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Antiviral Research 135 (2016) 1–14

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Review

Filovirus proteins for antiviral drug discovery: A structure/function analysis of surface glycoproteins and virus entry

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ARTICLE INFO

Article history:
Received 29 July 2016
Received in revised form
1 September 2016
Accepted 5 September 2016
Available online 14 September 2016

Keywords:
Ebola
Filovirus
Antiviral
Mononegavirales
Entry inhibitors
Glycoprotein

ABSTRACT

This review focuses on the recent progress in our understanding of filovirus protein structure/function and its impact on antiviral research. Here we focus on the surface glycoprotein $GP_{1,2}$ and its different roles in filovirus entry. We first describe the latest advances on the characterization of *GP* gene-overlapping proteins sGP, ssGP and Δ-peptide. Then, we compare filovirus surface $GP_{1,2}$ proteins in terms of structure, synthesis and function. As they bear potential in drug-design, the discovery of small organic compounds inhibiting filovirus entry is a currently very active field. Although it is at an early stage, the development of antiviral drugs against Ebola and Marburg virus entry might prove essential to reduce outbreak-associated fatality rates through post-exposure treatment of both suspected and confirmed cases.

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1. Introduction

The filovirus surface glycoprotein $GP_{1,2}$ triggers and drives virus

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http://dx.doi.org/10.1016/j.antiviral.2016.09.001
0166-3542/

entry into the host cell via a complex mechanism that is, to date, only partially understood. Drawing on the present knowledge, numerous research groups have recently developed antiviral strategies aiming to inhibit viral entry.

This review presents current knowledge on filovirus glycoproteins, and compares their structures and associated functions. After briefly placing filoviruses in viral classification and nomenclature, we describe the soluble GP derivatives sGP, ssGP and Δ-peptide, and review all structural features of the surface $GP_{1,2}$. We detail step by step the mechanism by which $GP_{1,2}$ triggers virus entry. In this part, the aim is to highlight the relationship between structural features and their role in the various phases of the filovirus entry process, with attention to idiosyncrasies within the family *Filoviridae*. Lastly, we recapitulate existing and ongoing antiviral strategies, in order to connect mechanisms of action to structure/function analysis aiming at potent anti-filovirus therapies.

2. Filovirus classification and genomic structure

The family *Filoviridae* belongs to the negative strand, non-segmented (NNS) RNA viruses of the *Mononegavirales* order. This family groups highly pathogenic viruses such as those found in the *Marburgvirus* and *Ebolavirus* genera (Ascenzi et al., 2008), responsible for severe hemorrhagic fevers, as well as the genus *Cuevavirus* (Negredo et al., 2011), the latter being found so far only in form of RNA sequenced from bats (Fig. 1). The *Marburgvirus* genus is represented by viruses within a single species, *Marburg marburgvirus* (Marburg virus - MARV). It was the first filovirus genus and species discovered in 1967 during related outbreaks in Frankfurt (Germany) and Belgrade (Yugoslavia) upon importation of infected monkeys from Uganda to Marburg (Germany) (Siegert et al., 1967). The *Ebolavirus* genus consists of five virus species. They are known as *Zaire ebolavirus* (Ebola virus - EBOV), which is the first ebolavirus species identified in 1976 in the Democratic Republic of the Congo (formerly northern Zaire) near the Ebola River, *Sudan ebolavirus* (Sudan virus - SUDV), *Taï Forest ebolavirus* (Taï Forest virus - TAFV), *Bundibugyo ebolavirus* (Bundibugyo virus - BDBV) and *Reston ebolavirus* (Reston virus - RESTV) according to the new nomenclature (Kuhn et al., 2010). While RESTV has not been described to cause human disease yet, the other species, including MARV, are highly pathogenic with fatality rates ranging from 25% up to 90% (Feldmann and Geisbert, 2011). The *Cuevavirus* genus was established after the discovery of sequences in 2002 most likely belonging to a new filovirus, *Lloviu cuevavirus* (Lloviu virus - LLOV), presumably infecting bats in Asturias (Spain) (Negredo et al., 2011). Since it is a novel entry in the filovirus phylogeny, only little is known about its biology and putative infectivity in humans.

With their high infectivity and their ability to impair the immune system (Feldmann and Geisbert, 2011; Ramanan et al., 2011), filoviruses trigger an abrupt onset of symptoms including fever, headache, myalgia and gastrointestinal disorders. Next, hemorrhagic manifestations can arise during the peak of illness. Shock, convulsions, coagulopathy and multi-organ failure appear later and are fatal in many cases (Feldmann and Geisbert, 2011; Nina, 2014). Unfortunately, there are no approved antivirals or vaccines available yet, although significant progress has been made lately in this respect (Mendoza et al., 2016), but supportive treatments such as rehydration and control of fever and pain might help patients to overcome infection. Lately, a lot of efforts have been put together to identify key viral targets in order to inhibit the viral cycle and help to cure the infection (Choi and Croyle, 2013).

Filoviruses share a common genomic organization. Their NNS RNA genome of around 19 kb carries seven main genes leading to the synthesis of the different viral proteins (Figs. 1 and 2) (Ascenzi et al., 2008). All these proteins are essential to establish an infection leading to efficient virus replication (Fig. 3). The sole surface protein $GP_{1,2}$ triggers the first steps of cell infection, which requires attachment to factors present at the surface of target dendritic cells (DCs) and monocytes/macrophages, and on endothelial cells of liver sinusoids and lymph node sinuses. Once attached, the virions are internalized, and endosomal events induce fusion (Feldmann et al., 1999) allowing the release of the viral particle content into the cytoplasm. The nucleocapsid is composed of the genomic RNA in complex with the nucleoprotein NP, the two cofactors VP30 and VP35, and the large protein L, which form a large macromolecular complex protecting the RNA genome and facilitating genome replication/transcription (reviewed by Mühlberger, 2007). The L protein harbors the RNA-dependent RNA polymerase (RdRp) activity, which is essential for both genome replication and transcription. In addition, this protein carries yet uncharacterized enzymatic activities involved in RNA transcriptional modifications such as RNA capping and polyadenylation, protecting viral mRNA from both degradation and detection by the host cell innate immunity guardians (Mühlberger, 2007; Liang et al., 2015). The nucleoprotein NP enwraps and protects the NNS RNA from host nucleases. The VP30 protein acts as a transcription cofactor, while VP35 is the polymerase cofactor (Mühlberger, 2007). After replication of the viral genome and RNA transcription, nascent viral particles are assembled in a process mediated by the matrix protein VP40, and virus budding occurs at the cell surface membrane in a process that involves hijacking the host ESCRT machinery (Hartlieb and Weissenhorn, 2006; Noda et al., 2006).

3. The multifaceted aspects of the filovirus *GP* gene

Early stages of cell infection have been shown to be mediated by the class I viral glycoprotein $GP_{1,2}$ exposed at the virus membrane surface (reviewed by Lee and Saphire, 2009). This protein is synthesized as a precursor GP_0 after translation of an edited *GP* open reading frame (Fig. 4), which is cleaved to yield an ectodomain GP_1

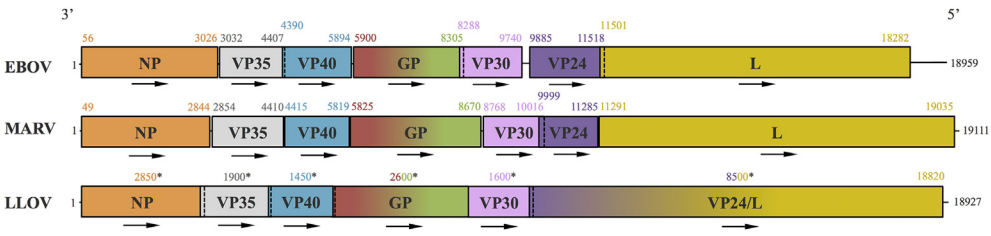

Fig. 1. Filovirus genome organization. Filoviruses are a family of non-segmented negative single stranded RNA viruses, including the genera *Ebolavirus*, *Marburgvirus*, and *Cuevavirus*, with the respective prototype viruses Ebola virus (EBOV), Marburg virus (MARV) and Lloviu virus (LLOV) sharing a common genome organization. Their genome of about 19 kb codes for at least 7 well defined monocistronic mRNAs with the exception of one bicistronic mRNA in the LLOV genome. For EBOV and MARV the first and last nucleotides in the mRNAs are indicated, whereas for LLOV exact mRNA ends are still unclear, but lengths are roughly estimated (*).

