Chapter 8

Strategies for the Development of Small Molecule Inhibitors of Ebola Viral Infection

Sebastian Pleško and Črtomir Podlipnik

Additional information is available at the end of the chapter

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Abstract

The recent outbreak of Ebola viral disease (EVD) in West Africa reminded us that an effective anti-viral treatment still does not exist, despite the significant progress that has recently been made in understanding biology and pathology of this lethal disease. Currently, there are no approved vaccine and/or prophylactic medication for the treatment of EVD in the market. However, the serious pandemic potential of EVD mobilized research teams in the academy and the pharmaceutical industry in the effort to find an Ebola cure as fast as possible. In this chapter, we are giving the condensed review of different approaches and strategies in search of a drug against Ebola. We have been focusing on the review of the targets that could be used for *in silico*, *in vitro*, and/or *in vivo* drug design of compounds that interact with the targets in different phases of the Ebola virus life cycle.

Keywords: small molecule inhibitors, Ebola virus, drug design, protein targets, structure and action

1. Introduction

Ebola virus (EBOV) is a (-)ssRNA filovirus, known for its extreme insidiousness. Case fatality rates of the current 2014 outbreak in West Africa are 50–70% [1]. Transmission of EBOV is predominantly via physical contact with bodily fluids of infected people or corpses and can be limited by a proper combination of early diagnosis, contact tracing, isolation of patients, infection control, and safe burial [2, 3].

The infection is characterized by suppression of the immune system and of the systemic inflammatory response, followed by the collapse of the vascular and immune systems, and

multi-organ failure. The patient dies from a combination of dehydration, massive bleeding, and shock. Currently, there are no approved drugs for the hemorrhagic fever caused by EBOV. However, there is some conflicting clinical evidence that antibodies isolated from survived patients may be effective in the treatment of the infection caused by EBOV [4, 5].

In this book chapter, we will review possible targets that are being used or could be used for structure-based design of small molecule inhibitors against EBOV. We will start the chapter with a brief review of the structure and action of EBOV, and then we will describe the targets along with possible hotspots. Additionally, we will present a short review of small molecules that could be used as medicaments against EBOV.

2. Structure and action of Ebola virus

Knowledge about the life cycle of EBOV, supported with structural information, is crucial for the successful design of antivirals. This is the reason why we will start our review with the structural information about EBOV.

The RNA genome of Ebola virus contains information for constructing seven proteins (GP, VP24, VP30, VP35, VP40, L-protein, nucleoprotein), which assemble with the genomic RNA to form one of the most lethal viruses [6]. EBOV's RNA exists in antisense form, which means that it cannot be used for proteins' production directly [7]. For protein building, the complementary copy of the negative RNA is required, which is produced with the help of the viral polymerase (L-protein). Not all genes are transcribed fully through. For example, transcription of GP gene could lead to three different proteins: GP, sGP, and ssGP. A small nonstructural sGP (secretory glycoprotein) is the protein that is efficiently secreted from infected cells. sGP acts as mimic of full GP that is presented at the surface of EBOV, this mimicry is one of the ways of how the Ebola virus deceive the immune system, by urging the body to develop antibodies to sGP instead of full GP [8, 9]. EBOV is enclosed by a membrane hijacked from an infected cell and covered with Ebola glycoproteins. A layer of matrix proteins supports the membrane on the inside and holds a cylindrical nucleocapsid at the center, which stores and delivers the RNA genome.

The main task of Ebola glycoprotein (GP) is binding to receptors located on a host-cell surface and getting the Ebola genome inside. GP is distributed throughout the whole viral membrane surface and the large proportion of oligosaccharides, which are attached to the GP making the virus unrecognizable for the adaptive immune system. GP is a highly dynamic protein that snaps into different shapes when it binds to a cell surface, driving the virus close enough to get fused with the membrane.

The viral matrix is composed of two proteins: VP40 and VP24. The function of VP40, known as the major matrix protein, is to assist in the process of budding. VP40 hexamers form layers that support the nucleocapsid in the middle of the virion. The minor matrix protein VP24 is involved in interferon antagonism.

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Another important structural element of EBOV is the nucleocapsid, which is located in the middle of the virion. The nucleocapsid is wound in a regular helix shape 50 nm in diameter. The nucleocapsid is composed of a series of viral proteins, which are attached to EBOV's genome, 19 kb linear, negative-sense RNA. The nucleocapsid is composed from the inner part where RNA is packed with NP protein, and the outer shell is composed of VP24, VP30, VP35, and polymerase (L-protein).

The detailed cross section through EBOV illustrated by Goodsell is shown in **Figure 1** [10].

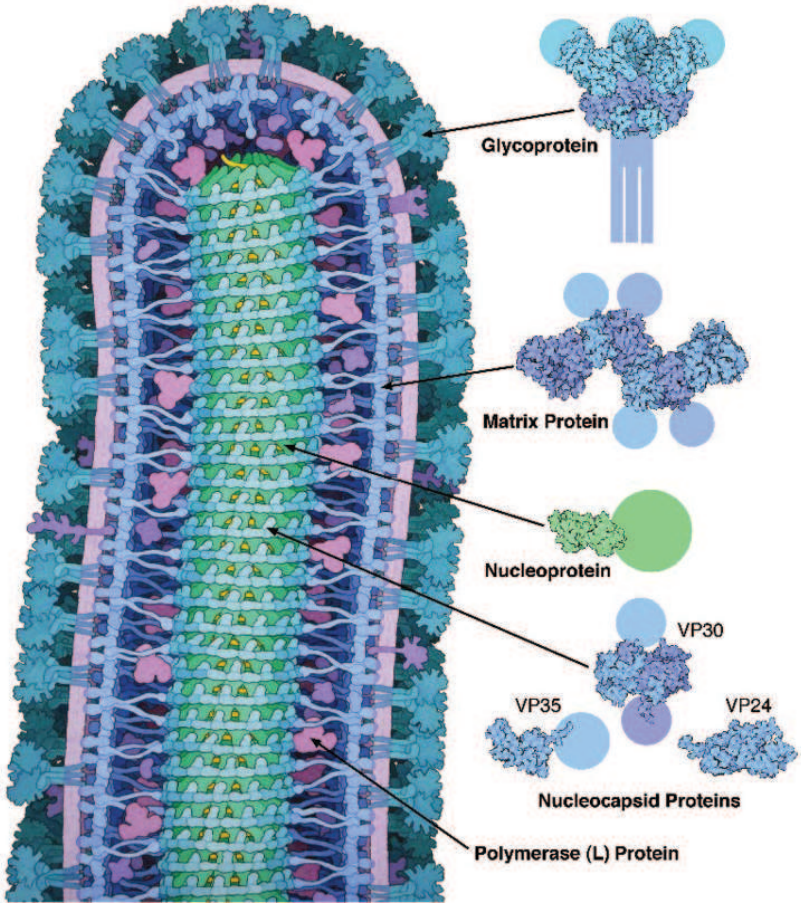

Figure 1. Cross section through of EBOV virion. The proteins are shown in blue, green, and magenta, the RNA genome in yellow, and the membrane in purple. The detailed structures are shown to the right. The nonresolved portions are shown with schematic circles (doi: 10.2210/rcsb_pdb/mom_2014_10).

The information about the function of EBOV's proteins about its structural data along with potential targets is collected in **Table 1**.

