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Emerging Targets and Novel Approaches to Ebola Virus Prophylaxis and Treatment

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Abstract

Ebola is a highly virulent pathogen causing severe hemorrhagic fever with a high case fatality rate in humans and non-human primates (NHPs). Although safe and effective vaccines or other medicinal agents to block Ebola infection are currently unavailable, a significant effort has been put forth to identify several promising candidates for the treatment and prevention of Ebola hemorrhagic fever. Among these, recombinant-virus based vectors have been identified as potent vaccine candidates with some affording both pre- and post-exposure protection from the virus. Recently, Investigational New Drug (IND) applications have been approved by the United States (U.S.) Food and Drug Administration (FDA) and Phase I clinical trials initiated for two small molecule therapeutics, 1) anti-sense phosphorodiamidate morphino oligomers (PMOs: AVI-6002, AVI-6003), and 2) lipid-nanoparticle/small interfering RNA (LNP/siRNA: TKM-Ebola). These potential alternatives to vector-based vaccines require multiple doses to achieve therapeutic efficacy which is not ideal with regard to patient compliance and outbreak scenarios. These concerns have fueled a quest for even better vaccination and treatment strategies. Here, we summarize recent advances in vaccines or post-exposure therapeutics for prevention of Ebola hemorrhagic fever. The utility of novel pharmaceutical approaches to refine and overcome barriers associated with the most promising therapeutic platforms will also be discussed.

Keywords

ebolavirus; vaccines; therapeutics; formulations

1. Introduction: Ebola Biology and Pathogenesis

Ebola virus is a filamentous, negative-stranded RNA virus of the *Filoviridae* family that causes a severe, often fatal viral hemorrhagic fever in humans and non-human primates (NHPs)¹. The single-stranded, negative sense 18.9 kb RNA genome encodes seven structural proteins and two non-structural proteins as shown in Figure 1A. The nucleoprotein (NP) is an essential component of the nucleocapsid that intimately binds to the virus genome. It, along with virion proteins (VPs) 30 and 35 and the RNA-dependent RNA polymerase (L) form the ribonucleoprotein (RNP) complex responsible for transcription and virus replication (Figure 1B) ^{2–4}. Matrix proteins VP40 and VP24, linked to the RNP

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complex and the inner surface of the viral envelope respectively, are also involved in nucleocapsid formation. They also play a role in viral budding, assembly, and host range determination $^{5-10}$. The virus particle is enclosed in a lipid bilayer envelope derived from the host cell membrane during the budding process (Figure 1B).

Ebola glycoprotein (GP), dispersed throughout the viral envelope as trimeric spikes, consists of two fragments; an extracellular protein (GP1) and a membrane-anchored protein (GP2). These are held together by disulfide bonds 11-14. Preferential binding of the Ebola virus to endothelial and monocytic cells is mediated by a 17 amino acid sequence within the GP1 domain that resembles an immunosuppressive motif in several human and animal retrovirus envelope proteins ^{15–21}. Interaction of this peptide sequence with target cells is thought to play a key role in apoptosis and the immunopathology of Ebola infection ²². Proteolysis of a precursor protein (pre-sGP) by furin generates a non-structural secretory glycoprotein (sGP) homodimer and a smaller -peptide. sGP shares neutralizing epitopes with the envelope $GP_{1,2}$ trimer spike and is released from cells in large quantity early in infection $^{23-25}$. This would suggest that it may be a decoy produced by the virus to bind circulating neutralizing antibodies (NABs). Additional studies evaluating the function of the -peptide have produced evidence that it plays a role in viral entry and prevents superinfection of cellular targets. It also prevents trapping of mature virions in the endoplasmic reticulum ²⁶. A third GP gene product, a smaller, soluble secreted glycoprotein (ssGP) has recently been discovered. Although its role in Ebola infection is currently unclear, it has very distinct properties from the sGP and -peptide²⁷.

Ebola virus infection in humans generally occurs through direct contact with mucosal surfaces, skin abrasions or contaminated needles²⁸. Antigen presenting cells (APCs), such as macrophages and dendritic cells (DCs) located at the site of infection, are primary targets of Ebola replication. Despite the fact that the virus enters immature DCs through typical C-type lectin (DC-SIGN) or other pattern recognition receptors, the cells become functionally deregulated and are unable to express co-stimulatory molecules or stimulate lymphocytes, namely naïve T cells ^{29, 30}. VP24 and 35 most likely play a pivotal role in preventing DCs from responding to infection as they block the type 1 interferon (IFN) anti-viral response in infected cellular targets by preventing nuclear accumulation of signal transducer and activator 1 (STAT1) and impeding the activity of interferon regulatory factor (IRF)-3 and 7^{31, 32}. This effect is further propagated by VP24 as it also blocks the p38 MAP kinase pathway in a JAK-STAT independent manner and by VP35 as it prevents activation of a dsRNA-dependent protein kinase required for production of IFN ^{33–35}. Unresponsiveness of DCs to Ebola infection most likely contributes to the massive lymphocyte apoptosis routinely observed during clinical cases of infection in humans ³⁶.

Ebola infection of monocytes and macrophages elicits the release of massive amounts of pro-inflammatory cytokines and chemokines including interleukins (IL)-1 , 2, 6, 8 and 10; tumor necrosis factor (TNF)- monocyte chemo-attractant protein (MCP)-1; regulated on activation normal T cell expressed and secreted (RANTES) and reactive and nitrogen oxygen species (ROS and NO, respectively) ^{37–39}. This "cytokine storm" recruits additional APCs to the site of infection, increasing the number of hosts to support virus replication. It also contributes to the pathogenesis at the late stage of disease by increasing endothelial permeability, and vascular leakage which, in turn, foster rapid dissemination of infected APCs throughout the systemic circulation to release Ebola in secondary lymphoid organs, lungs, liver and other ancillary sites of virus replication (Figure 2) ^{40–43}.

