with the body of the $GP_{1,2}$ trimer, and their insertion into the target endosomal membrane [46]. Further inferred conformational changes in GP_2 lead to the formation of a six-helix bundle (6HB) comprising three NHR and three CHR sequences. 6HB formation is proposed to drive the formation and expansion of fusion pores that is a prerequisite for cytoplasmic escape of the viral ribonuceoprotein (RNP) core [46]. The acidic environment of late endosomes plays a role in

GP_2-mediated fusion, triggering conformational changes in the fusion loops associated with target membrane insertion and the formation and/or stabilization of the 6HB [47-49]. Recent live-cell imaging studies delineate late endosomes and/or hybrid endolysosomes as the compartments where ebolaviral membrane fusion takes place [40, 41].

Ebola virus cell-entry inhibitors

Candidate MCMs, which include antibodies, small molecules, and peptides, can target various stages of the described EBOV cell-entry pathway. The majority of promising MCMs interrupt virion attachment to cell-surface attachment factors, the intracellular EBOV receptor NPC1, proteolytic processing of $GP_{1,2}$, or fusion of the viral envelope with the endosomal membrane. These therapeutic agents can be designed to act directly against the Ebola virion (e.g., antibody cocktails targeting the virion surface $GP_{1,2}$) or to act indirectly by targeting the host cell (e.g., prevent acidification of endosomes, inhibit cathepsins, block NPC1 receptor). Over the last decade, EBOV surrogate systems have been developed to isolate the EBOV cell-entry step without the need of biosafety level 4 (BSL-4) facilities. These systems include Ebola virion-like particles (VLPs), transcriptionally active Ebola virion-like particles (trVLPs), vesiculoviral or retroviral pseudotypes carrying EBOV $GP_{1,2}$, and recombinant rhabdoviruses expressing EBOV $GP_{1,2}$. Through the incorporation of reporter genes such as enhanced green fluorescent protein (eGFP) or luciferase, these surrogate systems were used in high-throughput screens for EBOV cell-entry inhibitors in biosafety level 2 (BSL-2) environments [50-55]. For instance, a recent high-throughput screen of 319,855 small molecules from the NIH Molecular Libraries Small Molecule Repository (MLSMR) library identified nine novel compounds that prevented EBOV infection *in vitro* by blocking either virion-cell surface attachment, macropinocytosis-mediated virion uptake, or endosomal trafficking [52].

To accelerate identification of anti-EBOV MCMs and their introduction into the clinic, several groups focused on screening compounds that have already been approved by the US Federal Drug Administration (FDA) for treatment of other human diseases [56-59]. For instance, Kouznetsova *et al*. recently identified 53 FDA-approved compounds that can inhibit entry of Ebola VLPs, including microtubule inhibitors (e.g., colchicine, nocodazole, vincristine), estrogen receptor modulators (e.g., raloxifene, tamoxifene, toremiphene), antihistamines (e.g., clemastine, maprotiline), antipsychotics/antidepressants (e.g., clomipramine, trifluoperazine), ion channel antagonists (e.g., digoxin, propafenone), and anticancer agents/antibiotics (e.g. azithromycin, clarithromycin) [56].

Johansen *et al*. identified 171 different anti-EBOV compounds in a high-throughput screen, 80 of which are FDA-approved with significant activity against Ebola VLPs. Two therapeutics, sertraline, a selective serotonin reuptake inhibitor, and bepridil, a calcium channel blocker, inhibited EBOV cell entry *in vitro* and *in vivo* [58]. C57Bl/6 laboratory mice injected with mouse-adapted EBOV and treated twice daily, starting one hour after inoculation, with either sertraline or bepridil (10 mg/kg and 12 mg/kg, respectively) for 10 days had a significant survival benefit (70% and 100% survival rate, respectively) compared to mice treated with a vehicle control [58]. The exact mechanism of action against EBOV of most of these compounds remains to be determined.

Virion-targeting antibodies

The possibility of using EBOV-neutralizing anti-$GP_{1,2}$ antibodies as possible therapeutic agents was first examined during an EVD outbreak in Zaire (today Democratic Republic of the Congo) in 1995. Of the eight patients with EVD treated with convalescent plasma from EVD survivors,

seven survived [60]. However, whether convalescent plasma directly led to recovery could never be determined [61] because the treated individuals also received supportive treatment.