Protein	Function	Structures	Potential target for the protein
NP	Nucleocapsid and inclusion of body formation; encapsulation of RNA genome, replication and transcription of viral genome. Binds VP35, and associates with VP30 and L.	PDB structures: 4z9p, 4ypi, 4zta	target interactions with or binding sites on VP35, VP30 and L
VP35	Associates in RNP complexes, binds RNA. Together with L, it forms replicase–trancriptase holoenzyme, initiates transcription. EBOV VP35 suppresses Type-I IFN production.	PDB structures: 4qaz,4qb0,4ypi, 4z9p,4zta,4ztg, 4zti	Target basic patch of VP35 around the Arg312 to prevent VP35 binding to double stranded DNA (dsRNA) and promote IFN production.
VP40	Viral matrix protein. Accumulates at cellular membrane proliferation sites, responsible for viral budding. Assocites with microtubules and outer cell membranes.	PDB structures: 1h2c, 1h2d, 2kq0, 4eje, 4ldb, 4ldd, 4ldi, 4ldm	Compound 5539-0062 inhibits interaction of VP40 with Tsg101 a factor involved in endosomal protein sorting.
GP1,2	Transmembrane protein that mediates viral attachment to the cell membrane and promote viral entry. Some domains of this protein may have immunosuppressive properties.	PDB structures: 2ebo, 2rlj, 3csy, 5f18,5f1b	Disruption of interaction between primed EBOV-GP and NPC1. Interaction of EBOV-GP with neutralizing antibodies (ZMAPP).
VP30	Secondary nucleoprotein, binds NP and ssRNA, part of ribonucleoprote-in complex. VP30 in oligomeric form is neded for the activation of transcription.	PDB structures: 2i8b, 3v7o, 5dvw	Target basic patch around LYS 180 to inhibit the activation of transcription.
VP24	Secondary matrix protein, it colocalizes with VP40 and has important function in nucleocapsid formation. VP24 inhibits cellular responses to IFN.	PDB structures: 3vne, 3vnf, 4d9o, 4m0q, 4or8, 4u2x	Inhibition of VP24 binding to karyopherin.
L protein	RNA dependent RNA polymerase; binds VP35 to form replicase transcriptional factor.	Only sequence	Poly-adenylation, profreading function or interaction with ZAP

This is based on a template by Shurtleff *et al*. [11].

Table 1. EBOV protein functions and availability of structural information[a].

3. Collection of targets for small molecule inhibitor design

In this section, we describe different targets and strategies for structure-based design of EBOV's small molecule inhibitors. Based on the knowledge of EBOV action, different strategies for

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curing Ebola viral disease are proposed: prevention of EBOV adhesion to host cells (monoclonal antibodies; inhibitors of host-cell receptors, …) [12]; inhibition of viral escape from endosome (inhibition of NPC1, protease inhibitors) [13]; reviving and enhancing intracellular innate immunity; orthogonal RNA destruction mechanism targeting essential Ebola genes; inhibiting viral RNA processing (inhibition of RNA polymerase) [14]; disruption of viral assembly (nucleoprotein, VP40, VP24, VP35), etc.

Some of these mechanisms will be described in this section along with proposed targets. The ideogram where the connection between Ebola virus life cycle and possible therapeutic targets is shown in **Figure 2**.

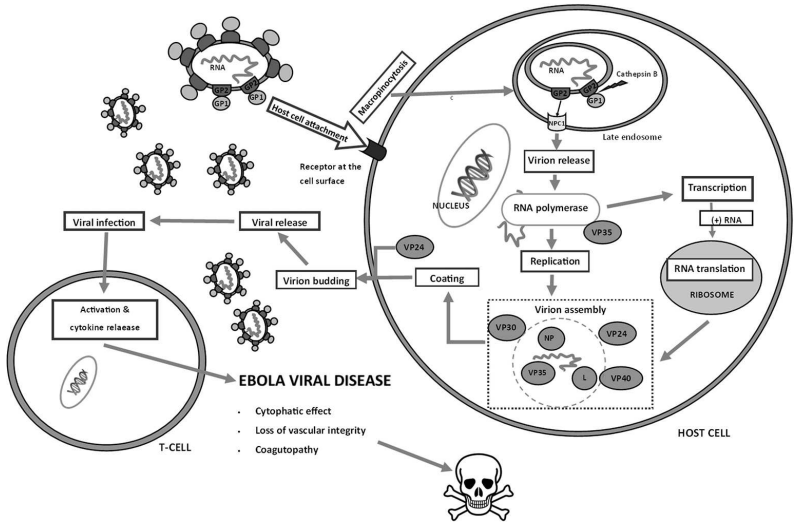

Figure 2. The ideogram of EBOV life cycle along with connections between possible therapeutic targets for the treatment EVD.

3.1. Nucleoprotein

The main function of nucleoprotein (NP) is encapsulation of the viral genome. EBOV's RNA located in the cage assembled mainly from nucleoprotein, which serves also as a scaffold for additional viral proteins forming nucleocapsid, is protected from the action of nucleases. The model of cross-section of RNA's cage composed from NP and VP35, based on recent X-ray diffraction structure (PDB-ID:4ypi) is shown in **Figure 3** [15]. A possible strategy to fight against EBOV is to prevent assembly of the RNA's cage. Binning *et al*. showed an elegant way to disrupt interaction between VP35 and nucleoprotein that is important for viral nucleocapsid's formation, by binding RNA aptamers [16]. Using electron microscopy technique, Noda *et al*. clearly showed that coexistence of NP and VP35 in proper ratio is important for nucleocapsid assembly [17]. Recently, Kirchdoerfer *et al*. have established that the N-terminal portion of VP35 acts as a chaperone for the viral nucleoprotein NP. They have also shown that VP35

prevents premature RNA binding and oligomerization of NP. Removal of VP35 peptide leads to NP self-assembly, which likely causes a conformational change between NP N- and C-terminal domains, facilitating RNA binding. The detailed knowledge of interactions between NP and VP35, supported with structural information (PDB-IDs: 4zta, 4zti, and 4ztg), which are conserved among filoviruses could provide key targets for structure-based drug design [18].

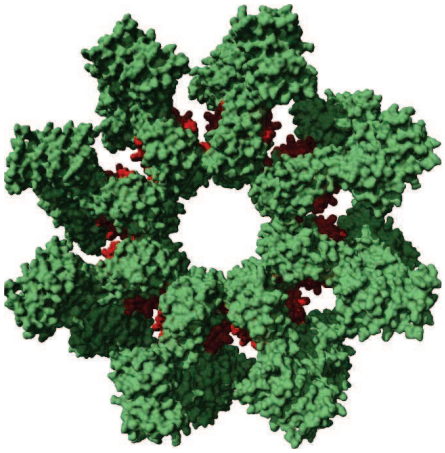

Figure 3. A model of axial cross section of the inner part of EBOV's RNA cage assembled from nucleoprotein (green) and VP35 (red). A model is based on PDB-ID:4ypi. YASARA (http://www.yasara.org) was used for visualization.

3.2. VP35 as a target for EVD therapeutics

EBOV's VP35 protein is a multifunctional protein, together with nucleoprotein and RNA, which is the main building block for the assembly of the nucleocapsid. Another important function of this protein is inhibition of interferon IFN-α/β production [19]. Additionally, VP35 is also a cofactor of the viral RNA polymerase. In this subsection, we will introduce the strategy for inhibition of VP35's polymerase activity with small molecules.

Using well-established *in silico* methods, followed by biological tests, NMR validation, and X-ray ligand:receptor structures, Brown *et al*. identified pyrrolidinone-based structures as promising inhibitors [20]. The ability of the identified compounds to inhibit interactions between VP35 and NP was evidently confirmed *in vitro* and by cell-based assay. The structure of inhibitor GA107 within the binding pocket of VP35 is shown in **Figure 4**. From the interaction map (**Figure 4A**), we may observe numerous contacts between GA107 and VP35's hydrophobic residues, including highly conserved Val 245, Ile 295, and Phe 328. This binding mode is also supported by two hydrogen bonds with Lys 251 and Gln 241. The authors of presented study have also shown that removal of either carboxylic acid or ketone moieties rapidly eliminates binding of certain ligands to the receptor. This fact is interesting from the aspect that Lys 251 is important for the VP35 polymerase cofactor function, as mutations at Lys 251 lead to loss of

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this function [21]. Detailed molecular dynamics study of interaction of complexes between inhibitors and VP35 have revealed induced structural changes of the VP35 protein during binding event [22].