Over the last 35 years, numerous Ebola outbreaks have been recorded⁴⁴. The Ebola virus was first identified during two near-simultaneous outbreaks in Central Africa in 1976 of two different species with case fatality rates of up to 90%: Zaire ebolavirus (EBOV) and Sudan

ebolavirus (SUDV). Since then, additional species have been identified: *Reston* (RESTV), Tai Forest (TAFV) and Bundigbugyo (BDBV) ⁴⁵. RESTV, isolated in 1989 from cynomolgus macaques exported from the Philippines to the United States, is the only species that has not been associated with human disease ^{46–48}. Although cases of Ebola virus infection have been primarily limited to Africa, the number of outbreaks and associated fatalities has slowly increased over time. This, coupled with documented reports that Ebola can be transmitted across species through aerosolization of virus particles ^{49, 50}, has raised significant concerns over its possible use as a biological weapon, makes the virus a NIAID Category A Priority Pathogen and restricts experiments using all Ebola species to biosafety level (BSL)-4 containment laboratories ^{50–52}.

There is generally a 2~21 day incubation period before symptoms of Ebola virus-induced hemorrhagic fever are noted. They initially manifest as non-specific flu-like symptoms (malaise, chills, fever) and rapidly progress to severe nausea, diarrhea, shortness of breath, hypotension, bleeding and coma ⁵³. Vascular injury due to endothelial cell damage, hepatocyte necrosis caused by virus replication, coagulation disorders and uncontrolled cytokine/chemokine secretion by infected monocytes and macrophages contribute to EBOV-induced hemorrhagic shock and eventual death of the patient (Figure 2) ^{36, 51, 54}. Although Ebola is the focus of many cutting-edge, coordinated, interdisciplinary research programs around the world, effective vaccines or medicinal agents to combat this deadly pathogen are currently unavailable for human use. These efforts, however, have accelerated identification of many new molecular targets and promising therapeutic candidates currently in preclinical testing.

2. Vaccine Targets: Ebola Proteins

The first Ebola vaccine consisted of whole virions inactivated by heat, formalin and gammairradiation ^{55, 56} and was largely ineffective in rodents and non-human primates. Since then,
overexpression of genes that encode Ebola virus proteins has been the primary approach to
vaccine development. The rationale behind this strategy was to induce cellular targets to
produce enough virus protein to elicit potent T and B cell-mediated immune responses that
would confer protection against Ebola (Figure 3). Because Ebola GP was known to play a
key role in virus entry and facilitate cell death and vascular permeability in the latter stages
of infection, most of the early recombinant vaccine platforms centered around
overexpression of GP alone or in combination with NP and other VPs (Figure 3). A variety
of viral and non-viral vectors have been used to deliver genes for these antigens and
encourage strong B and T cell-mediated responses (Table 1).

2.1 Recombinant Adenovirus and Plasmid DNA-Based Ebola Vaccines

The first vaccine platform that successfully protected NHPs from Ebola virus infection was a recombinant adenovirus serotype 5 (rAd5) vector expressing EBOV GP ⁵⁷. A single intramuscular (IM) dose of adenovirus after three consecutive priming doses of plasmid DNA encoding EBOV GP and NP, SUDV GP and TAFV GP fully protected primates against lethal challenge. This combinatorial approach, DNA prime/rAd5 boost, greatly improved circulating anti-GP antibody levels and generated notable antigen-specific CD4+ and CD8+ T cell proliferative responses in cynomolgus macaques. The high level of transgene expression and the inherent adjuvant properties of the adenovirus capsid were fully appreciated in subsequent studies in which a single IM injection of the virus alone could protect animals from lethal challenge ⁵⁸. Further refinements of the rAd5-based vaccine platform by Richardson et al. involved optimizing the GP expression cassette so that more antigen was produced ⁵⁹. As a result, the dose of this vaccine could be reduced 100 fold without compromising antigen-specific immune responses. This approach was so successful that a single IM injection of the re-engineered vaccine fully protected mice when

it was given 30 minutes after exposure to a lethal dose of EBOV, suggesting that this platform might be useful for both prophylaxis and post-exposure applications.

Despite these promising results, the concern remains that rAd5-based vaccines may have limited clinical utility due to the fact that a significant portion of the global population have considerable amounts of anti-Ad5 neutralizing antibodies (NABs) in their circulation ^{60, 61}. In the United States, approximately 30–60% of the population have measurable levels antiadenovirus NABs in their circulation, while 40-80% of those in Europe and Asia contain similar levels of NABs ^{62, 63}. The highest levels recorded to date are found in sub-Saharan Africa (80–100% positive) ⁶⁴. Increasing the vaccination dose can override pre-existing immunity (PEI) and achieve notable antigen expression. This approach, however, is not desirable since high doses of adenovirus particles can precipitate severe, toxic inflammatory responses in humans ⁶⁵. Another strategy to circumvent PEI to Ad5 involves immunization with rare adenovirus serotypes since anti-Ad5 NABs do not completely cross-react with and neutralize these viruses ^{66–69}. Vaccine platforms using these viruses have partially protected rodents and NHPs with PEI to Ad5 from lethal challenge (Table 1) 66. Mucosal administration of rAd5 has also been shown to avoid neutralization of the virus by anti-Ad5 NABs in the circulation. Although this route of immunization generally induces lower systemic antigen-specific T cell responses, it induces strong local T and B cell responses not impaired by PEI that afford full protection in rodent and NHP models of disease ^{60, 61}. Recently, a Phase I clinical trial conducted with 31 healthy adults demonstrated that a rAd5based Ebola vaccine is capable of inducing antigen-specific T cell and antibody responses without notable side effects, however, prior exposure to adenovirus did compromise immunogenicity of the vaccine when it was given by IM injection ⁷⁰.