The use of serum-based therapeutics is fraught with challenges such as possible transmission of blood-borne pathogens or graft-versus-host disease. Moreover, EVD convalescent plasma and serum may enhance EBOV infectivity at least *in vitro* [62]. Moreover, not all EVD survivors mount a strong neutralizing antibody response, necessitating the pre-screening and standardization of convalescent plasma batches prior to transfusion. To avoid these potential complications, researchers have focused their attention on developing therapies that use highly purified antibodies targeting neutralizing epitopes on EBOV $GP_{1,2}$.

The first purified anti-EBOV antibody to be extensively studied *in vitro* and *in vivo* was KZ52, which was isolated from a human survivor of the 1995 EVD outbreak [63-65]. KZ52 was found to bind the GP_1-GP_2 interface, rather than as expected to the more exposed surface of the $GP_{1,2}$ trimer [16]. Importantly, KZ52 protected guinea pigs (*Cavia porcellus*) from death after inoculation with guinea pig-adapted EBOV [64], but failed to have a beneficial effect on EBOV-exposed rhesus monkeys (*Macaca mulatta*) [65].

One promising monoclonal antibody, mAb114 isolated from a human survivor of EVD binds to an epitope that spans the EBOV GP_1 glycan cap and the GP_1 core. Importantly, mAb114 remains bound to $GP_{1,2}$ after cathepsin cleavage and inhibits binding of proteolytically cleaved $GP_{1,2}$ to NPC1 [66]. mAb114 protected rhesus monkeys when administered at 50 mg/kg starting 1 day or 5 days after EBOV injection, followed by two additional mAb doses at 24-hour intervals [67]. Another monoclonal antibody, FVM04, also showed promise. FVM04 binds to a surface-exposed portion of the EBOV GP_1 receptor-binding site, thereby blocking the interaction

between GP_1 and NPC1 [68]. FVM04 protected laboratory mice and guinea pigs infected with rodent-adapted EBOV or its antigenically distant relative, Sudan virus (SUDV) [68]. Identification of ebolavirus cross-neutralizing antibodies that are efficacious as antiviral agents in different animal models has been challenging due to the divergence of $GP_{1,2}$ between EBOV and related ebolaviruses. One reason may be that, during infection, conserved $GP_{1,2}$ epitopes may not be immunodominant. The host immune response may be monopolized by species-restricted epitopes [69, 70]. A second potential reason is the shielding of highly conserved viral epitopes, such as the receptor-binding site, in extracellular virions, with their exposure only occurring in endosomal compartments not accessible to antibodies [37, 71].

Recently, multiple research groups have isolated monoclonal antibodies that are cross-reactive for distinct ebolaviruses, and have some degree of cross-neutralization and cross-protection [68-70, 72-75]. However, such 'natural' cross-reactive antibodies are rare, and cross-neutralizing antibodies are rarer still. One potential solution to this problem is the recent development of bispecific antibodies that combine the specificities of mAbs recognizing a highly conserved (but non-neutralizing) surface-exposed $GP_{1,2}$ epitope and either the highly conserved (but inaccessible) GP_1 receptor-binding site or the endosomal NPC1 receptor [71]. Such "Trojan horse" bispecific antibodies could hitch a ride into endosomes with virions, where they could then engage the newly exposed GP_1 receptor-binding site or NPC1. Both available bispecific antibodies neutralized all five known ebolaviruses (including EBOV), and one provided post-exposure protection in mice against otherwise lethal exposure to EBOV or SUDV [71].

While antibody monotherapy may provide a simplified therapeutic strategy to treat EVD, an antibody cocktail therapy may be more potent and less brittle to viral neutralization escape. Potent GP_1-GP_2 interface-binding antibodies are therefore developed that work additively or

synergistically with GP_1 surface-binding antibodies. A direct result of this line of thinking was ZMab, the earliest anti-EBOV antibody cocktail. ZMab consists of three different murine antibodies (2G4, 4G7, and 1H3) that bind to three major $GP_{1,2}$ epitopes: the GP_1-GP_2 interface, the GP_1 glycan cap, and the GP_1 mucin-like domain [76]. Administered to crab-eating macaques (*Macaca fascicularis*) at a dosage of 25 mg/kg at 24 hours after EBOV exposure, ZMab provided 100% protection from disease or death. At 48 hours after EBOV inoculation, 50% of treated primates survived infection when ZMab [77]. These results indicated that ZMab could at the very least be developed as post-exposure therapy for laboratory workers.