Inspired by the success of Brown *et al*., we used Glide XP docking program to check the possibility of binding some small molecules, from our small in-house library of polyphenolic compounds [23], to the VP35 target. Surprisingly, we found that EGCG-green tea polyphenol could also nicely fit a druggable pocket of the protein. The pose of EGCG within VP35 IID is shown in **Figure 4B**. The number of hydrogen bonding contacts is greater than in the case of GA107 binding. The interaction between EGCG and VP35's Lys 251 is not detected in this case, but it is possible due to plasticity of the receptor site.

Ebola virus specifically inhibits the dsRNA (double stranded RNA) within cells via a sequestration process. The molecular basis of such sequestration is shown in **Figure 5**, where the complex between VP35 and model of dsRNA is presented. Mutagenesis studies have shown that critical residues for binding dsRNA are Phe 239, Arg 312, Arg 322, and Lys 339. Mutation of these residues to Ala results in VP35 complete loss of its ability to bind dsRNA, and thus were also unable to suppress IFN-β promoter [24]. Compounds like Ampligen, a immunomodulatory double stranded RNA, may be able to overcome this deficiency in host response [24, 25].

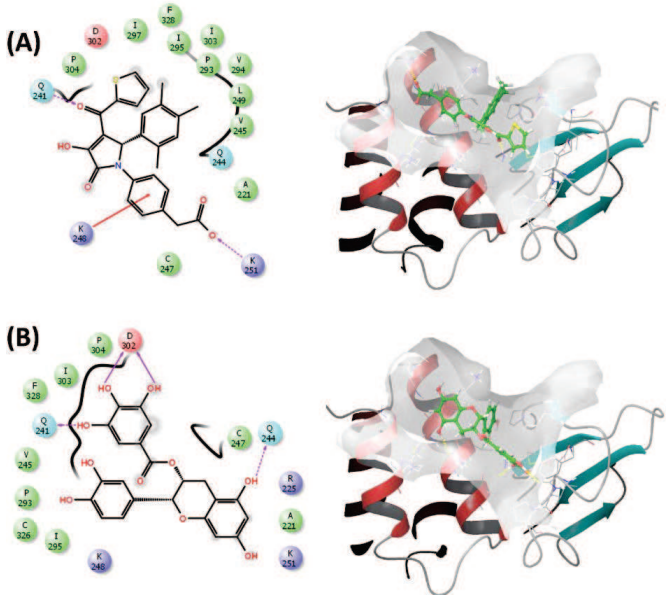

Figure 4. The interaction of compound GA017 (A) and EGCG (B) with proposed binding site of VP35. A docking protocol using Schrodinger's Glide XP docking is performed to get ligand: receptor complexes (http://www.schrodinger.com).

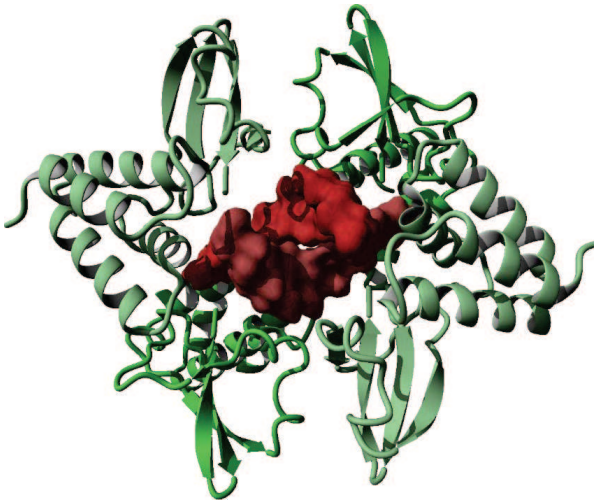

Figure 5. Interaction of VP35 tetramer with dsRNA. The picture shows how VP35 sequestrates dsRNA, which is vital for immunoresponse at cellular level.

3.3. VP24 is vital for budding and acts also as interferon antagonist

VP24 is a secondary matrix protein that is colocalized with VP40 in virions and it is important for intracellular nucleocapsid assembly. It is also a type I interferon antagonist. This protein plays an important role in the budding. VP24 by itself has features that are common to the viral matrix protein (VP40), such as hydrophobicity, membrane binding, and self-oligomerisation [26]. An important role of VP24 in the process of nucleocapsid formation has been demonstrated using electron microscopy [27].

Secondary matrix protein VP24 is one of the virion proteins that play a crucial role in Ebola virus disease pathology [28]. Recently, VP24 was recognized as a major virulent factor. The virulent action of VP24 is initiated by binding of interferon (IFN) to the IFN receptors located on the host cells' surface. The activation of interferon leads to activation of STAT1 protein via Janus tyrosine kinase (JAK1). Activated by phosphorylation, STAT1 forms a dimer that subsequently interacts with KPNA5 (karyopherin 5, also known as importin). KPNA5 is vital for translocation of STAT1 dimer into the nucleus where STAT1 acts as a transcription activator for the expression of IFN stimulated genes. Recent studies have also shown that VP24 competes with STAT1 to bind KPNA5. These studies have shown that KPNA5 binds to VP24 more than 100 times tighter than to the STAT1:STAT1 dimer [29]. The sequestration of free KPNA5 finally results in prevention of STAT1:STAT1 entrance into the nucleus and blocks the subsequent activation of numerous genes that are involved in antiviral activity. The mechanism of VP24 interferon signal path inhibitory action is shown in **Figure 6**.

Garcia-Dorival *et al*. have elucidated VP24's interactome to get better overview of protein functionality. Using label-free quantitative proteomics, they confirmed several known

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interactions between VP24 and cell proteins and discovered some new ones. They highlighted the interaction between VP24 and integral membrane protein ATP1A1, which is involved in osmoregulation and cell signaling. The researchers have deduced activity of EBOV by disruption of the interaction between ATP1A1 and VP24 with small molecule inhibitor. ATP1A1 could be a promising target for structure-based design of cure against EVD [30].

Another successful approach of protection against EBOV infection is to use phosphorodiamidate morpholino oligomers (PMOs), which are able to bind mRNA in a sequence-specific fashion. The study of Warren *et al*. revealed that PMOs targeting VP24 alone was sufficient to protect Rhesus monkey from lethal infection, while targeting VP35 alone resulted in no protection. The outcome of the study was to confirm VP24 as a key factor for virulence, and additionally, the researchers highlighted PMOs as promising therapeutics against EBOV infection [31].

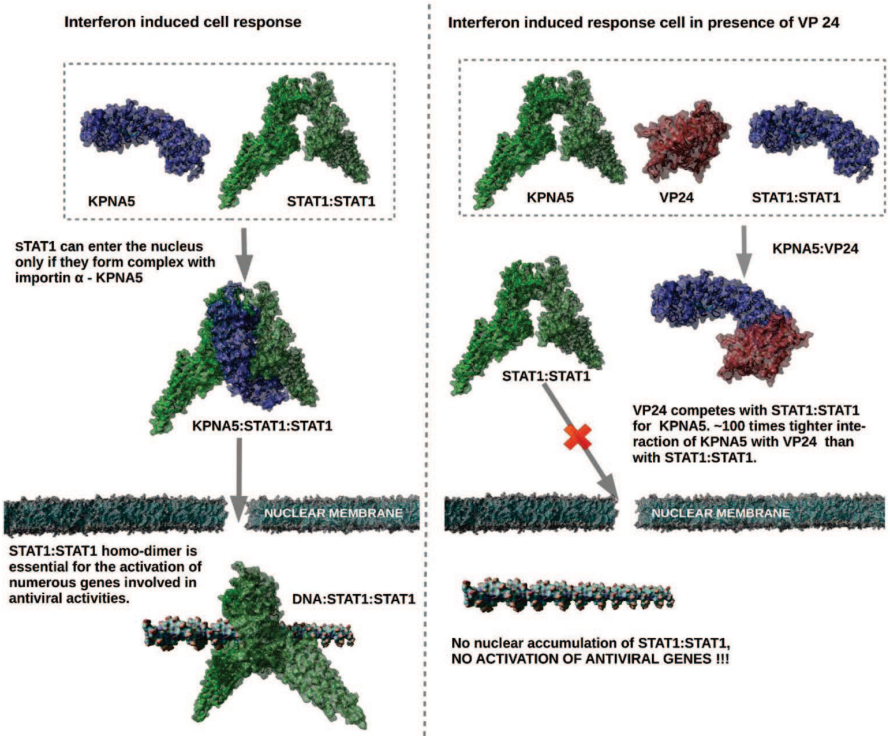

Figure 6. VP24 is an inhibitor of interferon-induced signal path. Normal interferon-induced cell response that results in expression of numerous genes involved in antiviral activity (left), while interferon-induced cell response in the presence of EBOV VP24 is presented on the right side.