2.2 Live Attenuated Virus-Based Ebola Vaccines

Another promising vaccine platform involves the use of live attenuated recombinant viruses bearing the Ebola GP (Figure 3). One particular candidate, a recombinant vesicular stomatitis virus (VSV) in which the wild-type VSV surface glycoprotein was replaced with EBOV GP demonstrated attenuated growth kinetics and tropism of EBOV in vitro 71. A single dose of the virus given by the IM, intranasal (IN) or oral (PO) route completely protected mice, guinea pigs and NHPs from lethal challenge in the absence of any clinical symptoms or measurable viremia (Table 1) ^{72–78}. In contrast, administration of a gammairradiated, inactive form of the virus did not protect animals, suggesting that replication is a critical component of the potency of this vaccine ⁷⁹. This vector is a promising therapeutic option for post-exposure therapy since a single intraperitoneal dose 24 hours after lethal Ebola infection fully protected mice ^{75, 76, 80}. Fifty percent of guinea pigs also survived lethal challenge when given the vector in a similar manner 24 hours after exposure 80. Fifty percent survival was also noted when rhesus macaques were given the vector 20~30 min after lethal challenge. These animals did develop notable clinical signs of disease (fever, lymphocytopenia) on day 6, but had low-level serum viremia that resolved 10 days later. High levels of GP-specific IgG NAB and relatively low IgM responses were also found in the serum of survivors.

Although more than 80 NHPs have been given this vaccine platform without notable toxicity 73 , progress of the VSV-based construct to the clinic has been limited by concerns about its safety. To resolve this issue, the vector was first evaluated in immune-compromised mice lacking functional B and T cell 77 and NHPs infected with simian/human immunodeficiency virus (SHIV) 81 . Administration of the vector to Non-Obese Diabetic/ Severe Combined Immunodeficiency (NOD-SCID) mice at a dose that was 10 times that previously given to healthy mice was well tolerated 77 . Four of six vaccinated SHIV infected NHPs survived Ebola challenge without vaccine-induced toxicity despite the fact that a relatively high dose of vaccine (1×10 7 PFU) was given to each animal 81 . In an effort to

address concerns associated with neurotoxicity of the VSV vector in healthy subjects, 21 NHPs were given either wild type VSV or recombinant VSVs containing either EBOV or Marburg GP on the surface by intrathalamic injection ⁸². Results from this study clearly indicate that recombinant VSV vectors lack neurovirulence properties associated with the wild type virus. An important observation made during this study was that, even though animals given the recombinant VSV vector did not elicit notable neurovirulence throughout the course of the study, the recombinant virus was detected in mucosal swabs, indicating that it could leave the central nervous system by an unknown mechanism. This vaccine was first used in humans when a laboratory scientist working with Ebola in a BSL-4 laboratory was exposed through an accidental needle-stick ⁸³. The vaccine was given 48 hours after exposure. The patient developed a mild fever and myalgia 12 hours after injection. Other laboratory parameters (blood chemistry, coagulation and hematology) remained normal. Although the protective efficacy of the vaccine could not be determined in this case since Ebola infection could not be confirmed through serological testing, the scientist remains healthy to date.

Recombinant human parainfluenza virus 3 (HPIV3) expressing EBOV GP alone (HPIV3/EboGP) or together with nucleoprotein (HPIV3/EboGP-NP) has also been developed as a live-attenuated vaccine platform. Each of these constructs has conferred complete protection in guinea pigs and NHPs after EBOV challenge (Table 1) ^{84–86}. Much like adenovirus, HPIV-3 is a common respiratory virus, making PEI to the vector in humans a major limitation of this platform. To address this issue, Bukreyev and colleagues developed a chimeric HPIV-3 vector expressing EBOV GP as the sole surface protein to circumvent impact of PEI on vaccine potency ⁸⁷. This vector, HPIV3/F-HN/EboGP, is resistant to HPIV-3-specific NABs *in vitro* and a single intranasal dose (4×10⁶ PFU) protected guinea pigs from EBOV infection. Additional studies in animals given the vaccine in the presence of PEI to HPIV-3 and evaluation of toxicity of the vaccine in NHPs are needed to evaluate the clinical utility of this platform more precisely.

2.3 Future Perspectives: Vaccine Development

Although many of the vaccine platforms under development have fully protected NHP against lethal Ebola infection and some have entered clinical testing 70, each contain antigen sequences for one species of Ebola. Because each species of Ebola is antigenically distinct ^{88, 89}, development of multivalent vaccines capable of conferring protection against several different species of Ebola would be practical since outbreaks are sporadic and difficult to predict. While a rAd5-based vector expressing both SUDV and EBOV GP and several different VSV-based vectors expressing GPs from SUDV, EBOV and Marburg virus (MARV) have been constructed, they have successfully protected a limited number of animals after lethal challenge with several different Ebola species 90-92. This multivalent approach is currently extremely labor intensive and limited by the cloning capacity of recombinant viral vectors. Development of mosaic antigens that contain protein fragments of potential T-cell epitopes from multiple Ebola species might be a good alternative strategy to overcome these issues. This concept has recently been illustrated with a recombinant viral vector expressing multivalent mosaic proteins that could elicit strong antigen-specific CD8+ T cell responses against several model antigens ^{93–95}. Even though the protective efficacy of this strategy has not yet been demonstrated in infectious disease models, recent studies that highlight the importance of the CD8⁺ T cell response in conferring immune protection against EBOV infection in NHPs support this approach for development of multivalent Ebola vaccines ^{96, 97}. As technology for production of protein-based vaccines progresses, large scale production of these novel antigens in combination with immunization scaffolds to ease purification and augment the immune response will be possible as recently

demonstrated with the production of a potent Ebola GP immune complex vaccine in N. benthamiana 98 .