Additional antibody cocktails have recently been developed and tested *in vivo* using nonhuman primates (NHPs) [68, 78, 79]. ZMapp consists of three antibodies, c13C6 from a previously developed cocktail called MB-003 [80] and 2G4 and 4G7 from ZMab [76]. All three monoclonal antibodies recognize conformational epitopes within the stem region of the $GP_{1,2}$ trimer or on GP_2. Administration of intravenous ZMapp at a dose of 50 mg/kg into EBOV-infected rhesus monkeys with detectable viremia (by qRT-PCR) at 5 days post-inoculation resulted in virus clearance at 21 days post-inoculation and animal survival [78]. ZMapp rose to prominence when it was incorporated into treatment given to aid workers during the 2013–2016 EVD outbreak in Western Africa. Two EBOV-infected healthcare providers were given three courses of ZMapp at a dose of 50 mg/kg each, three days apart. Both people fully recovered within 20 days of the initial ZMapp treatment. However, as the healthcare providers also received intensive fluid and electrolyte replacement therapy, their survival could not be attributed to ZMapp-therapy alone [81]. Phase I/II clinical trials of ZMapp were launched in early 2015 with two goals: (1) to assess the safety and pharmacokinetics of a single ZMapp dose of 50 mg/kg in healthy adult volunteers (NCT02389192) and (2) to evaluate the clinical and

antiviral effects of ZMapp treatment with standard-of-care (SOC) compared to SOC alone in patients who have been confirmed to be infected with EBOV in Guinea, Liberia, Sierra Leone, and the United States (NCT02363322).[82] ZMapp plus current SOC (e.g., replacement IV fluids, antiemetics, gastric acid inhibitors, antibiotics, antimalarials, antipyretics) were beneficial, but results with ZMapp alone did not meet threshold of superiority over supportive care alone. The estimated primary completion date for these trials is May 2017 and December 2016, respectively.

While no EBOV escape variants have been identified from EBOV-infected NHPs or human EVD patients that have received ZMapp, EBOV escape variants were detected in NHPs treated with the MB-003 antibody cocktail [83]. These findings are a reminder that even antibody cocktails may not prove to be the ultimate countermeasure against EBOV. Antibody-treated patients should be monitored for the emergence of mutations in the EBOV *GP* gene open reading frames that could lead to resistance to therapy. On the other hand, two of the three mAbs in ZMapp (2G4 and 4G7) target the same $GP_{1,2}$ epitope and EBOV could therefore escape from both via the same mutation (Q508R). Developers of next-generation mAb cocktails will therefore limit the possibility of virus escape by choosing antibodies all targeting separate epitopes.

Virion-targeting small molecule inhibitors

EBOV $GP_{1,2}$ is a highly *N*- and *O*-glycosylated glycoprotein. Consequently, glycan-binding molecules (lectins) have been pursued as potential steric disrupters of the $GP_{1,2}$ interaction with cell-surface attachment factors. For instance, a chimeric L-ficolin/mannose-binding lectin (MBL)

molecule, which binds *N*-glycans, inhibits EBOV infection *in vitro* [84], and MBL alone can protect laboratory mice from otherwise fatal infection with mouse-adapted EBOV [85].

A high throughput screen using HIV-1 pseudotypes carrying EBOV $GP_{1,2}$ has identified a benzodiazepine derivative termed Compound 7 as an effective transduction inhibitor. Compound 7 also inhibits entry of infectious EBOV with a 50% inhibitory concentration of 10 μM. Computational modeling and mutational analysis indicate that Compound 7 binds to a hydrophobic pocket at the GP_1/GP_2 interface in a prefusion conformation [86].

Salata *et al*. demonstrated that amiodarone, a multi-ion channel inhibitor that is used to treat irregular heart rhythm, inhibits cell transduction with vesiculoviral pseudotypes carrying EBOV $GP_{1,2}$ *in vitro*. When the pseudotypes were treated with thermolysin, an enzyme that can processes $GP_{1,2}$ into the 19 kDa fusogenic form, prior to exposure to amiodarone-treated cells, transduction was rescued. Amiodarone was also shown not to modify the total cell content of cathepsins B and L. These results suggest that amiodarone functions by either disrupting the processing of $GP_{1,2}$ into the 19 kDa fusogenic form or that it prevents trafficking of the virion to NPC1-positive cellular compartments [87]. Amiodarone and the related dronedarone were also shown to inhibit infectious EBOV entry in cell culture [88].