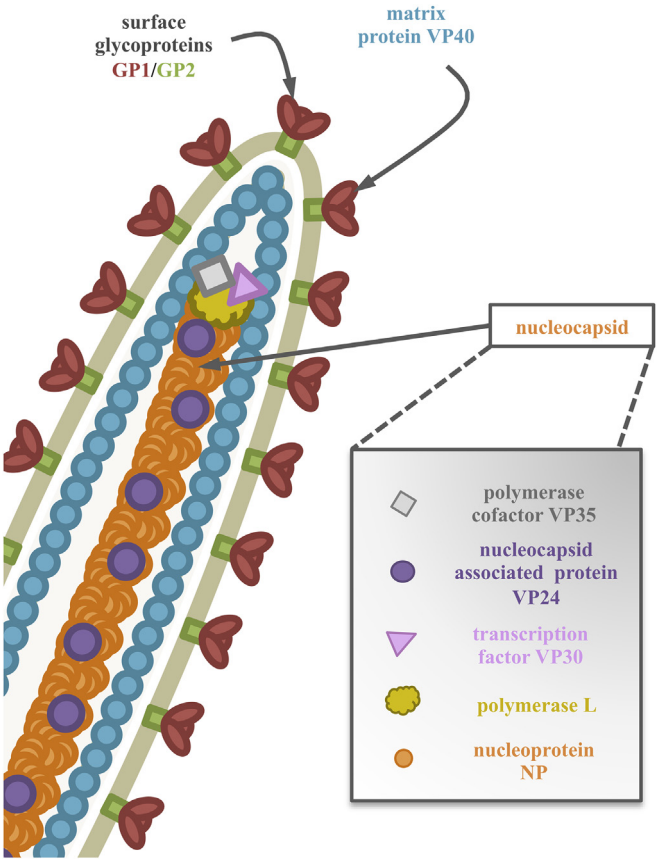

Fig. 2. Schematic ultrastructure of a filoviral particle. The viral protein assembly leads to the formation of filamentous viral particle able to infect host target cell and carrying the required material to complete a viral replication cycle. The surface glycoprotein $GP_{1,2}$ triggers viral attachment and entry. Then, the nucleocapsid components, the RNA-protecting nucleoprotein NP, the viral proteins (VP) 30 and 35 and the "large" (L) polymerase, are released into the cytoplasm for replication and transcription, resulting in synthesis of new viral genomes and proteins. VP24 aids in nucleocapsids assembly, while the matrix protein VP40 orchestrates the formation of new virions.

and a trans-membrane fusion domain GP_2. Viral cell entry being a critical point for infection, this step has been targeted for the design of antiviral molecules. It is also noteworthy that the *GP* gene codes for additional proteins, whose functions are not completely understood. The mechanism driving the expression of these proteins is described below (Fig. 4).

3.1. GP: one gene, many proteins

The *GP* gene is the fourth gene along the genome of every filovirus (Sanchez et al., 1993; Negredo et al., 2011). All filovirus *GP* genes encode a trans-membrane protein $GP_{1,2}$ localized at the virus surface. In ebolaviruses and presumably in cuevaviruses, unedited and edited transcripts produce several forms of GP, which, together with host furin-dependent proteolysis, lead to the expression of additional proteins: the soluble GP (sGP) described in ebolaviruses and cuevaviruses, the Δ−peptide, and the small soluble GP (ssGP) (Fig. 4). It has been proposed that this edition mechanism limits the surface GP-associated cytotoxicity (see 5.) (Volchkov et al., 2001; Mohan et al., 2015). The RNA editing of ebolaviruses involves a slippage region composed of seven consecutive template uridines where viral polymerase stuttering results in a frameshift in the middle of the *GP* sequence (Volchkov et al., 1995; Sanchez et al., 1996; Mehedi et al., 2011). Indeed, this editing mechanism has been recently shown to be regulated by neighboring sequences of the uridine template, probably in synergy with VP30 as a trans-acting factor (Mehedi et al., 2013). Thus, the ebolavirus *GP* gene is able to generate three different mRNAs coding for protein precursors pre-sGP, pre-GP and ssGP in a ratio of approximately 14:5:1, respectively, albeit the exact ratio is cell type dependent (Fig. 4) (Mehedi et al., 2011).

In ebolaviruses, the most abundant product is the unedited transcript pre-sGP mRNA, which leads to the synthesis of the protein precursor pre-sGP. Remarkably, it is not incorporated into the virion structure *per se*. This precursor of about 60 kDa is cleaved by cellular proteases of the furin family at the C-terminus of the R-X-R-R↓ conserved motif at position 324 (Volchkova et al., 1999). This cleavage forms the final sGP and the Δ-peptide (Fig. 4). The 40 amino-acid carboxy-terminal Δ-fragment is subsequently highly modified at the post-translational level (O-glycosylation) before being secreted. Although the Δ-peptide function has not completely been understood yet, it has been suggested that this peptide regulates filovirus entry since its expression limits infection on filovirus-permissive cells (Radoshitzky et al., 2011). Moreover, based on *in silico* analysis this peptide was also proposed to act as a virulence factor forming a lytic viroporin, although experimental evidence for such a function is lacking (Gallaher and Garry, 2015). Efforts have been devoted to characterize sGP because it shares its first N-terminal 295 residues with $GP_{1,2}$ and ssGP (Volchkov et al., 1995). After cleavage, sGP monomers bind to each other in a parallel orientation by means of two disulfide bonds involving residues Cys53 and Cys306 (Falzarano et al., 2006). Due to the lack of a transmembrane domain, sGP forms a soluble dimer, mainly N-glycosylated, of 110 kDa. Its roles have been recently investigated *in vitro* and *in vivo*. Indeed, sGP has been suggested as a virulence factor (Volchkova et al., 2015), although currently it is not entirely clear whether or not this is the case, with one study indicating that abolishment of sGP production did not lead to a noticeable attenuation of the virus (Hoenen et al., 2015). However, the fast emergence of revertants both *in vivo* and *in vitro* (in certain cell lines) when sGP production is reduced by introducing an 8A genotype (i.e. 8 uridine residues in the virus genome, leading to production of predominantly 8A mRNAs) in the editing site suggests an important role of sGP in the biology of ebolaviruses (Volchkova et al., 2011; Kugelman et al., 2012; Hoenen et al., 2015; Tsuda et al., 2015). *In vitro*, sGP exerts vascular effects, notably the restoration of the barrier function suggesting an anti-inflammatory role (Wahl-Jensen et al., 2005); however, the relevance of this finding is currently not clear. Also, there is increasing evidence that sGP might reduce viral cytotoxicity by limiting the amount of expressed $GP_{1,2}$ (see 5.) (Iwasa et al., 2011; Mohan et al., 2015). Finally, it has been shown that secreted sGP might also lead to immune subversion, and act as a decoy for antibodies directed against $GP_{1,2}$ (Ito et al., 2001; Mohan et al., 2012). However, for all these roles of sGP (reviewed by de La Vega et al., 2015) further investigations are required to ascertain what relevance they really have for ebolavirus biology, and whether this protein represents a potential antiviral target.

Another protein product of the ebolavirus and cuevavirus *GP* gene is ssGP, a small protein of 36 kDa that is synthesized from a transcript in which either one adenosine is deleted or two are added during transcriptional editing (Fig. 4). It has been shown that ssGP is secreted as a 100 kDa dimer carrying a disulfide linkage between Cys53 of each monomer, the latter being largely N-glycosylated (Mehedi et al., 2011). Although ssGP shares similar structural properties with sGP (and $GP_{1,2}$), it does not seem to exert the same anti-inflammatory function on endothelial cells (Mehedi et al., 2011), and its role in viral pathogenicity, as well as its potential as antiviral target, remains unclear.