3. Development of Novel Anti-Viral Molecules as Treatments for Ebola Infection

Even though Ebola has been identified as the cause of many lethal outbreaks of hemorrhagic fever for more than three decades, current treatment options for infected individuals are limited. Crude approaches like administration of convalescent serum from infected survivors ⁹⁹ or equine anti-Ebola immunoglobulin with interferon ¹⁰⁰ have been successfully used to reduce the severity of infection in laboratory settings (Figure 4A). Although each person that received these preparations survived, the mechanism for protection is not clear since the composition of each product was complex and one specific component could not reproducibly be linked to survival. For example, it was initially thought that anti-Ebola IgG was responsible for the protection of 8 patients with Ebola hemorrhagic fever who survived after they were given whole blood known to contain anti-EBOV IgG antibodies 101. While this association seemed logical and straightforward, it has not been successfully reproduced in a controlled laboratory setting. In a study conducted by Jahrling et al., naïve rhesus macaques given convalescent-phase whole blood from 3 macaques that survived EBOV infection developed notable serum anti-EBOV IgG antibody titers ¹⁰². This, however, was not sufficient to control virus replication and disease in these animals progressed in a manner similar to that seen in untreated macaques. The efficacy of a highly characterized human anti-EBOV monoclonal antibody (KZ52) has been evaluated in guinea pigs and NHPs (Tables 2 and 3) 103 . Even though this antibody had a reported IC₅₀ of 0.5~2 µg/ml in vitro and in vivo, administration of a dose of 25 mg/kg, one hour after EBOV challenge conferred only partial protection (9 of 15) in guinea pigs. This preparation also failed to control virus replication and protect NHPs at a dose of 50 mg/kg ¹⁰³. Additional detailed molecular studies revealed that monoclonal neutralizing antibodies interact with Ebola GP at different sites and combinations of monoclonal isolates are most effective for pre- and postchallenge applications ^{104–107}. Once it was clear that direct transfer of immunity from infected survivors was an unreliable approach to combat Ebola infection, large multi-center collaborative research projects were initiated to screen libraries of well characterized and novel compounds to identify cellular and molecular targets associated with virus entry and replication. Compounds that have currently been evaluated in rodents and NHPs are summarized in Tables 2 and 3.

3.1 Small Molecule Inhibitors of Virus Entry and Endosomal Escape

Virus entry is an essential step in the virus life cycle and is often an attractive target for therapy since inhibition of this process blocks replication at an early stage, significantly reducing the chance for the virus to evolve and develop drug resistance. High-throughput cell-based screening (HTS) assays performed in tandem with recombinant EBOV expressing green fluorescent protein or recombinant pseudotyped viruses coated with EBOV GP that do not require high-level containment have been used to identify proteins that mediate filovirus entry ¹⁰⁸. This approach, used in combination with structural analysis of Ebola GP, has led to the discovery of several small molecules that may be useful therapeutics for EBOV hemorrhagic fever. A benzodiazepine derivative (compound 7) was identified from HTS of libraries of novel molecules as a compound that could prevent infection of a recombinant human immunodeficiency virus (HIV) pseudotyped with EBOV GP (HIV/EBOV-GP) ¹⁰⁹. Computational analysis of the crystal structure of EBOV GP was subsequently used to determine how compound 7 interacted with the virus. Results from this study indicated that compound 7 could fit within the hydrophobic pocket between the GP1 and GP2 subunits in a manner that could interfere with virus entry (Figure 4A).

Ebola must also closely interact with multiple host proteins for endosomal escape, replication, assembly, budding and release processes. HTS also identified a compound derived from benzylpiperazine adamantane diamide(2-((3r,5r,7r)-adamantan-1-vl)-N-(2-(4benzylpiperazin-1-yl)-2-oxoethyl)acetamide), known as compound 3.47, that inhibited EBOV infection in vivo 110. Biochemical studies revealed that this molecule binds to the Niemann-Pick C1 (NPC1) protein, a cholesterol transporter protein responsible for removal of cholesterol from late-endosomes/lysosomes and intracellular cholesterol homeostasis 111. Further analysis with this and other chemically similar compounds revealed that this protein, which mediates a devastating neurodegenerative condition, Niemann-Pick Type C disease, also plays a role in EBOV entry 112, 113. In a similar manner, efforts to characterize EBOVhost protein interactions identified two novel molecules: EBOV -peptide conjugated to the Fc region of a human IgG1 antibody and the endosome targeting C-peptide derived from native C-terminal heptad repeat regions of EBOV GP2 conjugated to the arginine rich sequence of HIV-1 Tat as promising candidates to prevent virus release from the endosome (Figure 4B) ¹¹⁴. In a somewhat opposing manner, Spargers and colleagues identified several host proteins (HSPA-5, RPL18) by liquid chromatography-linked tandem mass spectrometry that were key in Ebola replication and confirmed their findings by using small interfering RNA sequences (siRNA) to target host protein expression ¹¹⁵. Various protease inhibitors have also been evaluated as potential anti-viral compounds for EBOV. This strategy is based upon the fact that EBOV GP is processed by endosomal cysteine proteases; cathepsin, CatB and CatL ^{116–118}. Addition of cysteine protease inhibitors E64 and CA074 in culture media effectively blocked cellular cathepsins and suppressed EBOV replication and virus-induced cytopathic effects in vitro 117. These compounds also demonstrated anti-viral activity against many other viruses (Marburg, Rift Valley fever, Lassa) in vitro and protected mice from challenge with mouse-adapted EBOV when given as prophylaxis (80%) or post-exposure (50%) therapy (Figure 4B).

3.2 Compounds that Block Virus Replication

For many years, ribavirin, a common anti-viral drug that interferes with the replication of many RNA viruses like influenza and polio by fostering mutations in the viral genome with increasing incidence ("error catastrophe") and through other contributing mechanisms, has been used in hemorrhagic fever ¹¹⁹. Despite its success in mitigating hemorrhagic fevers arising from arenaviruses and bunyaviruses ^{120, 121}, ribavirin did not control EBOV replication and failed to protect animals from lethal challenge. An alternative to this approach is to block virus transcription and/or replication with antisense oligonucleotides complementary to sequences in the EBOV genome or within the RNA polymerase complex (Figure 4C) 122, 123. Geisbert and colleagues have identified siRNAs that specifically bind to sequences within the EBOV polymerase L (EK-1), VP24 (VP-24-1160) and VP35 (VP-35-855) regions ¹²³. When these compounds were combined and formulated as stable nucleic acid-lipid particles (SNALPs; LNP/siRNA: TKM-Ebola) and given to NHPs in 4 separate doses of 2 mg/kg each intravenously, 66% of the population survived lethal challenge with EBOV (Table 3). A seven-dose regimen effectively halted virus replication with a moderate increase in serum aspartate aminotransferase levels noted. All animals given this regimen survived challenge.