Another promising small molecule is LJ001 that functions by intercalating into the viral membrane of enveloped virions, thereby preventing virion-cell fusion. The survival rate of laboratory mice exposed to mouse-adapted EBOV pretreated with LJ001 was 80%, whereas mice that received an inactive version of LJ001 or a control vehicle did not survive. However, LJ001 was not efficacious as a post-exposure therapeutic. LJ001 could be developed as an effective therapeutic if the formulation potency and/or pharmacokinetic properties can be

improved [89]. Another membrane intercalator that inhibits EBOV infection *in vitro* is teicoplanin [90], and arbidol, which also is highly effective against EBOV, may work in a similar manner [91].

Finally, C-peptide inhibitors, synthetic peptides that are modeled to interact with specific domains of a targeted fusion protein, have been generated to counter EBOV entry. These C-peptides inhibit Ebola virion-host cell membrane fusion by binding to an NHR region of GP_2, thereby preventing the NHR and CHR interaction and arresting the GP_2 conformational switch to the 6HB. A modified C-peptide inhibitor, conjugated to an arginine-rich domain of endosome-targeting HIV-1 Tat ("Tat-Ebo") reduced the number of EBOV-infected cells by greater than 90% after 48 hours post-inoculation [92].

Host cell-targeting antiviral agents and small molecule inhibitors

Not much progress has been made with host cell-targeting strategies aimed at preventing EBOV particle adsorption. This failure is likely due to EBOV particles binding to multiple, highly diverse attachment factors [20-28]. However, proof-of-concept studies demonstrated that by targeting individual attachment factors, EBOV infection can indeed be curtailed. For instance, giant globular multivalent glycofullerenes, such as compounds 17a and 17c, successfully prevent the EBOV particle interaction with dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) [93].

Interrupting the cellular endocytotic pathway is a promising avenue to counter EBOV infection because EBOV is critically dependent on endocytosis to gain entry into host cells. Tyrosine kinase inhibitors such as genistein and tyrphostin AG1478, which are known disrupters of endocytosis, indeed inhibit EBOV infection *in vitro* [94]. Likewise, molecules that prevent

acidification or lower the pH of the endosome (e.g., chloroquine, esomeprazole, omeprazole) have shown promise in cell culture assays [57, 95].

Host-cell endosomal cysteine proteases, which process EBOV $GP_{1,2}$ into the fusogenic form in late endosomal compartments, are also tempting cellular targets for interruption of EBOV infection. The cathepsin L inhibitor K11777 and synthesized vinylsulfone analogs indeed inhibit transduction of target cells by EBOV $GP_{1,2}$-pseudotyped vesiculoviruses *in vitro* [96]. The pharmacokinetic profile of K11777 in rodents, dogs, and nonhuman primates is suggestive of its safety in humans [97]. In addition, the cysteine-serine protease inhibitor leupeptin [34, 98], broad-spectrum cysteine protease inhibitors (e.g., E64c[98], E-64d [33] and E-64 [99]), cathepsin B inhibitors CA-074 [33, 98], CA-074Me [34, 98], cathepsin B downregulator nafamostat mesilate [100], cathepsin L inhibitors (e.g., cathepsin L inhibitor III [98], Z-FY(tBu)-DMK [34], CID 23631927 [101], 5705213 [102], 7402683 [102]), cathepsin B/L inhibitor FY-DMK [33], and broad-spectrum cathepsin inhibitors, R11Et, R11P, R7Et, and R23Et [103] inhibit both cell transduction with $GP_{1,2}$-carrying pseudotypes and infection with wild-type EBOV. However, evaluation of these compounds in appropriate *in vivo* models of EVD has not yet been reported.

By targeting the function of the intracellular filovirus receptor NPC1, the EBOV GP_1-NPC1 interaction is blocked. The small molecules 3.0 [104], 3.47 [104, 105], imipramine [38, 106], MBX2254 [54], MBX2270 [54], Ro47-8071 [107], and U18666A [107, 108] all target NPC1 and reduce EBOV infectivity *in vitro*. While the mechanism of action of U18666A is not completely understood, a high concentration of U18666A is hypothesized to interact with low affinity with domain C of NPC1, the site at which EBOV GP_1 binds to NPC1 [108]. Basu *et al.* hypothesized that MBX2254 and MBX2270 function in a manner similar to U18666A [54].