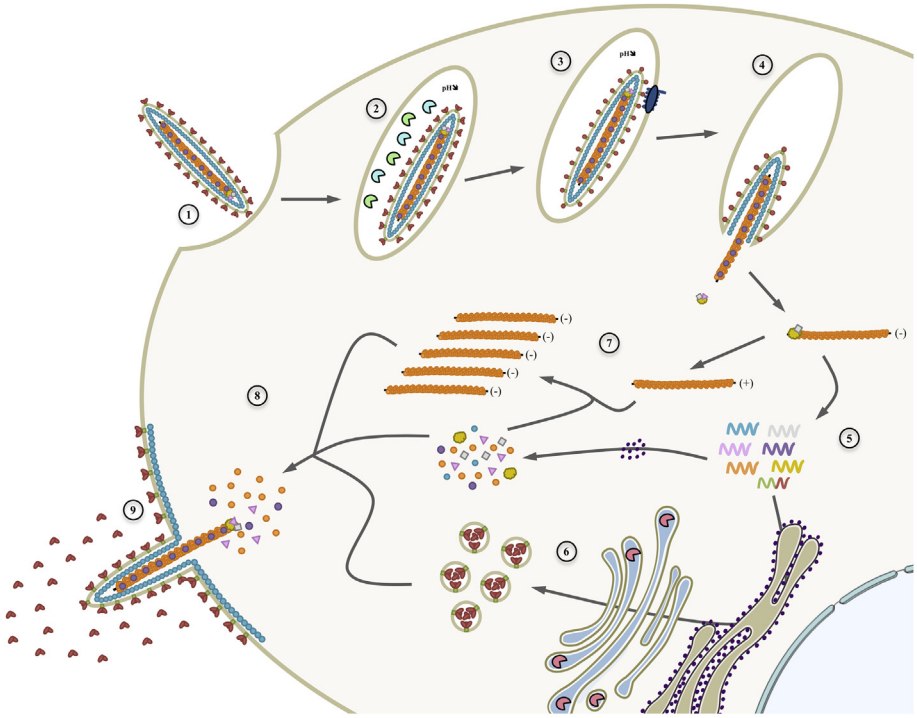

Fig. 3. Viral life cycle of filoviruses. After attachment (1), the viral particle is processed in the endosome by proteases (2) leading to receptor recognition (3) that triggers fusion and release of nucleocapsids into the host cytoplasm (4). Negative strand RNA is transcribed into messenger RNAs (5), allowing translation and protein synthesis to occur, which facilitates further secondary transcription, as well as replication through a complimentary positive sense RNA (7). $GP_{1,2}$ transits through the rough endoplasmic reticulum/Golgi apparatus pathway (6). Then, budding occurs by diverting host trafficking machinery (8), leading to the formation of new virions (9).

3.2. The viral surface glycoprotein: main structural features

The $GP_{1,2}$ RNA transcript codes for the GP_0 precursor. This transcript results from a polymerase slippage on its template resulting in an additional adenosine on ebolavirus (and presumably cuevavirus) $GP_{1,2}$ mRNAs, unlike in the case of the unedited marburgvirus mRNAs (Fig. 4) (Volchkov et al., 1995; Sanchez et al., 1996). mRNAs are then translated into the GP_0 precursor, which transits through the endoplasmic reticulum and the Golgi apparatus, where it is cleaved by furin-like protease(s) into two proteins, GP_1 and GP_2. The position of the conserved cleavage site is variable inside the family *Filoviridae,* but gene structure and functional organization are homologous (Volchkov et al., 2000; Manicassamy et al., 2007; Maruyama et al., 2014). Indeed, these two proteins together form a trimeric chalice structure made of three GP_1 and three GP_2 subunits (Figs. 4 and 5) assembled by GP_1/GP_2 and GP_2/GP_2 interactions (Simmons, 2013). The bowl of the chalice is shaped by the GP_1 subunits, while GP_2 organizes and anchors the complex to the membrane. In the trimer, $GP_{1,2}$s are bound to each other by disulfide bonds - between the Cys53 of GP_1 and the Cys609 of GP_2 as described for ebolaviruses (Lee et al., 2008) - leading to a complex and metastable intricacy.

The ectodomain GP_1 is constituted of a core protein and a mucin-like domain (MLD), which is largely glycosylated (Simmons, 2013). This MLD has not been structurally defined yet, but there are slight differences in terms of sequence, length, and position relative to the cleavage site (Volchkov et al., 1995; Sanchez et al., 1996) between different filovirus genera. Indeed, ebolaviruses hold their MLD as a single unit on GP_1 whereas marburgviruses and cuevaviruses carry it in two blocks, one in GP_1 and the other in GP_2. Despite these differences, it has been proposed that this feature plays a common role both in the attachment of the virus via lectins and in immune escape by hiding potential conserved epitopes (Simmons, 2013). Actually, known epitopes targeted by neutralizing anti-ebolavirus antibodies such as KZ52 and 16F6 have been shown to lie in an uncovered domain present at the interface between GP_1 and GP_2 (Lee et al., 2008; Dias et al., 2011). Similarly, the antibody MR78, which was identified in a marburgvirus disease survivor, targets key residues for receptor binding, which are masked in case of ebolaviruses by the MLD, but more accessible in case of marburgviruses (Hashiguchi et al., 2015). The fact that such a cross-reactive antibody targeting the receptor-binding site (RBS) was found in a marburgvirus disease survivor, but not in ebolavirus disease survivors, is used as a further argument to strengthen the case for a function of the MLD in immune escape.

The core of GP_1 is subdivided into three domains: the glycan cap, the head, and the base (Lee et al., 2008). The glycan cap is the outer part of GP_1 forming the chalice. The head supposedly helps structuring the metastable pre-fusion conformation. This part is exposed to the host membrane surface carrying the putative RBS. The base subdomain supports the linkage with GP_2 and stabilizes the metastable pre-fusion conformation. Thus, GP_1 has the required structural features to mediate viral attachment to cell receptors (see 4.1).

The trans-membrane GP_2 protein anchors the complex to the viral membrane, but also manages virus entry and fusion (Figs. 4 and 6) (Simmons, 2013). Its structure/function complexity has been well described for ebolaviruses (Lee et al., 2008). Briefly, its structure incorporates a transmembrane domain, a short cytoplasmic tail, an internal fusion loop defined by a disulfide bound between GP_2 Cys511 and Cys556, and two heptad repeat regions (HRR1 and HRR2) surrounding the fusion peptide. This domain constitutes the unstable pre-fusion conformation of GP_2, which rearranges itself at low pH to trigger fusion. To maintain the structure in the pre-fusion state, the GP_1 head packs the GP_2 hydrophobic fusion peptide and stabilizes GP_2. Such features have not