Molecules that bind to the VP24 surface where KPNA5 interacts could be inhibitors of viral action of VP24. Pleško *et al*. suggested some compounds from Mediterranean plants as possible

inhibitors of interaction between VP24 and KPNA5 [23]. Some of these compounds are listed in **Figure 7**.

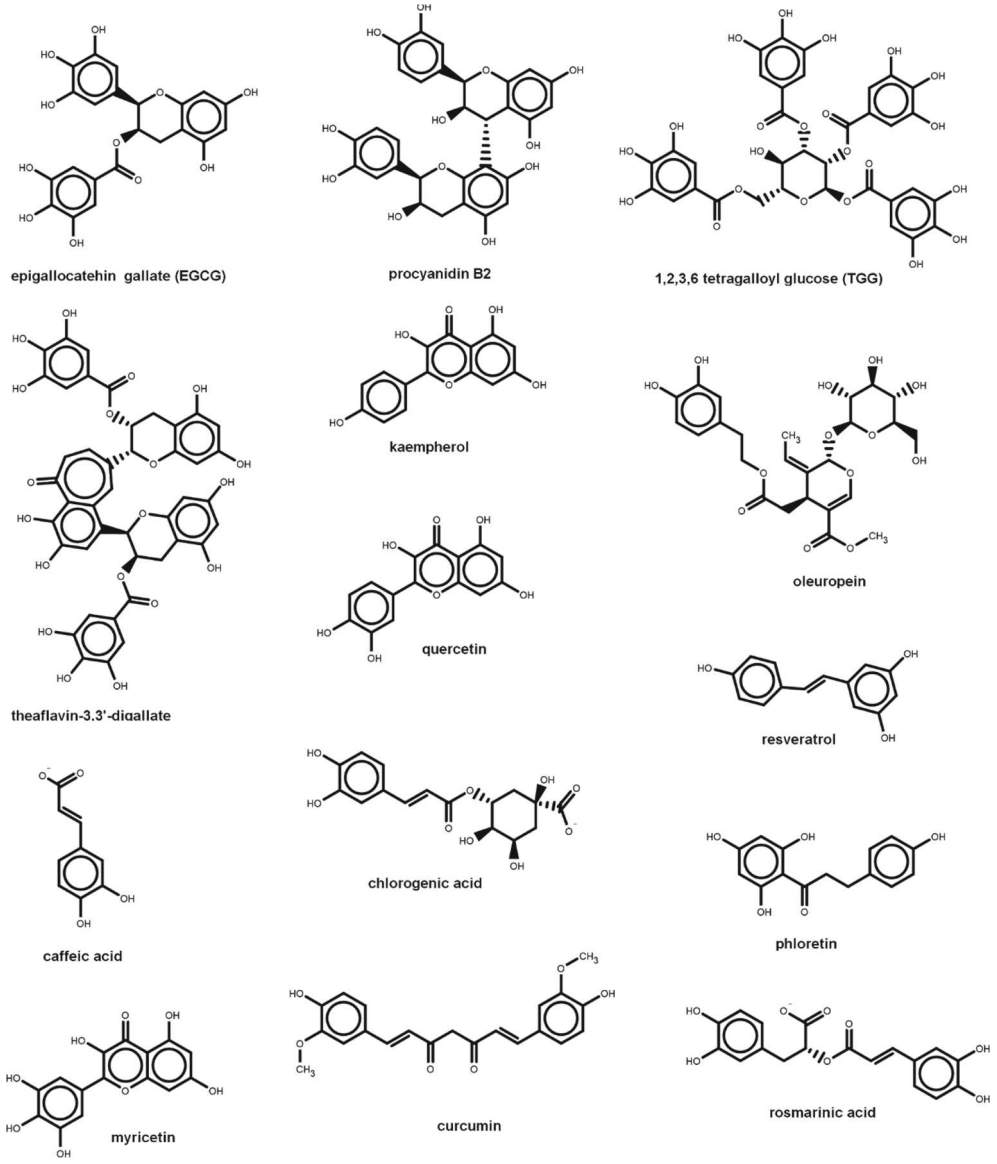

Figure 7. The collection of natural compounds selected for *in silico* search for inhibitors of the interaction between KPNA5 and Ebola's VP24 [23].

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This study by Pleško *et al*. has shown that several plant polyphenols, such as epigallocatechin gallate (EGCG), 1,2,3,6-tetragalloyl glucose, theaflavin-3,3'-digallate, and oleuropein, have relevant *in silico* affinity to VP24 [23]. *In silico* studies of Kasmi *et al*. have also indicated that oleuropein, an active ingredient of olive leafs, can interact with EBOV's proteins VP24 and VP30 [32]. Poses of EGCG and oleuropein within binding site of VP24 are shown in **Figure 8**.

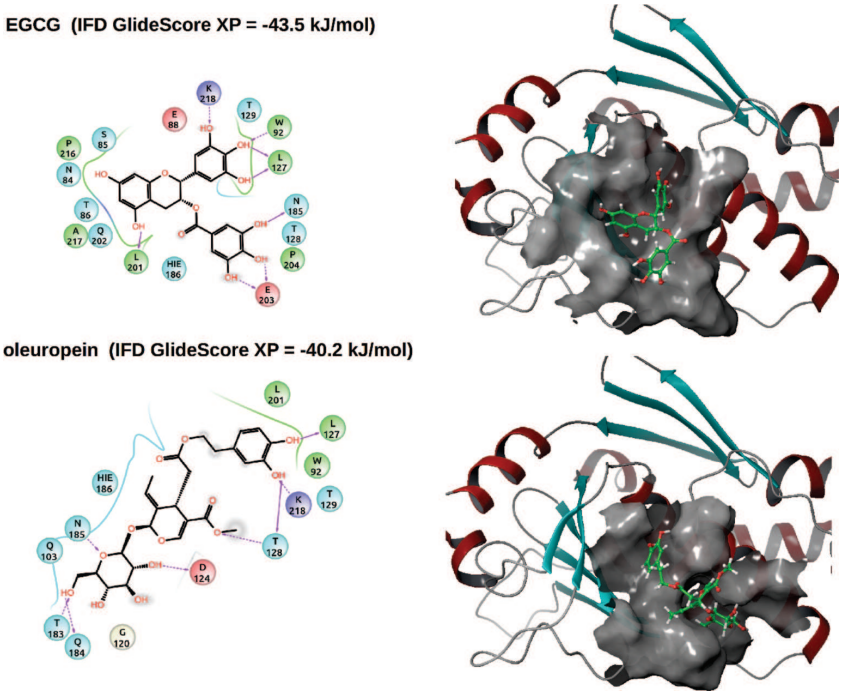

Figure 8. Poses of EGCG and Oleuropein within binding site of VP24. Poses were obtained using Schrodinger's Prime/Glide-Induced Fit Docking protocol [33] (http://www.schrodinger.com).