Selective estrogen receptor modulators (i.e., clomiphene, raloxifene, tamoxifene, toremifene) also inhibit EBOV cell entry *in vitro* and *in vivo* [56, 59, 107]. C57Bl/6 laboratory mice exposed to mouse-adapted EBOV and given clomiphene or toremifene (60 mg/kg) one hour later had survival rates of 90% and 50%, respectively [59]. Both compounds induce cholesterol accumulation in endosomes, similar as in Niemann-Pick disease. However, neither compound disrupted the EBOV GP_1/NPC1 interaction, pointing towards a novel mechanism to inhibit the EBOV cell entry pathway [107]. Recent evidence indicates that toremifene binds to a cavity between the GP_1 and GP_2 subunits, inducing a conformational rearrangement. This rearrangement might result in the premature release of the GP_2 subunit and conversion to a post-fusion conformation that prevents fusion of the virion envelope with the endosomal membrane [109].

TPCs, which are cation-selective ion channels, have also been implicated in promoting EBOV cell entry. Disruption of calcium signaling pathways and TPCs *in vitro* and *in vivo* with calcium signaling therapeutic agents such as verapamil, siRNAs RNAs, or small-molecule inhibitors such as tetrandrine, significantly inhibit EBOV infection [43, 88]. Tetrandrine prevented EBOV infection of human monocyte-derived macrophages *in vitro*. Furthermore, 50% of BALB/c laboratory mice treated with tetrandrine (90 mg/kg) starting one day after exposure to mouse-adapted EBOV survived [43].

Conclusion and future perspective

Inhibition of EBOV cell entry can be accomplished in a multitude of manners: disruption of the virion interaction with the target cell; blocking the processing of $GP_{1,2}$ by host proteases; prevention of the GP_1 interaction with EBOV attachment factors or the endosomal receptor

NPC1; and prevention of virus-host cell membrane fusion. In this paper, we reviewed several distinct candidate therapeutics targeting EBOV cell entry that have been identified through *in vitro* and *in vivo* studies.

Unfortunately, most discussed candidate therapeutics (with the notable exception of antibodies), have shown to be efficacious in *in vitro* or in rodent studies. As rodent models of EVD do not fully capture the extent of manifestations observed in patients with EVD (e.g., coagulopathy, immune responses), additional studies in NHPs should be performed. Some of the compounds were efficacious in rodents but not in the more stringent nonhuman primate models of EVD. Additionally, the majority of rodent and the few nonhuman primate studies that have been performed have administered the initial dose of candidate therapeutic relatively shortly after virus exposure. Since EBOV-infected people may not receive treatment until after developing clinical signs of EVD or until they test positive for EBOV infection, future studies should examine the efficacy of anti-cell entry drugs at much later time points of infection. Currently, only ZMapp has been shown to be efficacious in nonhuman primates after EBOV RNA was detected in serum.

While all candidate therapeutics discussed here target the EBOV cell entry pathway, the exact mechanisms of action of many compounds have yet to be determined. Some promising anti-cell-entry compounds, such as the potassium channel inhibitor noricumazole A [110] or G protein-coupled receptor antagonists [111], appear to be cell entry inhibitors as well, but their mechanism of action is unclear. Additional studies evaluating molecular mechanisms of action are needed to fully characterize these compounds and determine therapeutic potential.

In vitro and *in vivo* studies evaluating potential additive or even synergistic effects of multiple compounds with different mechanisms of action have not been published thus far. However, most EVD patients with access to modern healthcare will be treated following multiple successive or parallel therapeutic avenues based on changing clinical parameters. Performing studies with multiple compounds, possibly with the aim of developing synergistic drug cocktails, should therefore become a priority if they can be performed in a statistically significant and reproducible manner.

Although understanding of EBOV cell entry has increased substantially in recent years, a number of questions remain. For example, several cell surface attachment factors have been identified, but their roles in uptake of EBOV have not been fully defined. The interaction between $GP_{1,2}$ and NPC1 is required for viral fusion and release of viral genome into the cytoplasm, but additional cellular fusion trigger factor(s) have not been fully elucidated. As steps in the EBOV cell entry pathway become more defined, the identification of targets of inhibitors will become more precise. When the mechanisms of action of the compounds described here with anti-EBOV activity are more fully characterized, then analogs with greater target specificity and potency than the original parent compound can be designed and tested.

Figure 1. Ebola virion cell entry. The Ebola virion initiates cell entry by binding to various cell-surface attachment factor, thereby inducing macropinocytosis (1). As the endosome matures, the environment turns acidic, thereby activating cysteine proteases (cathepsins) that proteolytically process EBOV $GP_{1,2}$ (2). The processed $GP_{1,2}$ subsequently interacts with the endosomal filovirus receptor NPC1 (3), triggering fusion of the virion envelope with the endosomal membrane and the release of the Ebola viral ribonucleocapsid into the cytoplasm (4).