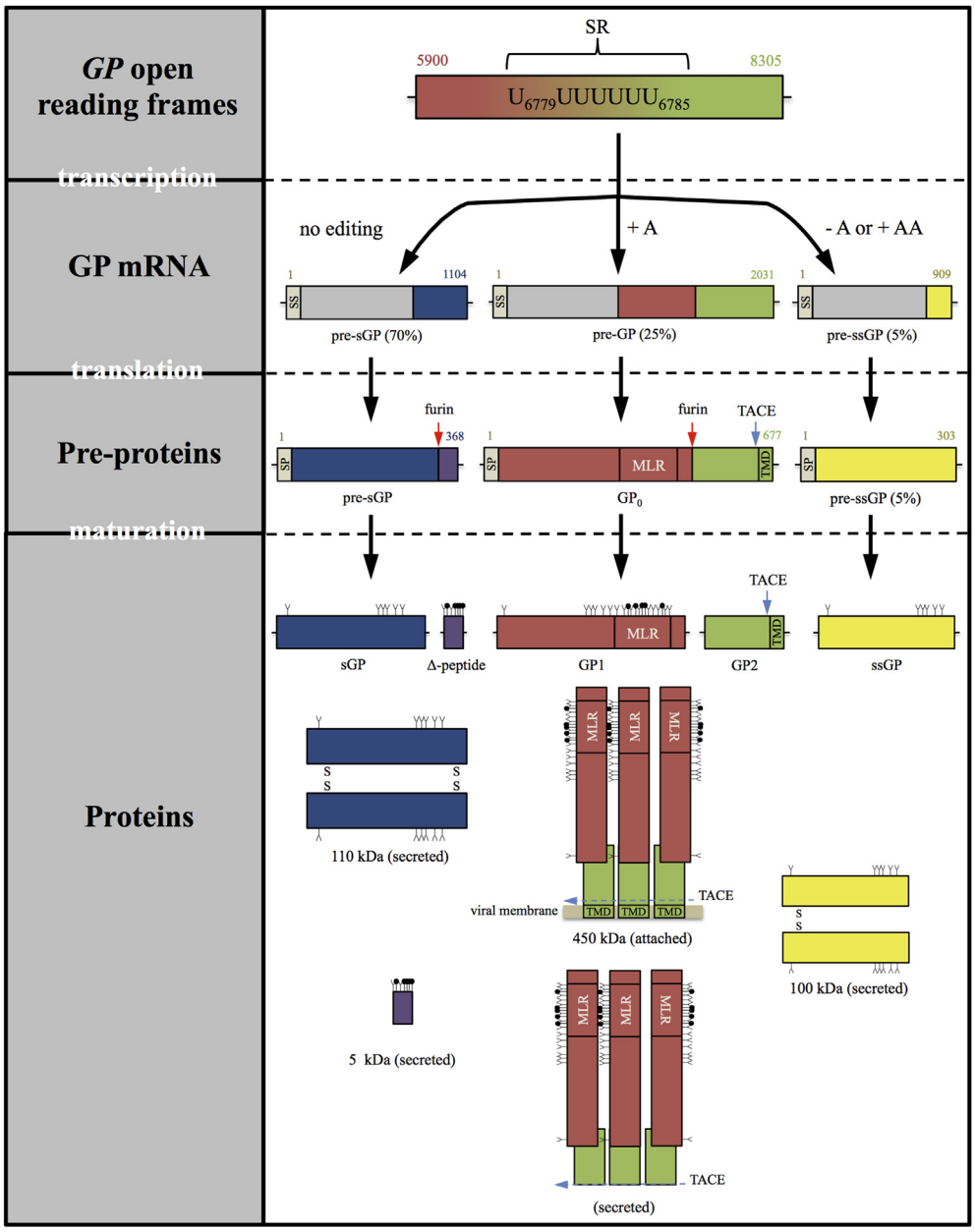

Fig. 4. Ebolavirus *GP* gene products. *GP* genes are roughly similar for ebolaviruses, marburgviruses, and cuevoviruses, with the notable exception that the marburgvirus *GP* gene does not undergo transcriptional editing, but only encodes GP_0. The ebolavirus *GP* gene, like the cuevovirus *GP* gene, contains a poly-U repeat (stuttering region, SR), facilitating an editing mechanism that results in the synthesis of three different mRNAs, leading to the synthesis of sGP (shown on the left), GP_0 (shown in the center), and ssGP (shown on the right). All these mRNAs contain a signal sequence (SS) and the coding sequence for the different proteins. They share a common 5′ sequence (grey) leading to an identical amino-terminus for all GP-proteins. mRNAs are translated into pre-proteins transiting through the endoplasmic reticulum and the Golgi apparatus. During this intracellular trafficking, the signal peptide (SP) is removed; the protein is glycosylated (N- and O-glycosylations), and GP_0 matures by cleavage by furin-like proteases in GP_1 (red) and GP_2 (green). The mucin-like domain is part of GP_1 (red) for ebolaviruses, whereas for marburgviruses and cuevoviruses it is part of both GP_1 and GP_2. Together, GP_1 and GP_2 form $GP_{1,2}$, which assembles further into trimers. The surface $GP_{1,2}$ can shed as a soluble trimer upon cleavage by the host TNFα-converting enzyme (TACE). Three other proteins, sGP (blue), the Δ-peptide (purple), and ssGP (yellow) are synthetized by ebolaviruses, and presumably also by cuevoviruses.

been clearly described yet for marburgviruses and cuevaviruses, but sequence alignments and *in vitro* data suggest similar conformations and entry/fusion mechanisms (Manicassamy et al., 2007; Maruyama et al., 2014; Liu et al., 2015).

4. GP, a fusion protein mediating filovirus cell entry

$GP_{1,2}$ orchestrates viral entry, which can be seen as a three step mechanism: attachment, uptake, and fusion (Fig. 6) (Olejnik et al., 2011).

4.1. Filovirus attachment

The attachment between a filovirus and its target cell is mediated by GP_1, but the identity of the main cellular attachment factor remains unclear (reviewed by Takada, 2012), and in fact it appears that there are numerous proteins at the cell surface that can fulfill this function.

First, C-type lectin family members such as hMGL on immature dendritic cells and macrophages (Takada et al., 2004; Matsuno et al., 2010a) or asialo-glycoprotein, DC-SIGN, L-SIGN, L-SECtin

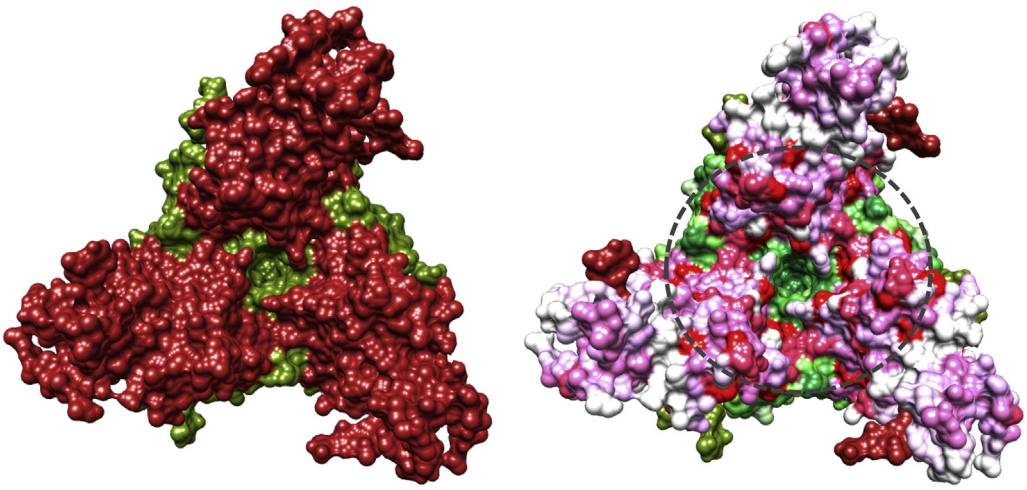

Fig. 5. Ebolavirus surface $GP_{1,2}$ and its conservation among the family *Filoviridae*. Surface $GP_{1,2}$ (PDB: 3CSY) is a trimer composed of three dimers of GP_1 (red) and GP_2 (green), forming a chalice at the viral envelope. The left panel presents a surface representation of the three-dimensional structure of ebolavirus $GP_{1,2}$, and GP_1 and GP_2 are shown in red and green, respectively. The right panel highlights the conserved residues (from dark red to light red or dark green to light green according to their conservation) derived from a sequence alignment of every filovirus species using hierarchical clustering (MultiAlin server). The figure shows that conserved residues are localized at the center of the trimeric complex (indicated with a grey dotted circle), which contains all features for priming and fusion, as well as in the external domain targeted by the cross-genus neutralizing antibody MR78.

and DC-SIGN(R) on liver endothelial cell and lymphocytes (Becker et al., 1995; Alvarez et al., 2002; Lin et al., 2003; Simmons et al., 2003; Marzi et al., 2004; Gramberg et al., 2005, 2008; Dominguez-Soto et al., 2007; Powlesland et al., 2008; Maruyama et al., 2014) are expected to interact with a set of N- and O-linked glycans on the MLD and the glycan cap of GP_1, since these lectins increase filovirus attachment. However, the binding to such molecules does not seem to be sufficient to trigger virus internalization (Simmons et al., 2003; Marzi et al., 2007; Matsuno et al., 2010b).