3.4. Secondary nucleoprotein VP30

The Ebola protein VP30 is known as a secondary nucleoprotein, the protein is colocalized with the nucleoprotein (NP) in inclusion bodies when both proteins are coexpressed *in vitro*. It is evident, from a crystal structure, that the VP30 C-terminal domain is responsible for the interaction with the NP [34]. It has been also shown that VP30 acts as a transcriptional activator only in oligomeric form [35], while binding to the NP oligomerization of VP30 is not required. *In vitro* experiments have shown that the N-terminal domain of the protein is involved in interaction with filoviruses' single-stranded RNAs. Mutagenesis studies have demonstrated Glu197, Arg179, Lys180, and Lys183 in VP30 as key residues essential for nucleocapsid association and transcription activation [8]. The Lys180 centered binding site may be appropriate as a target for small molecules that will act as inhibitors of transcriptional activation and

thus behave as effective antiviral agents. The structure of VP30 in its dimeric form that is responsible for activation of transcriptional process is shown in **Figure 9**.

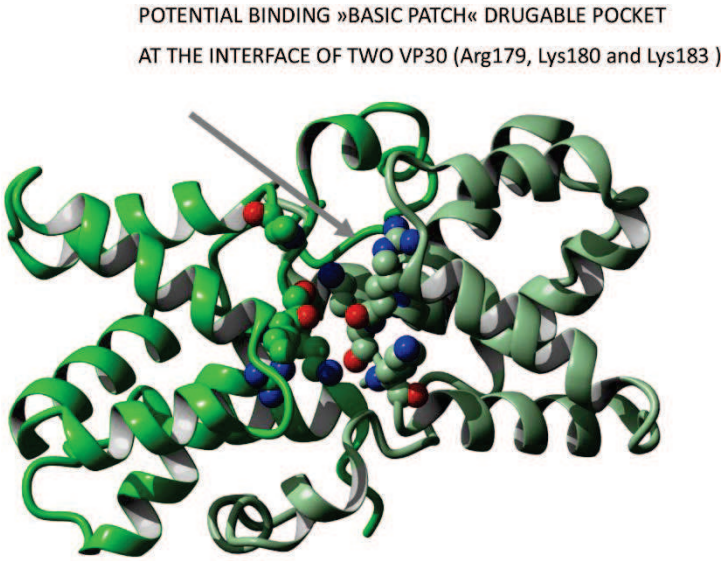

Figure 9. VP30 dimer is required for the activation of transcriptional activity.

The study of Modrof *et al*. has shown that phosphorylation of VP30 impairs transcription of EVD. Only slightly phosphorylated VP30(6A), processed by replacement of six phosphoserines with alanines, is still able to activate EBOV-specific transcription. Mutated VP30 is not able to form inclusion bodies induced by the NP. The authors have also observed that inhibition of intracellular phosphatases with okadaic acid has similar negative impact on the transcriptional activity than replacement of VP30's phosphoserines with aspartate residues. Okadaic acid has no impact on the transcriptional activation by VP30(6A) [36].

3.5. The viral matrix protein VP40

The viral matrix protein VP40 is the most abundant among Ebola virus' proteins, and it is encoded by the most conserved filovirus gene. VP40 is responsible for numerous functions of Ebola virus by rearranging into different oligomeric structures [37]. Each of the structure has different role in the EV life cycle. VP40 dimer traffics to the cellular membrane, where the electrostatic interactions induce its rearrangement into a linear hexamer. VP40 hexamers form a multilayered matrix filament that is critical for budding of virion. Another structural rearrangement of VP40 is in the form of an octameric ring. The RNA-binding VP40 octameric ring is important for the regulation of viral transcription [38]. Different assemblies of VP40 are shown in **Figure 10**.

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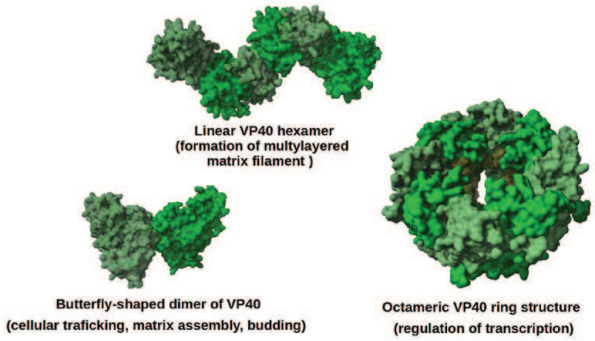

Figure 10. Different assembly of VP40 is required for its distinct functions [39].

The VP40 dimer with some of the potential sites for inhibition of viral budding is shown in **Figure 11** [39]. The area inside the magenta sphere represents the N-terminal domain interface that is responsible for the dimer formation. This interface domain involves two alpha helices 52-65 and 108-117, it is mainly composed of hydrophobic residues, thus the hydrophobic effect could be the driving force in the dimerization. It has been shown that mutation of Leu 117 disrupts the formation of VP40 dimers and inhibits viral budding. The C-terminal domain hexameric interface is shown in red. Met 241 and Ile 307 are residues that are mainly involved in the formation of hexamers. Disruption of the hexameric domain by a set of mutations reduces viral budding from plasma membranes. The cationic patch (blue area) consists of numerous positively charged residues: Lys 224, Lys 225, Lys 274, and Lys 275, which may interact via electrostatic interactions with the anionic leaflet of the plasma membrane. Mutation of these Lys residues reduces the viral budding. The area within the green sphere represents a hydrophobic loop, which penetrates into the plasma membrane—this step is necessary for viral budding.

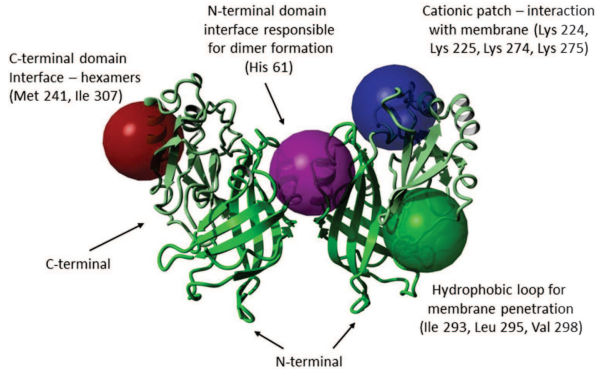

Figure 11. Potential binding sites of small molecules to block viral budding activity of VP40 dimer.

3.6. Ebola virus surface's glycoprotein

The EBOV glycoprotein decorates the surface of virions, and it is responsible for the attachment of virus to the receptor cells and subsequent entry into the cells. Glycoprotein ($GP_{1,2}$) is a transmembrane protein composed of two subunits, GP_1 and GP_2, linked by a disulfide bond. N- and O-linked glycosylation accounts for about one-third of the molecular mass [40]. The posttranslational cleavage of the precursor GP into GP_1 (~160 kDa) and GP_2 (~38-45 kDa) is required for successful infection. Both subunits have an important role in the mechanism of viral infection: heavily glycosylated GP_1 is responsible for the attachment to host cells, while GP_2 is responsible for fusion of the viral and host-cell membrane once the virion has entered the endosome (lysosome). Both subunits have functional domains that are reported to have activities not connected with binding and entry. GP_1, for example, has a domain at the N-terminus, which suppresses lymphocyte blastogenesis *in vitro*. Released GP_1 also has a mucin-like domain (MLD) decorated with glycans. Its function is to prevent neutralizing antibodies from binding to $GP_{1,2}$ at the viral surface. GP_2 has N-terminal motif that exerts immunosuppressive activity. A variety of host cell factors have been connected with EBOV binding and entry: DC-SIGN, L-SIGN, B-integrins, folate receptor A, etc. [41, 42]. Once the virion is inside the endosome, endosomal proteases, mainly cathepsin B, are required for removal of the GP_1 subunit from GP_2. The interaction between NPC-1 and GP_2 has been identified as vital for fusion of the viral and cellular membrane. The role of NPC-1 in EBOV life cycle and the possibilty to use NPC1 as a valuable target for the inhibition of EBOV's escape from the late endosome will be described in a separate subchapter.