In a similar approach, c-Abl1 and related tyrosine kinases, known to affect replication of certain DNA viruses and bacteria, were evaluated for their role in EBOV replication. A series of silencing studies revealed that phosphorylation of the VP40 protein by c-Abl1 is necessary for transport of the nucleocapsid complex to the cell membrane and release of complete virions from the cell ¹²⁴. Blocking this process with compounds that have been approved for the treatment of leukemia in humans such as imatinib (Gleevec®) and nilotinib (Tasigna®) which target this enzyme significantly limited the amount of infectious Ebola

virions released in culture medium (Figure 4C) ¹²⁴. Use of compounds that target host gene products rather than the virus itself in larger models of Ebola infection may effectively prevent the development of drug-resistant escape mutants over time.

Another therapeutic platform which holds promise in preventing EBOV replication involves the use of third generation synthetic antisense oligonucleotides, phosphorodiamidate morphino oligomers (PMOs), which are RNase H incompetent and arrest translation and mRNA processing through steric hindrance ¹²⁵. These molecules, in which the ribose rings are replaced with 6 membered morpholine rings and traditional phosphodiester bonds replaced with phosphorodiamidate linkages, demonstrate improved solubility and are more chemically stable in biological fluids and during storage with respect to their first generation counterparts ¹²⁶. Several recent reports have demonstrated that these molecules could be valuable therapeutics for filovirus infection ^{115, 127–129}. Administration of positively charged EBOV-specific PMOs (AVI-6002) targeting mRNA sequences within the VP24 and VP35 regions 30-60 minutes after challenge suppressed virus replication and subsequent inflammatory responses and fully protected 5 of 8 macaques (Figure 4C, Table 3) 130. Postexposure administration of Marburg virus-specific PMOs (AVI-6003) targeting MARV VP24, 35 and L protein also fully protected NHPs from lethal challenge ¹³⁰. Although New Investigational Drug applications are on file with the United States Food and Drug Administration and Phase I clinical trials are in progress to evaluate the safety of these compounds ¹²⁶, additional studies must be performed to accurately define the timeframes within which they offer post-exposure protection before they can be used in post-exposure therapeutic regimens.

High throughput screening systems have also aided in identifying three small molecule inhibitors of EBOV infection: FGI-103 ¹³¹, FGI-104 ¹³² and FGI-106 ¹³³. These compounds have provided 80~100% protection in mice challenged with EBOV (Table 2). Although the mechanisms of anti-viral activity of FGI-103 and FGI-104 are not completely understood, the broad activity of FGI-106 against Ebola, Marburg, Rift Valley fever, Dengue, HIV and hepatitis C viruses suggests that this compound targets a host cellular pathway involved in and common to the replication of many different viruses.

3.3 Compounds for the Symptoms of Ebola Infection: Inflammatory Modulators

To date, non-adapted strains of EBOV have been found to only induce hemorrhagic fever in humans and non-human primates 41, 134–136. Although this species-specific restriction has been problematic since adapted strains are required for modeling disease in rodents ¹³⁷, studies conducted in rodents have been pivotal in identifying ways to augment the immune response and block virus replication. For example, NOD-SCID, IFN- / receptor knockout and immune-competent mice treated with anti-mouse IFN- / antibodies succumb to nonadapted (wild-type) EBOV infection ¹³⁸. In a separate series of studies, treatment with sadenosyl-homocysteine (SAH) hydrolase inhibitors, 3-deazaadenosine (c3-NpcA) ¹³⁹ and carbocyclic 3-dezaadenosine (C-c3Ado) ¹⁴⁰, which inhibit replication of a variety DNA and RNA viruses, fully protected immune-competent mice during lethal infection with mouseadapted-EBOV (Table 2). Further investigation revealed that the protective effect could be completely eliminated by co-administration of SAH hydrolase inhibitors and anti-mouse IFN- / antibodies ¹³⁸. Although these results strongly suggest that resistance and/or susceptibility to wild type ZEBOV infection is mediated by the type I interferon response, re-invigoration of the anti-viral response in this manner has not been therapeutically effective in rhesus macaques ¹⁴¹, baboons ¹⁴² and African green monkeys ¹⁴³. This might be improved by using species-specific interferon. Further evaluation of this approach has not yet been performed.

In a recent effort to identify novel therapeutics to treat and mitigate the pathological symptoms associated with filovirus hemorrhagic fevers, compound NSC62914, an antioxidant that acts as a scavenger of reactive oxygen species (ROS), was found to inhibit replication of EBOV, Marburg, Lassa and Rift Valley Fever viruses *in vitro* ¹⁴⁴. Although the role of ROS in the pathogenesis of filovirus infection is not currently understood, this compound protected mice from lethal challenge with EBOV (Table 2). This was significant, given that other known antioxidants have had no impact on EBOV infection. It also suggests that NSC62914 and other compounds with antioxidant properties may maintain other cell signaling pathways common to many viruses that are arrested during EBOV infection.

3.4 Compounds for the Symptoms of Ebola Infection: Coagulation Modulators

Overproduction of pro-coagulant tissue factors during Ebola infection facilitate clotting disorders that progress to multi-organ failure, often indicated by a reduction in circulating serum protein C ¹⁴⁵. Thus, stimulating coagulation through the tissue factor pathway by administering a factor VIIa/tissue factor inhibitor (recombinant nematode anticoagulant protein c2: rNAPc2) ¹⁴⁶ or by activating the natural anticoagulant protein C pathway with recombinant human activated protein C (rhAPC) ¹⁴⁷ were logical choices in the design of supportive post-exposure therapeutic regimens. Each of these compounds significantly decreased production of pro-inflammatory cytokines and extended the mean time to death with respect to non-treated controls in animal models of infection (Table 3). Despite these promising results, routine use of these agents in humans has been hindered by conflicting reports from Phase II trials designed to evaluate their clinical efficacy for treatment of septic shock ¹⁴⁸, ¹⁴⁹ and subsequent withdrawal of one product from the U.S. market ¹⁵⁰.