Additionally, β_1-integrins have been proposed to serve as attachment factors for ebolaviruses, since infection decreases in the presence of antibodies targeting these proteins in cell lines and primary cell types (Takada et al., 2000; Simmons et al., 2002). However, no direct interaction between both molecules has been demonstrated yet, and recent studies suggest that $\alpha_5\beta_1$-integrin is not required for GP-mediated binding of internalization, but rather is a positive regulator of cathepsins, which play an important role in processing GP_1 into its fusion-competent form within the endosomes of infected cells (Schornberg et al., 2009).

Axl, member of the Tyro3/Axl/Mer (TAM) receptor family, has also been proposed as a co-receptor for EBOV attachment but, similarly to β_1-integrins, it may promote viral entry indirectly (Shimojima et al., 2006; Schornberg et al., 2009; Brindley et al., 2011; Hunt et al., 2011). Other cell-surface molecules, the T-cell immunoglobulin mucin domain-1 and 4 (TIM-1 and TIM-4), have been described to interact with ebolavirus $GP_{1,2}$, leading to virus internalization (Kondratowicz et al., 2011; Moller-Tank et al., 2013; Yuan et al., 2015; Rhein et al., 2016). Nevertheless, despite this clearly demonstrated role, only epithelial cells and some antigen-presenting cells subsets significantly express TIM-1 and TIM-4 respectively, suggesting that there are other attachment factors involved in other filovirus-susceptible cell types. TAM, TIM-1 and TIM-4 could target phosphatidyl-serine (PtdSer), which is exposed on the outer leaflet of the filovirus membrane, strengthening an interplay promoting efficient attachment (reviewed in Moller-Tank and Maury, 2014).

Finally, a last attachment mechanism has been described, reminiscent to an antibody-dependent enhancement (ADE) process, by which filoviruses divert virus-specific antibodies to get attached to immune system cells through cellular Fc receptors or via the complement component C1q and its ligands identified in most mammalian cells (Takada and Kawaoka, 2001; Takada et al., 2003, 2007; Nakayama et al., 2010). Interestingly, viral pathogenicity seems to correlate with filovirus ADE (Takada and Kawaoka, 2001; Nakayama et al., 2011).

In all cases, it appears that attachment requires a set of proteins that interact in a complex manner to promote entry.

4.2. Uptake and proteolytic processing

The uptake is a key step in filovirus entry, as it serves to transform the pre-fusion $GP_{1,2}$ conformation into a primed $GP_{1,2}$ that triggers fusion events (Fig. 6).

First, the internalization has been proposed to involve different endocytic pathways. The precise mechanisms were controversial in the past, since clathrin-dependent and caveolin-dependent uptakes have been shown to occur (Bavari et al., 2002; Empig and Goldsmith, 2002; Sanchez, 2007; Bhattacharyya et al., 2010, 2011). However, latest data support that the filovirus uptake mechanism is mainly mediated by macropinocytosis and depends among other factors on the host cell and virus particle size (Nanbo et al., 2010; Saeed et al., 2010; Aleksandrowicz et al., 2011).

After internalization, macropinocytosis vesicles are routed to endosomal vesicles of the host cell, where proteolytic events occur to prime $GP_{1,2}$ (Fig. 6). Once in the endosome, EBOV $GP_{1,2}$ is sequentially processed by the cysteine proteases cathepsin B (catB) and/or cathepsin L (catL) under acidic pH and reducing conditions (Chandran et al., 2005; Schornberg et al., 2006; Brecher et al., 2012). Concisely, it appears that, for EBOV, BDBV, and TAFV, catB removes the major part of GP_1 (glycan cap and MLD). The proteolytic events are slightly different for SUDV, RESTV, MARV and LLOV, as catB has been shown to be dispensable (Chan et al., 2000; Gnirss et al., 2012; Xia et al., 2012; Maruyama et al., 2014; Ng et al., 2014). It is thought that catL partially removes a part of the GP_1 cap and subsequent proteolysis – by catB, catL and/or other proteases – results in a smaller GP_1 form of less than 20 kDa. With latest structural inputs, further information regarding cathepsin cleavage have become available. Indeed, amongst the minor differences between EBOV and MARV $GP_{1,2}$, a catB cleavage site identified on EBOV has been shown to be disordered, and thus potentially easily accessible,

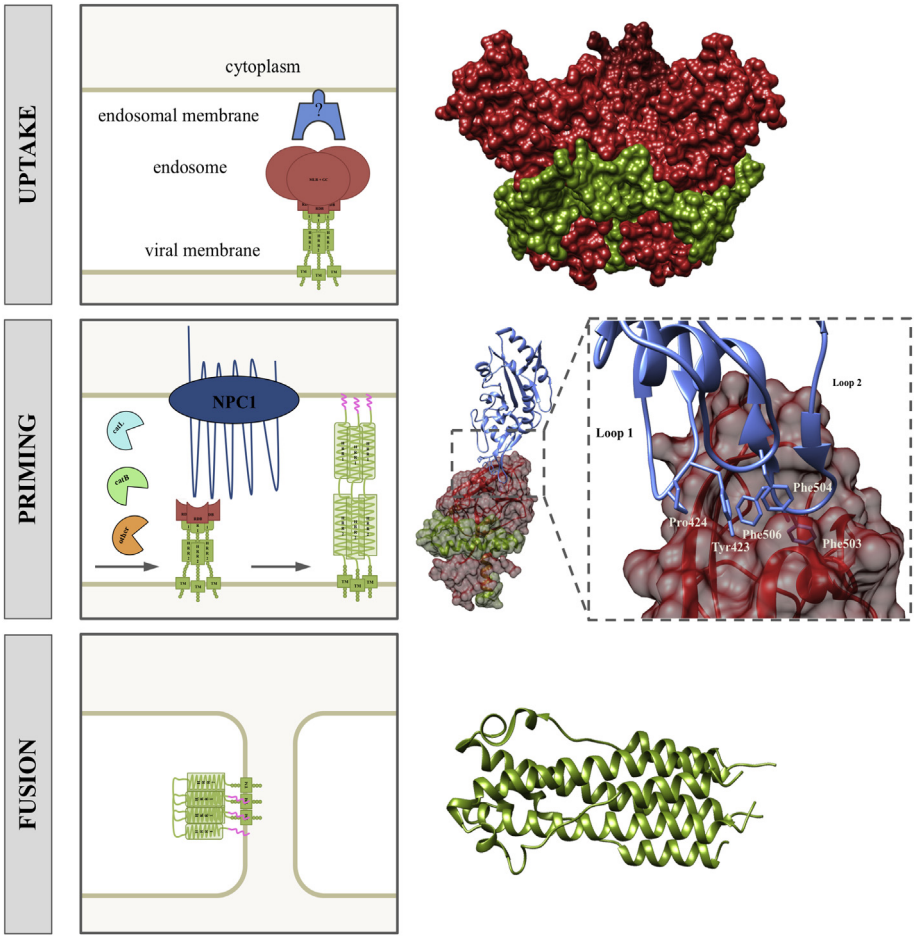

Fig. 6. Surface GP endosomal processing. After attachment mediated by interaction between the filovirus surface protein $GP_{1,2}$ (PDB: 3CSY) and various attachment factors, the complex is internalized and routed to the endosome, where $GP_{1,2}$ is processed to trigger fusion of viral and host membranes. First, in the endosomal low pH environment, cathepsin proteases L&B (catL & catB) and others remove the mucin-like region (MLD) and the glycan cap (GC) domain of GP_1 (red). A receptor binding domain (RDB), also carried by GP_1, is unmasked, leading to the interaction with a mainly hydrophobic pocket in the N-terminal domain (blue) of the endosomal protein Niemann-Pick C1 (NPC1) (PDB: 5F1B). This interaction together with other only partially understood molecular events remove GP_1 constraints on GP_2 (green), forming the primed-$GP_{1,2}$ capable to induce fusion. The GP_2 heptad repeat regions 1 and 2 (HRR1 and HRR2) then rearrange themselves, pushing out the fusion peptide (pink) to anchor it in the host membrane. This intermediate pre-hairpin conformation destabilizes membrane bilayers, and a folding-back into a six-bundle helices conformation (PDB: 2EBO) merges membranes, opening a fusion pore for the release of viral nucleocapsids.

while in MARV the homologous region is an α-helix (Hashiguchi et al., 2015). However, there remain open questions regarding filovirus GP proteolysis processing, since other proteases were also proposed to participate to this process. Further, while much of the cathepsin work was done using GP-pseudotyped VSV or retrovirus particles, work with infectious ebolaviruses challenges the importance of cathepsins for the viral life cycle. Particularly, virus replication *in vitro* as well as *in vivo* was shown to be independent of catB and catL, and cathepsin knockout mice succumbed to ebolavirus challenge, albeit not to VSV infection (Marzi et al., 2012).