An innovative approach to prevent adhesion of EBOV to host cell is blocking receptors (DC-SIGN, L-SIGN) located at the cell surface with glycodendritic structures. DC-SIGN (dendritic cell specific intercellular adhesion molecule-3-grabbing non-integrin) is one of the most important pathogen recognition receptors [43]. This lectin is specific for the recognition of highly mannosylated branched oligosaccharides of EBOV's glycoprotein. Since the affinity between the single copy of the oligosaccharide and its respective receptor is often weak, researchers used the approach of multivalency to enhance the activity. Researchers have developed a series of dendritic glycoconjugates such as Boltorn-type glycodendrimers, glycodendrofullerenes, and virus-like gycodendronanoparticles [44–46]. Some representatives

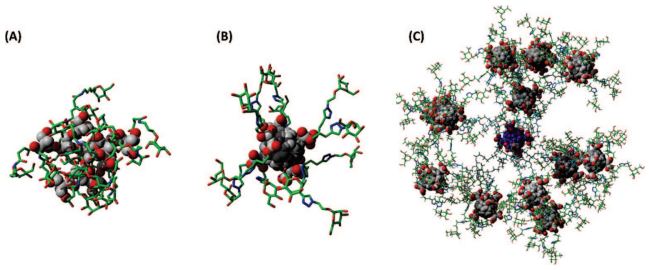

Figure 12. Dendrimeric glycoconjugates: (A) Boltorn-type (16 copies of mannose); (B) glycodendrofullerene (12 copies of mannose); (C) tridecafullerene's superstructure decorated with 120 copies of mannose.

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of dendrimeric structures that could successfully bind to the receptors at the host cell surface with high affinity are depicted in **Figure 12**.

In the most recent approach, researchers have synthesized globular multivalent glycofullerenes that act as potent inhibitors in a model of EBOV infection [11]. They recognize hexakis adducts of 60-fullerene as useful building blocks since the obtained products maintain globular shape and with the aspect that it is relatively easy to control the size and multivalency.

Targeting GPs exposed at the surface of EBOV with neutralizing antibodies is one of the most often used strategies in the fight against Ebola. The GP structure represented in **Figure 13** includes neutralizing antibodies from a person who survived infection by the virus. The antibodies bind to the bottom side of the glycoprotein, the portion of protein essential for the process of fusion, which is not usually decorated by oligosaccharidic chains.

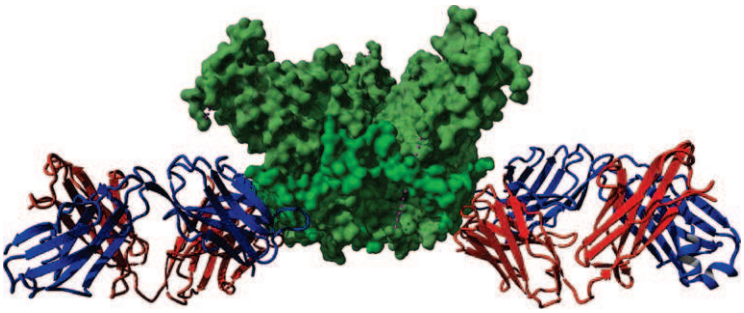

Figure 13. Interaction of glycoprotein with a monoclonal antibody from survived patient (PDB-ID:3csv).

3.7. The human Niemann-Pick disease type C1 protein

The human Niemann-Pick disease type C1 (NPC1) is a membrane protein that is predominantly required for intracellular transport of cholesterol and lipids in mammals. A deficiency of this protein leads to abnormal accumulation of lipids and cholesterol within cells. Recent studies of Côté *et al*. indicated NPC1 protein as an important step for cellular entry of Ebola virus and some other filoviruses [47]. The authors have shown that cultured cells with mutant NPC1 are more resistant to infection with Ebola or Marburg (MARV) filovirus than wild-type cells. Another important finding was that cells treated with imipramine, a tricyclic antidepressant that causes a cellular phenotype similar to NPC1 deficiency, are also more resistant to the infection with EBOV and MARV than untreated cells. In mouse models of EBOV and MARV infection, the animals with deficiency of NPC1 had significantly greater survival ratio than wild type mice. Cunnigham *et al*. screened a small library of compounds and showed that a small molecule inhibitor based on benzylpiperazine-adamantane scaffold almost completely inhibits EBOV infection by blocking interaction between human NPC1 and viral GP_2 [47].

The proposed model for Ebola virus entry via binding of Ebola virus to NPC1 is presented in **Figure 14**. Binding of EBOV to the receptors (DC-SIGN [48, 49], TIM-1 [50], …) at the cell surface

is the first and essential step in the viral infection. After successful attachment, the viruses undergo endocytosis and enter the cell internalized in late endosomes. In the next phase, cysteine proteases, primarily cathepsin B, cleave EBOV-GP to a 19-kD fragment. The cleaved EBOV-GP serves as a ligand for NPC1, a multimembrane spanning cholesterol transport protein. After binding of EBOV-GP to NPC1, EBOV nucleocapsid is released into the cell cytosol.

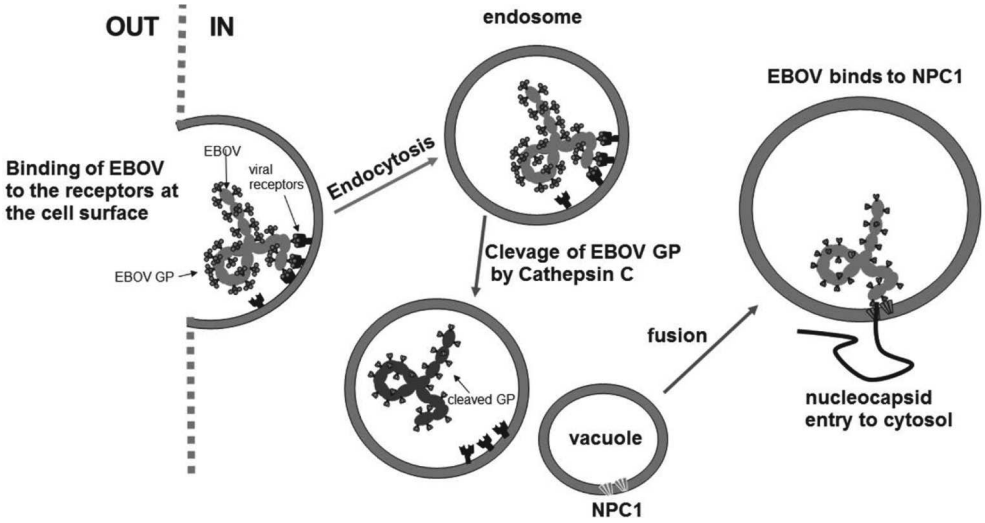

Figure 14. In the first phase, EBOV-GP mediates viral attachment to the cell membrane, which is followed by endocytosis. The glycoproteins that cover the surface of EBOV are cleaved by cathepsin B protease that removes highly glycosylated domains (GP1) to expose the putative receptor-binding domain (GP2) of the glycoprotein. In the next phase, the vacuole that contains NPC1 protein and endosome containing virus fuse together. The virus binds to NPC1 and then is released into the cytoplasm.

We are expecting further development of antivirals for blocking endosomal escape since the structure of the EBOV's GP_2 to its endosomal receptor Niemann-Pick C1 has been recently solved [51].

4. Recent status of EBOV drugs

The most recent outbreak of Ebola viral disease in West Africa 2014 initiated a worldwide activity of searching for an effective cure against one of the most threatening diseases. Numerous bio/med/pharm researches are devoted to the investigation of the action of EBOV at the molecular level as a way to find optimal strategies to combat the virus. In this section, we will describe some small molecule inhibitors that have proven *in vitro* or *in vivo* activity against EBOV infection. In this section, we have followed compound classifications proposed

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by Picazo *et al*. [52], which is based on their reported mechanism of action (e.g. inhibition of viral replication) and/or documented molecular mechanism (e.g. kinase inhibitors). This section summarizes the identification of numerous compounds with a promising anti-Ebola activity. The reader could find more detailed information related to EBOV small molecule inhibitors in the review articles of Shurtleff *et al*. [11] and others [52–57].