4. Future Directions: Development of Small Molecule Therapeutics for Ebola Infection

Although there is a clear need for effective regimens for prevention and protection against Ebola-mediated hemorrhagic fevers, many significant hurdles have limited the ability of promising candidates to reach those in need of them. One of the primary reasons for the modest progress made in this area over the last two decades is the fact that meaningful "proof-of-principle" studies can only be handled in maximum-containment biosafety level 4 (BSL-4) laboratories. Significant progress in the development of high throughput systems that allow rapid screening of potential anti-viral compounds at a much lower containment level (BSL-2) and the use of RNA interference technology to confirm these findings has led to the discovery of several novel compounds that have demonstrated protective efficacy in animal models of infection (Tables 2 and 3) ¹²³, ¹²⁸, ¹³⁰, ¹³¹. Since this approach will significantly accelerate the discovery of new therapeutics to combat Ebola, it will also heighten the need for studies in larger animal models, as data obtained from rodents may not accurately reflect the pharmacological and toxicological responses of humans.

Most of the recently discovered small molecule therapeutics to treat filovirus hemorrhagic fevers have limited serum half-lives and poor bioavailability in target tissues, making administration of excessively large amounts of compound in multiple dosing regimens necessary to achieve an optimal therapeutic effect ¹¹⁵, ¹²⁷, ¹²⁸, ¹³⁰, ¹³¹, ¹⁴⁴. It is also well established that the potency of any therapeutic or vaccine is heavily influenced by the physical stability of the active compound and the chosen delivery system. Half-life and bioavailability can be greatly extended through formulations that maintain the structural integrity of a medicinal agent and protect it from nucleases and other degradative enzymes *in vivo* and during long-term storage ¹⁵¹, ¹⁵². Thus, focused efforts in development of novel formulations that can stabilize or alter the physical conformation of these promising compounds will be vital to improve their efficacy and reduce their toxicity as they progress

to the clinic. Incorporating these formulations in the proper delivery platform will then move them toward single dose regimens that are easy to administer in outbreak and post-exposure scenarios. Some of the most relevant formulation/delivery strategies for novel Ebola therapeutics are discussed in detail below and have application to both small molecule and vaccine platforms.

4.1. Formulation Development: Mucoadhesive/Absorption Enhancers

Mucoadhesive agents bring therapeutic molecules in close contact with the mucosal cell surface and prolong their residence time along the surface of the airways, oral cavity, digestive and genitourinary tracts and the skin ¹⁵³. They also suppress mucociliary clearance (MCC) processes that rapidly remove foreign particulates from these areas ¹⁵⁴. Mucoadhesive compounds can be divided into three categories ¹⁵⁵. The first group includes hydrophilic polymers like sodium alginate, sodium carboxymethylcellulose, hydroxypropyl methylcellulose, and Carbopol that can form covalent hydrogen bonds with the mucus layer ¹⁵⁶. The second group consists of cationic polymers like chitosan and synthetic polymethacrylates that interact with the negatively charged mucin through the formation of ionic or hydrogen bonds. The third group is made up of thiolated polymers, or thiomers, that form covalent bonds with free sulfhydryl groups in mucin ¹⁵⁷. The thiomers are currently the strongest mucoadhesives available for delivery of drugs to mucosal surfaces ¹⁵⁸. While mucoadhesives clearly facilitate direct contact with the mucosal surface, most but not all are relatively poor at getting therapeutic molecules across the underlying epithelial cell monolayer, which is impermeable to most compounds in the absence of specific transporters. Thus, mucoadhesives are often paired with an absorption enhancer, a compound that gently weakens cellular membranes or loosens tight junctions to allow the medicinal agent to be absorbed through transcellular or paracellular pathways ¹⁵⁹. Examples of absorption enhancers commonly used in therapeutic formulations approved for human use include carbohydrates, surfactants, bile salts and their derivatives, phospholipids, cyclodextrins and poly(ethylene) glycols ¹⁶⁰. Cell penetrating peptides, derived from the human immunodeficiency virus (HIV-1) transactivator of transcription (Tat) protein ¹⁶¹ and the *Drosophilia melanogaster* Antennapedia homeodomain (penetratin) ¹⁶² have also been used to increase cellular uptake of large molecules. Although the exact mechanism by which they exert their effect is not clearly understood, several recent in vivo studies demonstrate that these novel molecules can improve delivery of siRNA molecules with minimal toxicity ¹⁶³.

4.2. Formulation Development: Lipid-based Carriers

Liposomes are vesicles consisting of lipid or phospholipid bilayers with an aqueous core. Depending upon the manufacturing process, these particles are composed of a single (unilamellar) or several concentric (multi-lamellar) lipid bilayers and range in size from 50 to 2,000 nm ¹⁶⁴. Lipid micelles are monolayer structures composed of poly(ethylene) glycol (PEG)-conjugated phospholipids that self-assemble spontaneously at concentrations above their critical micelle concentrations (CMCs) in aqueous solution ¹⁶⁵. In these relatively small particles (7–35 nm), the hydrophobic acyl chains of the lipids form the micelle core while the polar head groups make up the outer hydrophilic corona. Solid lipid nanoparticles consist of sub-micron sized lipid emulsions where the liquid lipid (oil) has been replaced by a solid lipid dispersed in an aqueous surfactant solution ¹⁶⁶. Each of these systems employ phospholipids, triglycerides and cholesterol derivatized or extracted from natural sources that are biocompatible and biodegradable *in vivo* ¹⁶⁷. Of these systems, liposomes have been the most extensively studied with over 40 years of documented research describing their suitability as carriers for hydrophilic and hydrophobic small molecules and antigens for vaccines.