In any case, these cleavages trigger an increase in binding and infectivity for Ebola virus by unmasking the potential RBS, which was firstly described as a 6 residue peptide on GP_1 (K114, K115, K140, G143, P146, K147) (Lee et al., 2008). Recently, the Niemann-Pick C1 protein (NPC1), which is an anchored late endosomal/lysosomal protein physiologically implicated in cholesterol absorption and homeostasis, has been shown to interact with this region of GP_1 (Fig. 6) (Carette et al., 2011; Côté et al., 2011) downstream both attachment and uptake events independently from its proper role in cholesterol trafficking (E. H. E.H. Miller et al., 2012; White and Schornberg, 2012). Indeed, the latest structural data define the RBS as a negatively charged crest and a hydrophobic trough at the apex of GP_1 trimer that interact mostly with the domain C (loops 1 and 2) and partially with the domain NTD of NPC1 (Bornholdt et al., 2016; Gong et al., 2016; Wang et al., 2016; Zhao et al., 2016). This structural characterization of the NPC1/GP_1 interaction provides key information to design promising antiviral strategies. More importantly, further analyses described NPC1 as a crucial receptor that potentially determines the species susceptibility to filoviruses (Ng et al., 2015; Hoffmann et al., 2016; Ndungo et al., 2016), paving the way towards filovirus-specific antiviral molecules (**see 6.2.**).

4.3. Membrane fusion

Even if deeper investigation is needed into the mechanism of NPC1 during filovirus entry, its interaction with cleaved GP_1 and the endosomal environment triggers a conformational change of GP_2 initiating membrane fusion (Fig. 6) (Lee et al., 2008; Kuroda et al., 2015). Fusion necessitates large conformation changes representing a high-energy barrier. With GP_1 cleavages and a low-pH environment, it is hypothesized that both GP_1 constraint removal from the metastable GP_2 and histidine protonation generate the energy triggering the fusion events (Kampmann et al., 2006; Lee et al.,

2008, 2016; Markosyan et al., 2016). At this point, the disordered HRRs rearrange themselves into α-helices. These helices push through the host membrane, and then anchor the hydrophobic fusion peptide in target membrane. Within the membrane, key residues, amongst them Pro537, trigger bilayer destabilization with an extended pre-hairpin intermediate conformation (Fig. 6). Therefore, elongated GP_2 spans and thus branches both host and viral membranes. The fusion finally occurs by the collapse of this intermediate into a folding-back conformation that distorts both viral and host membranes with their simultaneous rapprochement. GP_2 acts as a clamp to reach its low energy state characterized as a six helices bundle, which is the GP_2 hairpin post-fusion structure (Fig. 6). This novel conformation leads to a merge into a hemi-fusion stalk and then to the opening of a fusion pore allowing the release of the nucleocapsid into the host cytoplasm (Fig. 6).

5. Modulation of cytotoxicity and inflammation

While the principle function of $GP_{1,2}$ is cell infection, several lines of evidence suggest that it might also be involved in pathogenesis. Cellular $GP_{1,2}$-related cytotoxic events occur in infected cells. This cytotoxicity is reflected in rounding of cells *in vitro*, due to masking of various host cell surface molecules such as β1-integrins (Takada et al., 2000; Francica et al., 2009). This masking has been shown to be a "glycan umbrella" mediated steric shielding of these adhesion proteins mediated by surface-expressed $GP_{1,2}$, leading to a loss of accessibility and function of these host surface proteins (Reynard et al., 2009; Francica et al., 2010). The masking of such adhesion molecules leads to cell detachment, and may contribute to the disruption of blood vessel integrity and hemorrhages developed during a filovirus infection. Indeed, in primary endothelial cell cultures overexpression of $GP_{1,2}$ after transduction with an adenovirus vector resulted in a loss of adherence resulting in apoptosis (Ray et al., 2003). Additionally, steric shielding by $GP_{1,2}$ also affects MHC-I surface expression (Francica et al., 2009), a phenomenon also known to occur in infected primary endothelial cells (Harcourt et al., 1999). This might alter immune cells recruitment, and thus may participate in the immune suppression and inflammatory dysfunction linked to a filovirus infection (Harcourt et al., 1999; Reynard et al., 2009).

As mentioned in **3.1.**, sGP has been shown to regulate $GP_{1,2}$ expression thanks to transcriptional editing in the *GP* gene (Volchkov et al., 2001; Mohan et al., 2015). Interestingly, when EBOV is serially passaged in certain cell lines like Vero cells, the *GP* gene gets modified to predominantly generate $GP_{1,2}$ by addition of an 8th U in the editing site (n.b., in contrast to transcriptional editing this occurs on the genome level) (Volchkova et al., 2011). However, when this EBOV is further passaged in animals or in other cell lines like Huh7 cells, the genotype reverts back so that sGP is again produced as the main product of transcription (Volchkova et al., 2011; Hoenen et al., 2015; Tsuda et al., 2015). This observation suggests that either the importance of the function of sGP or the importance of *GP* gene modulation is dependent on the host cell environment.

Additionally, if $GP_{1,2}$ is over-expressed, the cellular glycosylation machinery is overwhelmed, which competitively inhibits physiological processes (Volchkov et al., 2001; Mohan et al., 2015). Nevertheless, at later stages of viral infection, $GP_{1,2}$ gets highly expressed, which is associated with a massive release of viral particles by infected cells. Thus, cytotoxicity appears even in these situations to be under control by transcriptional editing. However, viral particles produced at this point have been shown to be less pathogenic, suggesting that a quantitative balance in $GP_{1,2}$ expression might also be required to regulate infectivity (Mohan et al., 2015).

Finally, another form of $GP_{1,2}$, known as shed GP or $GP_{1,2}\Delta TM$, has been shown to be released during ebolavirus infection (Dolnik et al., 2004, 2015). Indeed, $GP_{1,2}\Delta TM$ is released from surface-expressed $GP_{1,2}$ by the host TNFα-converting enzyme (TACE), a member of the disintegrin and metalloproteinase (ADAM) family, leaving only the transmembrane and cytoplasmic parts of GP_2 on the cell surface (Fig. 4). This secreted protein may play a role in viral pathogenesis, blocking neutralizing antibodies or stimulating ADE. It might also contribute to an impaired inflammatory response (Escudero-Pérez et al., 2014). A last potential role of shed GP might be the modulation of endothelium homeostasis, thus triggering increase of vascular permeability, and disseminated intravascular coagulation, ultimately causing multi-organ failure and death (Escudero-Pérez et al., 2014).