4.1. Viral transcription modulators

An example of small molecules that alter the process of transcription of EBOV's genome is favipiravir (**Figure 15**), which was developed as a selective inhibitor of influenza virus replication (inhibits the viral RNA-dependent RNA polymerase). The antiviral potential against EBOV of favipiravir has been recently tested in a small animal model. Total prevention of mortality of the small animals subjected to the EBOV infection was achieved in these experiments [14]. The nucleoside analog BCX4430 (**Figure 15**) is another compound from the class of viral transcription modulators. BCX4430 is active in vitro against negative-sense RNA-viruses including EBOV. Laboratory tests of BCX4430's anti-EBOV activity have shown a survival rate between 90% and 100 % of experimentally infected mice. Further experiments reported success with a protective action of BCX4430 in EBOV infection of non human primates [58]. C-c3Ado and c3Nep, which were first reported as S-adenosylhomocysteine hydrolase (SAH) inhibitors, are additional examples of compounds with protective action against small animal experimental EBOV infection [59].

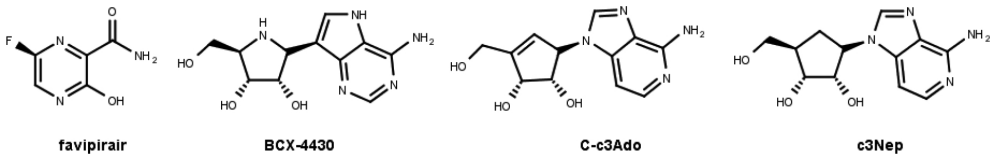

Figure 15. Representative compounds that modulate viral transcription with reported anti-Ebola viral activity.

4.2. Viral entry and fusion modulators

The fusion of EBOV and host cell membrane represents the first phase of EBOV infection [60]. It has been shown that proteolysis of EBOV's glycoprotein $GP_{1,2}$ represents an obligatory step in virus' life cycle. Proteolytic degradation of $GP_{1,2}$ was successfully blocked using inhibitors of cysteine proteases, among which E-64d (unselective protease inhibitor), CA-074 (selective cathepsin B inhibitor), FY-DMK (Cathepsin B/L inhibitor) and Z-FY-(t-Bu)-DMK (Cathepsin L inhibitor), which are shown in **Figure 16** [60, 61]. It was shown that Leupeptin (inhibitor of serine protease) and CIS23631927 – Cat L inhibitor were able to reduce EBOV infection in macrophages and human embryonic cells [62].

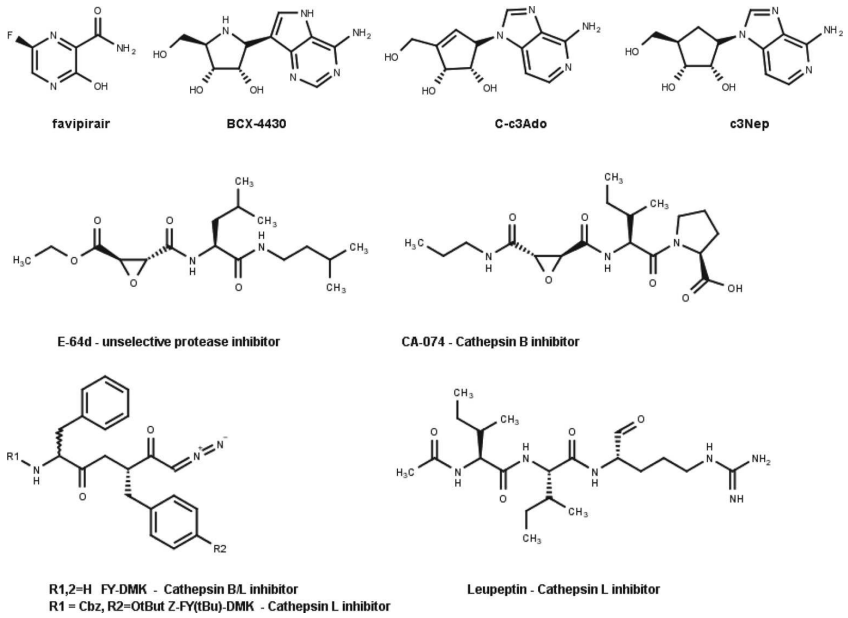

Figure 16. Protease inhibitors with anti-Ebola activity.

Basu *et al*. reported that compounds based on benzodiazepinic scaffold could be used as inhibitors of EBOV entry process. The authors hypothesized that benzodiazepines interfere with proteases by binding to a hydrophobic pocket of the EBOV GP_1-GP_2 interface. A representative benzodiazepine is shown at **Figure 17** [63].

After fusion, the entry of viral particles is followed by endocytosis, which is dependent on a functional cytoskeleton. The research of Yonezawa *et al*. showed the importance of microtubule stabilisation in the process of viral entry. They observed enhanced virus entry in the presence of microtubule stabilizer taxol, on the other hand microtubules' destabilization using nocodazole and colchicine significantly impaired entry process [64].

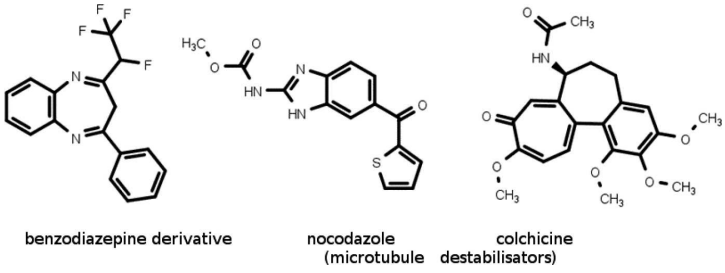

Figure 17. The benzodiazepine derivatives hinder proteases and two microtubules' destabilization.

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After fusion with the membrane and subsequent macropinocytosis, the Ebola virus is catched in late endosomes. Carette *et al*. indicated that Niemann-Pick C1 (cholesterol transporter) protein assists in virus escape from endosome to host cell's cytosol [65]. They also showed that impairment of NPC1 (NPC1 phenotype) function of the cell by genetic manipulation leads to complete resistance to EBOV infection. Further research showed that resistance to EBOV infection could be achieved also with U18666A and imipramine, two agents that are known to induce the NPC1 phenotype. In line with these researches, Cote *et al*. indicated piperazine derivative 3.47 as an effective inhibitor of cellular entry by viruses pseudotyped with EBOV-$GP_{1,2}$ [47]. Screen of database of FDA-approved drugs revealed that clomiphene and toremifene (estrogen receptor modulators) strongly inhibited EBOV infection *in vitro*. The action of these two compounds is resulted in accumulation of cholesterol in endosomes, which is typical for impaired NPC1 [66]. The most representatives of NPC-1 inhibitors are shown in **Figure 18**.

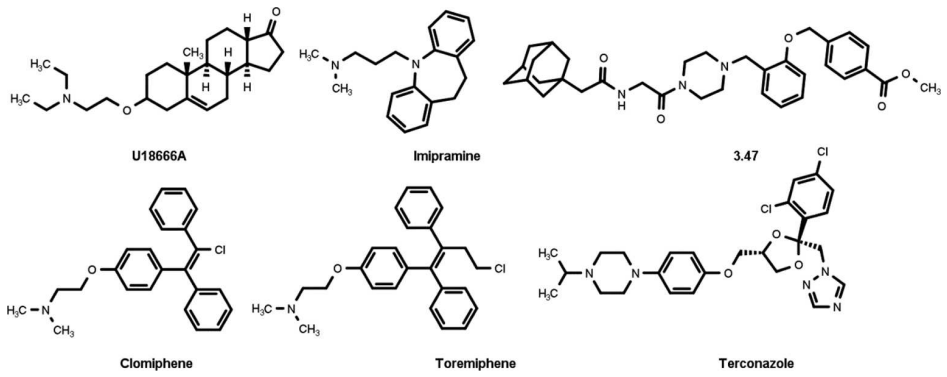

Figure 18. NPC-1 inhibitors are useful as medicaments against EBOV infection.