Early studies to evaluate the ability of liposome formulations to improve drug absorption revealed that these particles were efficiently taken up by the reticuloendothelial system (RES), thus making them suitable candidates for vaccine development ^{168, 169}. Since then, liposome-antigen preparations have been shown to induce Th1 and Th2 type responses with respect to lipid composition ^{170, 171}. Liposomes have been found to be quite versatile for vaccine development in that lipid compositions can easily be tailored to the type of immune response desired, they are compatible with most adjuvants and can accommodate antigens of varying size ¹⁷², ¹⁷³. They also serve as the platform for virosomes which can be used for immunization or drug targeting ^{174, 175}. While the inherent uptake of liposomes in the RES was highly regarded within the vaccine field, it was not acceptable for most small molecule therapeutics as they were rapidly cleared from the circulation before they could exert their therapeutic effect. Development of "stealth" liposomes in which PEG and other biocompatible polymers have been placed on the liposome surface to prevent recognition by opsonins have greatly reduced uptake by the RES and resulted in the first liposomal formulation to be approved for clinical use in the United States and Europe 164, 176. Incorporation of these polymers in liposome preparations has also been of additional benefit in that they allow for chemical attachment of a wide array of ligands to redirect the liposome from the RES to specific organs and cell types ^{164, 167, 177}.

4.3. Formulation Development: Biocompatible Polymers

Liposome preparations are limited by poor physical and chemical stability in biological fluids and as formulated products at ambient temperatures. Large-scale manufacturing of these products is also difficult with batch-to batch reproducibility a significant concern ¹⁷⁸. To address these issues, "entrapment and encapsulation" methods to embed small molecules, proteins and peptides within nanoparticles made from biodegradable polymers were developed and have been widely used for several decades in the field of pharmaceutical science ¹⁷⁹. These preparations allow continuous release of compound over extended periods of time to improve the half-life of drugs with poor bioavailability profiles ^{180, 181}. They also minimize the overall surface charge of a therapeutic compound and foster interaction with target tissues and organs to improve bioavailability profiles ¹⁸². Many naturally occurring polymers such as alginate, chitosan, gelatin, albumin, pullulan, gliadin and dextran have been the focus of many pioneering studies evaluating this carrier system for vaccine and drug delivery ¹⁷⁹. Synthetic polymers like poly(caprolactone), poly(methyl acrylate) and poly(lactic-co-glycolic acid) are less immunogenic than those listed above. Nanoparticles consisting of these polymers in various combinations and molecular weights can be prepared in a highly reproducible manner. Of these polymers, poly(lactic-co-glycolic acid) (PLGA) has been studied and characterized extensively and is approved by the U.S. FDA and European Medicine Agency (EMA) in various drug delivery systems in humans ¹⁸³. As with liposome carriers, proteins, antibodies and other known ligands can be placed on the surface of these particles to direct them to specific physiological targets. Recent studies have elucidated methods where targeting molecules can be imprinted directly in the polymer matrix to minimize the need for additional complex modifications of the surface of these particles once the therapeutic compound is embedded in them ^{184, 185}. Advances in polymer chemistry have also facilitated the development of "smart" particles capable of delivering their therapeutic payload in response to changes in temperature, oxygen content, pH, and light which may be useful in the future development of therapeutics to treat Ebola hemorrhagic fevers ^{186–188}.

5. Final Thoughts

From what we have summarized here, it is clear that the development of an effective vaccine against Ebola has progressed further than efforts to identify small molecule therapeutics to treat infection. Although several vaccine platforms have entered the early stages of clinical

testing, the longevity of the immune response elicited by each is not fully characterized. This is of some concern since recurrent immunization programs for Ebola hemorrhagic fever seem unrealistic and costly since the disease burden is currently limited to a specific region of the world. Although the exact correlates of protection for Ebola in humans continue to be an issue of debate, a systems vaccinology approach employing microarrays, mass spectrometry-based proteomics, metabolomics and computational modeling would define the immune responses necessary for protection. Data obtained from these types of studies will also allow for further refinement of current vaccine platforms with formulations that foster specific types of immune responses that are long lasting. It is also important to note that most of the potential post-exposure treatments described above have shown protective efficacy in animals treated very soon (30~60 minutes) after exposure to Ebola. These are impractical with respect to natural outbreaks where infected individuals will not seek treatment for days. Studies evaluating gene expression profiles during active Ebola infection would be a valuable tool in developing post-exposure treatments for those that have progressed to symptomatic illness, however, facilities, funding and reagents to support them are very limited. Although discovery of small molecule therapeutics to combat Ebola hemorrhagic fever has been lagging behind that of vaccine development, recent efforts to develop high-throughput screening systems which do not require use of maximum containment facilities will foster an exponential increase in the discovery and development new therapeutics for Ebola-mediated and other hemorrhagic fevers in the next five years. This trend may reinvigorate interest and funding to support clinical testing of highly promising therapeutic compounds and solidify plans for how a given vaccine or therapeutic would be used once it is licensed as well as for the infrastructure necessary to deliver it to regions where it is needed the most.

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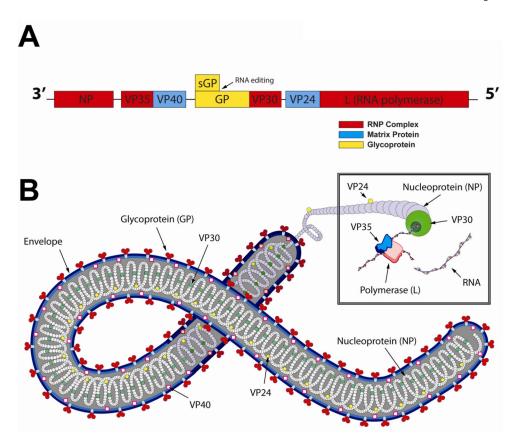


Figure 1. The Ebola Virus

A. Schematic Representation of the Zaire Ebola (EBOV) Genome. The non-segmented negative-stranded RNA genome contains seven structural proteins (NP, VP24, VP30, VP35, VP40, L, GP) and two non-structural proteins (secreted GP (sGP) and small soluble sGP (ssGP) not shown). **B. Configuration of the Ebola Virus Particle.** During replication, NP, VP30, VP35, VP24 and L protein form the ribonucleoprotein (RNP) complex with the viral genomic RNA. The rod-shaped virus is 80 nm in diameter. The length of the virion, ranging from 1,028 to 1,978 nm is dictated by the number and length of the genomes that are incorporated into a single virus capsid during replication and assembly.