6. Potential of GP targeting antivirals

Since filovirus $GP_{1,2}$ plays a key role in virus entry, strategies targeting different key points in this process are supposed to block replication at an early stage, thus reducing the chance of virus spread and its potential evolution towards drug resistance. Therefore, numerous methods targeting filovirus entry have been investigated. (i) Immune-based therapies have been rapidly developed (reviewed by Choi and Croyle, 2013) leading to efficient monoclonal antibody cocktails (Olinger et al., 2012; Qiu et al., 2012, 2013a, 2013b) such as the promising antibody-based drug against ebolaviruses ZMapp® (Qiu et al., 2014). (ii) Alternatively, peptide-based antiviral molecules like Tat-Ebo and analogs were designed in order to block cell membrane fusion (Miller et al., 2011; Higgins et al., 2013). The inhibition is based on peptides limiting HRR1 and 2 interactions to block GP_2 extension. Such compounds have still to be optimized in terms of treatment window and dosage. (iii) Other strategies based on a broad range of small molecules have been built up to disrupt the entry step. These potential antiviral compounds have been heterogeneously characterized from *in vitro* high throughput screening hits to *in vivo* studies (reviewed by Nyakatura et al., 2015; Rhein and Maury, 2015). Here are reported the latest advances related to small entry inhibitors, which can be sorted as broad-spectrum molecules, filovirus-specific compounds, and FDA-approved therapeutics (summarized in Table 1). With regards to antibody-based therapies the interested reader is referred to (Zeitlin et al., 2016).

6.1. Broad-spectrum molecules

As broad-spectrum molecules we define inhibitors targeting key points of the entry process that are effective against multiple virus multiple RNA viruses Indeed, filoviruses share entry steps with other viruses using class I envelope glycoproteins such as HIV or influenza.

The first target that has been considered is attachment. Various soluble mannose-specific lectins from plants (e.g., concanavalin A and cyanovirin N) have been proposed because of their potential antiviral effect on HIV-1 (Balzarini et al., 2004). Another example is griffithsin, a lectin of terminal mannose residues of asparagine (N)-linked Man 5-9 GlcNAc2 structures purified from red-algae, which might also have antiviral potential since these carbohydrate residues are found on HIV-1 and 2, HCV, SARS-CoV and, relevant to our discussion, on ebolaviruses (Barton et al., 2014). Similarly, recombinant human mannose binding lectin has been shown in a mouse model to be protective against ebolaviruses (Michelow et al., 2011); however, the mouse model, while a necessary and important early step in drug evaluation for ebolaviruses, has only limited predictive value regarding the effectivity of a treatment in other, more stringent animal models of EVD, or even human patients.

Table 1
Antiviral compounds acting against filovirus entry.

Category	Target	Drug activity	Molecules	References
Broad spectrum molecules	Attachment	Mannose-specific lectins	griffithsin, concanavalin A, cyanovirin N	(Balzarini et al., 2004; Barton et al., 2014)
	Endosomal processing	Protease inhibitors	Cys&Ser protease inhibitors (leupeptin) Cys protease inhibitors (E-64 and E-64d) catL&catB inhibitors (FY-dmk) catB inhibitors (CA-074, CA-074Me, nafamostat mesilate) catL inhibitors (oxobarzate, ZY(t-Bu)-dmk, triazine derivatives 5705213 and 7402683, and K11777)	(Chandran et al., 2005; Schornberg et al., 2006; Barrientos et al., 2007; Shah et al., 2010; Gnirss et al., 2012; Elshabrawy et al., 2014; Nishimura et al., 2015; Zhou et al., 2015; van der Linden et al., 2016)
		Endosome disruption	genistein, tyrphostin AG1478, chloroquine	(Savarino et al., 2001; Keyaerts et al., 2004; Keyaerts et al., 2009; Madrid et al., 2013)
	Fusion	Intercaling agents	LJ001, dUY11, arbidol	(St Vincent et al., 2010; Wolf et al., 2010; Pécheur et al., 2016)
		Unclear	25HC oxysterols, teicoplanin	(Liu et al., 2013; Schoggins et al., 2013; Wang et al., 2016)
Filovirus-specific compounds	Attachment	Lectin competitors	tridecafullerenes	(Muñoz et al., 2016)
	Fusion	NPC1 inhibitors	U18666A, imipramine, Ro48-8071, compounds 3.47 and 3.0, and MBX2254 and MBX2270	(Cenedella, 2009; Côté et al., 2011; Kolokoltsov et al., 2012; Miller et al., 2012; Lee et al., 2013; Shoemaker et al., 2013; Basu et al., 2015)
		GP2 inhibitors	compound 7	(Basu et al., 2011)
Approved drugs against other targets	Attachment	Glycosaminoglycan competitor	heparin	(Salvador et al., 2013; Cheng et al., 2015; O'Hearn et al., 2015)
	Uptake	Cytoskeleton inhibitors	vinblastine, vincristine, colchicine, nocodazole, cytochalasin B and D, latrunculin A, chondramides	(Kouznetsova et al., 2014; Beck et al., 2016)
	Endosomal processing	G protein-coupled receptor antagonsits	benztropine mesylate	(Cheng et al., 2015)
		Endosomal pH increase	omeprazol, esomeprazol	(Long et al., 2015)
	Unclear	Estrogen receptor modulators	clomiphene, toremiphene, raloxifene, taxomifene	(Johansen et al., 2013; Shoemaker et al., 2013; Gehring et al., 2014; Kouznetsova et al., 2014; Sakurai et al., 2015; Zhao et al., 2016; Beck et al., 2016)
		Ion channel inhibitors	amiodarone, dronedarone, verapamil, tetrandine, nimodipine, diltiazem, digoxin, rottlerin, noricumazole A)	

In addition to serological and peptide-based approaches, numerous studies report potential small anti-filovirus molecules. These compounds can be sorted in three categories (broad-spectrum molecules, filovirus-specific compounds and repurposed FDA-approved therapeutics). In each category, molecules have been arranged according to their targeted entry process and then following their specific activity when described.

The inhibition of catL and catB, involved in EBOV $GP_{1,2}$ endosomal processing, could also have a broad-spectrum antiviral effect. Various more or less specific inhibitors have been described: the unselective Cys and Ser protease inhibitor leupeptin, unselective Cys protease inhibitors E-64 and E-64d and its recent derivatives, the mixed catL and B inhibitor FY-dmk, the specific catB inhibitors CA-074, CA-074Me and nafamostat mesilate (which has a dual action also targeting factor VIIa for an anticoagulation action), and catL-specific inhibitors oxobarzate, ZY(t-Bu)-dmk (also known as CID23631927), triazine derivatives 5705213 and 7402683, and K11777 (Chandran et al., 2005; Schornberg et al., 2006; Barrientos and Rollin, 2007; Shah et al., 2010; Gnirss et al., 2012; Elshabrawy et al., 2014; Nishimura and Yamaya, 2015; Zhou et al., 2015; van der Linden et al., 2016). All these compounds have been characterized in ebolavirus entry modelling systems, such as pseudotypes, and/or studies with infectious virus. They are mechanism-based suicide inhibitors, since they carry an epoxide or diazomethane functional group. They are not only active on filoviruses, but also on SARS-CoV as well as Hendra and Nipah viruses (Elshabrawy et al., 2014). As far as both anti-filovirus attachment and anti-uptake are concerned, these inhibitors need further optimization, especially concerning their toxicity.

Molecules targeting fusion constitute another class of inhibitors. Viruses being unable to repair membrane damage, the use of membrane intercalating agents such as aryl methyldiene rhodamine derivative LJ001 (Wolf et al., 2010), dUY11 (St Vincent et al., 2010), a rigid amphipathic fusion inhibitor (RAFI), the indole based hydrophobic molecule arbidol (Pécheur et al., 2016), presumably synthetic 25HC oxysterols (Liu et al., 2013; Schoggins and Randall, 2013) and teicoplanin (Y. Wang et al., 2016) may impair virus/host cell membrane fusion. To date, development is at a proof-of-concept stage, but the strategy showed a broad efficiency against ebolaviruses, influenza, HIV, pox-, arena-, bunya-, herpes-, paramyxo- and flaviviruses (Wolf et al., 2010).