EBOV-GP, as the key recognition element, represents a crucial mediator of viral budding; therefore, suppression of protein glycosylation is another option to decrease EBOV infection. An example of such treatment is the use of tunicamycin, a N-glycosylation suppressor, which decreases EBOV infection of HeLa cells by more than 90%. Another way to reduce EBOV infectivity is to use imino sugars (IHVR11029, IHVR17028, and IHVR19029) as inhibitors of -glucosidase I, a glycosyl hydrolase from endoplasmic reticulum, which is responsible for proper folding and maturation of nascent proteins [67].

4.3. Signal pathway modulators

Perturbation of cell signaling pathways involved in EBOV infection is another option to inhibit the devastating action of the virus. Here we will describe a few examples where existing kinase and protease inhibitors were used as a cure for EVD.

siRNA screening of human kinome identified mitogen-activated kinase (MAPK), phosphoinositide 3 kinase (PI3K) [68], and calcium/calmodulin kinases (CAMK2) as novel cellular targets for therapeutic intervention against EBOV infection [69]. Garcia *et al*. have shown that

c-Abl1 tyrosine kinase is involved in phosphorylation and activation of VP40, the viral protein required for the transport of the viral genome-protein complex to the cell surface and subsequent budding. They reported that inhibition of c-Abl1 kinase with nilotinib and imatinib significantly reduce EBOV replication *in vitro* [70].

By contrast, only dephosphorylated form of VP30, part of EBOV nucleocapsid complex, is required for viral transcription. Studies of Modrof *et al*. exposed that okadaic acid, inhibitor of protein phosphatases 1 (PP1) and 2A (PP2A), both responsible for VP30 dephosphorylation, significantly blocked EBOV growth *in vitro* [36].

The use of ion channel blockers was also used to affect the complex process of viral entry, an example of such approach is to use multiple ion channel blockers amiodarone, dronedarone, and the calcium channel blocker verapamil for inhibition of Ebola virus $GP_{1,2}$-mediated cell entry [71].

Structures of representative signal pathway modulators are presented in **Figure 19**.

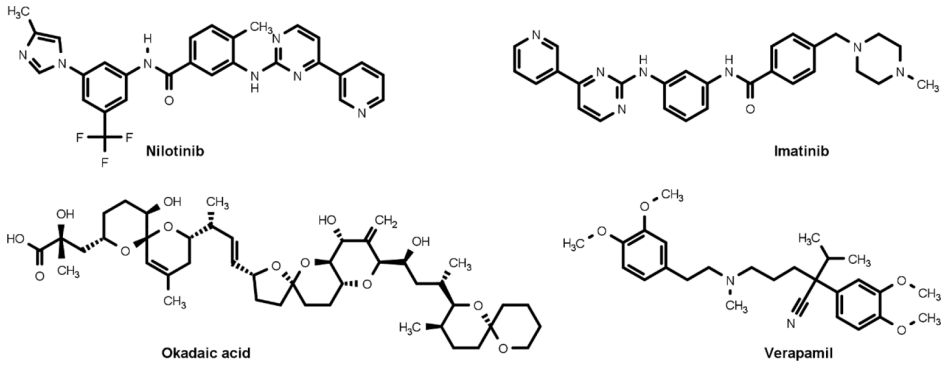

Figure 19. Structures of signal pathway modulators.

4.4. Small molecule Ebola virus modulators *in vitro* and *in vivo*

The review of Picazo *et al*. has collected several different chemotypes, which were shown to impair EVD infection *in vitro* [52]. The collection of structurally diverse compounds, targeting different proteins that are related to pathology of EVD, is shown in **Figure 20**. This collection contains: compound 17-AAG, which represents a heat shock protein 90 (HSP90) inhibitor; ouabain the ATP1A1 (sodium/potassium-transporting ATPase subunit alpha-1) inhibitor, which probably affects the function of VP24; glycyrrhizic acid that acts as the 11beta-hydroxysteroid dehydrogenase inhibitor; bafilomycin A1 and concanamycin A the inhibitors of V-ATPase; and retinazone, which disrupts EBOV infection via binding to glucocorticoid receptor (**Figure 20**).

Representatives of *in vivo* inhibitors are FGI-104 (derivate of amodiaquine, an antimalarial drug) and chloroquine (**Figure 20**). FGI-104 has a broad-spectrum of antiviral activities *in*

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vitro, including EBOV, Hepatitis virus B and C, and Cowpox virus among others. The *in vivo* study of experimental Ebola infection of mice has shown excellent prophylactic action of FGI-104. A 10 mg/kg dose of FGI-104 (2 hours before infection) yielded 100% survival [72]. Nowadays, chloroquine is one of the best *in vitro* EBOV inhibitors. Moreover, *in vivo* studies using chloroquine against EBOV infection have shown a promising surviving potential. This compound was able to reduce mortality by 90% when dosage (90 mg/kg) was given four hours before infection [73].

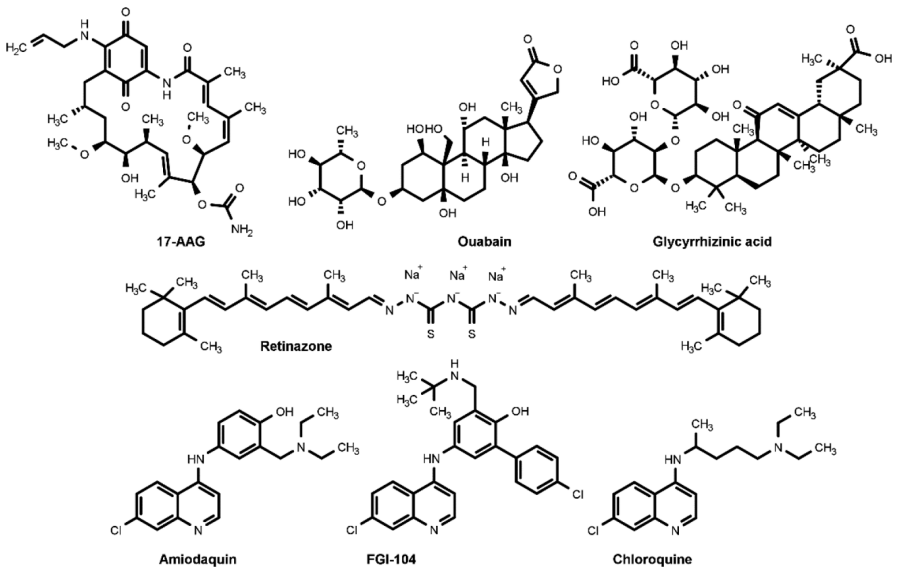

Figure 20. Representatives of *in vitro* and *in vivo* inhibitors of EBOV.

5. Conclusion

Recent outbreak of extremely lethal Ebola hemorrhagic fever in West Africa motivated scientists from all over the world to research EBOV life cycle and the pathology of EVD. The interest to find a successful treatment of Ebola hemorrhagic fever is currently one of the hottest topic in the academy and industry. Our review presents different strategies that could be used for the design of anti-EBOV medications. We have collected what is known about EBOV life cycle, structural information about EBOV protein targets, and some interesting inhibitors in one place. We believe that better knowledge of EBOV life cycle, supported with high quality structural information, could be a deciding factor in accelerating the task of finding a suitable cure against Ebola. Our review has shown that despite a huge collection of data (PDB structures, genome analysis, *in vitro* and in *vivo tests*, etc.) there is still a lot of unknowns about Ebola.

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Author details

Sebastian Pleško and Črtomir Podlipnik*

*Address all correspondence to: crtomir.podlipnik@fkkt.uni-lj.si

Faculty of Chemistry and Chemical Technology, University of Ljubljana, Ljubljana, Slovenia

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