EXECUTIVE SUMMARY

EBOV particle attachment to cells and internalization

- EBOV glycoprotein ($GP_{1,2}$) trimers on virions attach to host-cells via attachment factors.
- Virions internalizes into endosomes through a macropinocytosis-like process.
- Acidic environment in endosomes triggers cysteine proteases to remove the glycan cap of the GP_1 subunit.
- Trimmed $GP_{1,2}$ trimers bind to endosomal NPC1.
- GP_2-mediated fusion to endosome occurs after NPC1 binding.

Entry inhibitors as antiviral agents

- Sertraline and bepridil inhibit EBOV particle cell entry and protect laboratory mice from lethal disease.

Monoclonal antibodies

- Certain antibodies block surface-exposed $GP_{1,2}$ epitopes or the internal GP_1-NPC1 receptor-binding site.
- ZMapp, a monoclonal antibody cocktail, plus supportive care were beneficial in clinical phase I-II trials, but ZMapp alone was not superior over supportive care alone.

Host-cell targets

- Disruption of endosomal calcium channels with tetrandrine increases survival of EBOV-infected laboratory mice.
- Toremifene and LJ001 increases survival of infected laboratory mice by preventing fusion to endosomes.

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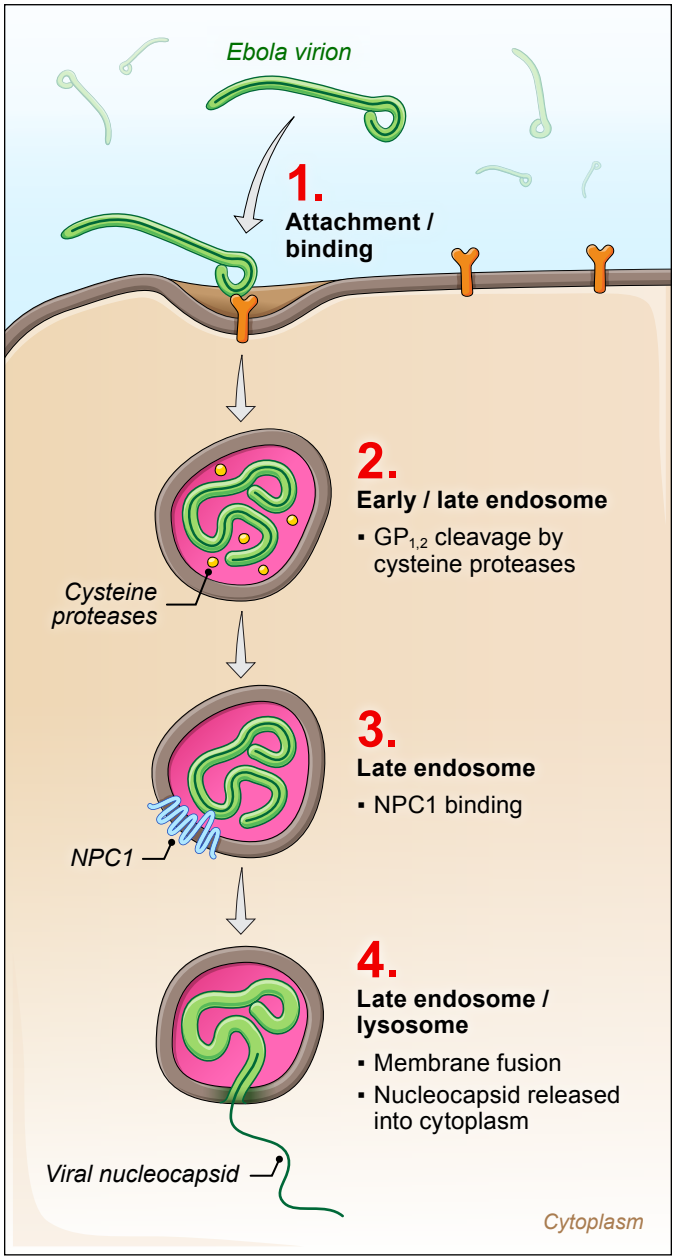
Figure 1


Ebola virion
1.
Attachment / binding
2.
Early / late endosome
▪ GP1,2 cleavage by cysteine proteases
Cysteine proteases
3.
Late endosome
▪ NPC1 binding
NPC1
4.
Late endosome / lysosome
▪ Membrane fusion
▪ Nucleocapsid released into cytoplasm
Viral nucleocapsid
Cytoplasm