Other broad-spectrum compounds can be mentioned such as genistein, a broad tyrosine kinase inhibitor, or tyrphostin AG1478, an epidermal growth factor receptor tyrosine kinase blocker. Both trigger the disruption of endocytosis and endosome formation used by numerous viruses (Chandran et al., 2005). The 9-aminoquinoline, long known as the antimalarial agent chloroquine (Savarino et al., 2003), showed beneficial effects, from endocytosis to exocytosis with increase of endosomal pH, diminution of INF-γ and TNF-α production, on HIV-1, SARS-CoV and ebolaviruses *in vitro* (Savarino et al., 2001; Keyaerts et al., 2004, 2009; Madrid et al., 2013).

Unfortunately, for some of these compounds animal studies have shown that they are not effective against ebolaviruses *in vivo* (Dowall et al., 2015; Falzarano et al., 2015; Akpovwa, 2016), whereas other compounds still need to be tested in such models, before any conclusions regarding their effectiveness can be made. Importantly, different *in vitro* and *in vivo* models have different predictive values regarding the effectiveness of drugs, with *in vitro* experiments providing the weakest evidence (although they remain an essential starting point), followed by the various mouse models, whereas guinea pigs provide a more stringent model, and non-human primates constitute the most stringent model for

ebolavirus and marburgvirus disease. These characteristics have to be kept in mind when evaluating these and other antiviral drugs against filoviruses, or when interpreting study results. Further, all these compounds target a broad spectrum of pathogenic viruses; however, since they often target the cellular machinery, selectivity and toxicity will inevitably remain an important issue.

6.2. Filovirus-specific inhibitors

An alternate strategy would be to select molecules targeting filoviruses specifically. The GP_2/NPC1 interaction that triggers fusion events in filoviruses has been highly considered and recently characterized (Bornholdt et al., 2016; Gong et al., 2016; Wang et al., 2016; Zhao et al., 2016). Such a strategy is challenging, since the interface between NPC1 and GP_2 is large, dynamic and mainly hydrophobic, but also very promising for the development of filovirus specific antiviral molecules.

Development of protein-protein interaction inhibitors towards both GP_2 and NPC1 has been reported. Several compounds have been described targeting NPC1, such as U18666A, imipramine, cathionic amphiphiles Ro48-8071 and the terconazole adamantane class of compounds like benzylpiperazine adamantane diamides 3.47 and 3.0, or the lately discovered sulfonamide derivative MBX2254 and the triazole thioether derivative MBX2270 (Rodriguez-Lafrasse et al., 1990; Cenedella, 2009; Côté et al., 2011; Lee et al., 2013; Shoemaker et al., 2013; Basu et al., 2015). Most of these inhibitors helped to identify NPC1 as a critical factor for filovirus entry. Interestingly, the tertiary amine imipramine inhibits both NPC1 and sphingomyelinase, resulting in ebolavirus entry inhibition (M.E. Miller et al., 2012). As NPC1 is involved in other cellular functions, its inhibition disrupts its primary role in cholesterol homeostasis and triggers cholesterol accumulation in endosomes, as is the case in Niemann-Pick disease type C (Lee et al., 2013; Shoemaker et al., 2013). Therefore, further development might be required to uncouple the NPC1 function in cholesterol traffic from its implication in filovirus entry. This unbundling should be tricky, since both cholesterol management and ebolavirus binding are facilitated by the same domains of NPC1. However, as ebolavirus disease constitutes an acute infection, it should be possible to keep the treatment time relatively short, so that this side effect might have limited detrimental effects in practice.

An alternative strategy avoiding unwanted host-directed effects might be to target GP_2, since it is a viral protein with no cellular homolog. Indeed, a benzodiazepine derivative (referred to as compound 7) has been shown - by computer calculation and mutagenesis - to mask the hydrophobic conserved S2 pocket defined at the GP_1/GP_2 interface, inhibiting ebolavirus replication (Basu et al., 2011). These results also show that this S2 pocket does not correspond to the trough of the RBS, suggesting that it might be another binding site or a steric regulation region of the NCP1 binding site. Although the mechanism of inhibition has yet to be clarified, S2 pocket and RBS pharmacophores might be key targets to design filovirus-specific antiviral molecules.

Lately, another filovirus-specific strategy has been described using tridecafullerene derivative compounds (17a and 17c) (Muñoz et al., 2016). These compounds are giant globular multivalent molecules carrying sugar motifs able to compete with lectins. Dependent on the sugar motifs linked on such “superballs”, it is possible to target specific lectins. Targeting DC-SIGN for example resulted in inhibition of ebolavirus *in vitro* at subnanomolar concentrations with no cytotoxicity notable, and perfect solubility in water. As a very new technology, these tridecafullerene derivatives require deeper investigations to depict their mechanism of action *in vitro* and their effectiveness *in vivo,* as well as their specificity and their delivery to target organs.

6.3. Repurposing of approved drugs

Given that the development of new therapeutics is a long and expensive process, one approach to rapidly develop drugs against filovirus infection is to reposition compounds that have been already approved by national and international health agencies (Kouznetsova et al., 2014).

Using this strategy, cationic amphiphiles such as the estrogen receptor modulators clomiphene (infertility treatment to induce ovulation), toremiphene (breast cancer chemotherapeutic) raloxifene (osteoporosis prevention) and taxomifene (breast cancer chemotherapeutic), and ion channel inhibitors (cardiac arrhythmias and vascular modulation) such as amiodarone, dronedarone, verapamil, tetrandine, nimodipine, diltiazem, digoxin, and rottlerin have been shown to inhibit filovirus entry (Johansen et al., 2013; Shoemaker et al., 2013; Gehring et al., 2014; Kouznetsova et al., 2014; Sakurai et al., 2015; Nelson et al., 2016). Other channel blocking compounds such as noricumazole A from myxobacteria have been also described as potent inhibitors (Beck et al., 2016). Their inhibition mechanisms are not yet understood, but are distinct from GP_2/NPC1 interaction-based inhibitors.

Another group of therapeutics targeting the cytoskeleton has been described to have anti-EBOV potential (vinblastine, vincristine, colchicine, nocodazole, cytochalasin B and D, latrunculin A and others) (Kouznetsova et al., 2014; Beck et al., 2016). However, these molecules are supposed to be quite toxic, since they are mostly used for anti-cancer chemotherapies.

Additionally, as a large class of approved therapeutics, G protein-coupled receptor (GPCR) antagonists have been screened. Benzotropine mesylate, initially used against Parkinson's disease, exhibits *in vitro* anti-filovirus activity targeting a post-attachment step (Cheng et al., 2015). Such molecules deserve a thorough mechanistic investigation, since there is no other literature mentioning the involvement of GPCR in filovirus entry.

Other drugs such as omeprazole and esomeprazole (acid reflux disease) have off-target inhibitory activity triggering increase of endosomal pH, which inhibits late entry events during a filovirus infection (Long et al., 2015). Also, several studies described heparin as a potent inhibitor of early attachment and co-reception via glycosaminoglycans (Salvador et al., 2013; Cheng et al., 2015; O'Hearn et al., 2015).

However, further studies are needed to unravel the antiviral mechanism of those FDA-approved compounds. Although these compounds have already passed safety tests, it is important to underline that dosing and treatment window will likely be a critical issue before use.

7. Conclusion

Antibodies such as ZMapp® have demonstrated that inhibitors targeting filovirus entry might provide a potent antiviral strategy. However the development of small molecules inhibiting this crucial viral step is still hampered by both the insufficient molecular characterization of the attachment/uptake mechanism, and the multiple molecular events occurring in endosomal compartments. To date, the identification of the main characters is clear, but the fine-tuning of each step has to be clarified. The inhibitors presented herein appear to function at several entry steps, but the most susceptible one has yet to be identified. Targeting pre-fusion $GP_{1,2}$ or the GP_2/NPC1 interface might represent the best strategy to develop filovirus-specific treatments. With a more integrative vision in mind, antiviral therapies might advantageously complement vaccines and serological therapies during filovirus outbreaks with their simple logistics especially in West and Central Africa.

Acknowledgements

This work was supported by a DGA-MRIS and Aix-Marseille Université scholarship and by the ANR grant ANR-ST14-ASTR-0026 for the project VMTaseIn.

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