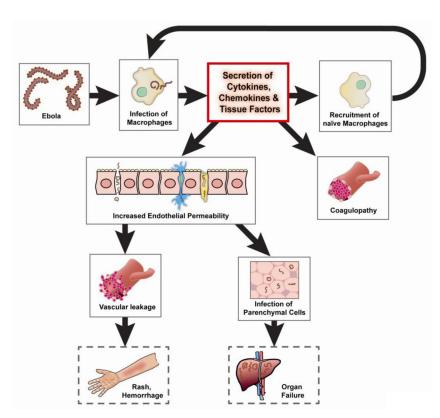


Figure 2. Ebola Pathogenesis

Ebola infects a variety of cellular targets (endothelial cells, fibroblasts, hepatocytes and adrenal cortical cells) as well as macrophages, monocytes and dendritic cells. While infected dendritic cells fail to activate naïve T cells to combat infection, infected macrophages and monocytes release a large number of cytokines and chemokines in a "cytokine storm". This cytokine storm supports virus replication and dissemination as it recruits new hosts (naïve antigen presenting cells) to the site of infection. Excess cytokines tissue factor released from macrophages also interfere with the coagulation cascade and increase endothelial permeability that leads to vascular leakage, hemorrhage and a notable maculopapular rash. Repeated cycles of rapid virus replication in parenchymal cells eventually overcome a dysregulated immune response and lead to severe tissue damage, necrosis, septic shock, multi-organ failure and eventually death.

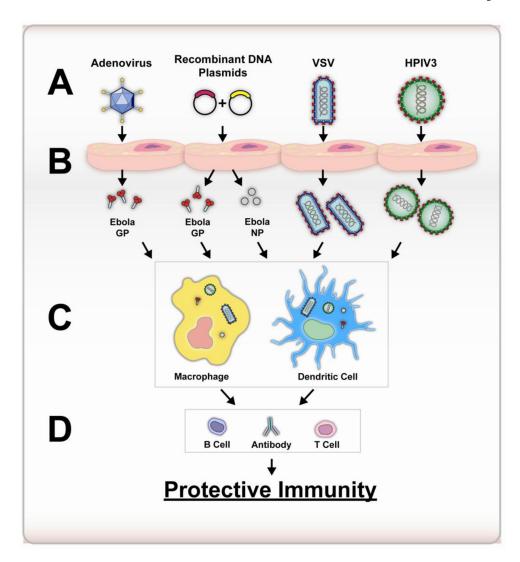


Figure 3. Generalized Approach to Ebola Vaccine Development

The most promising platforms under development for clinical testing involve overexpression of Ebola glycoprotein and/or nucleoprotein. This is accomplished through administration of replication deficient recombinant adenoviruses or plasmid vectors (Panel A) that transduce various cellular targets (Panel B) to make large amounts of the Ebola antigens that enter the general circulation. Other platforms use attenuated recombinant viruses, such as VSV or HPIV3, that bear Ebola glycoprotein on their surface. These viruses can replicate in cellular targets (Panel B). These particles, like the glycoprotein and nucleoprotein made from cells transduced with adenovirus or plasmid, are taken up and processed by macrophages and dendritic cells (Panel C) to generate strong B and T cell-mediated immune responses against Ebola (Panel D).

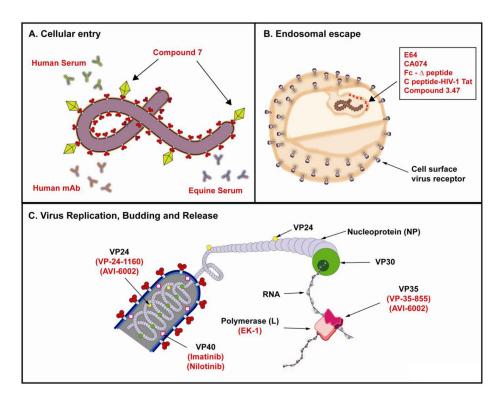


Figure 4. Common Targets of Small Molecules for Treatment of Ebola Infection **A.** Cellular Entry. Early attempts to mitigate the progress of Ebola infection involved administration of serum collected from convalescent survivors. Moderate success with this approach led to the development of monoclonal antibodies specific for Ebola glycoprotein that should, in theory, prevent virus entry into cellular targets. Additional work demonstrated that polyclonal antibodies or combinations of monoclonal isolates capable of interacting with Ebola GP at multiple sites are most effective for pre- and post-challenge treatments. High throughput screens identified one molecule, Compound 7, which could prevent virus entry *in vitro*. Computational analysis revealed that it fit in a pocket in the GP_{1,2} trimer. **B.** Endosomal Escape. High throughput analyses have identified several compounds that are effective in preventing endosomal escape of the virus particle. Other screens to identify host proteins that interact with Ebola throughout infection have led to the development of silencing technologies that make these proteins inaccessible to prevent release of virus particles from the endosome. **C.** Virus Replication, Budding and Release.

Compounds that have demonstrated efficacy in stopping Ebola replication target various

components of the RNP complex. Compounds that target VP 40 and 24 affect cellular

transport of the virus particle and budding and release from the cellular host.

Table 1

Ebola Vaccine Platforms Currently Tested In Non-Human Primates

Platform	Targets in vaccine	Prophylactic efficacy	Therapeutic efficacy	Concerns	References
Recombinant adenovirus serotype 5 (rAd5)	GP, GP+NP	Yes, $1 \times 10^{10} \sim 2 \times 10^{12} \text{ VP}$	Not tested	Pre-existing immunity	58, 189
Rare adenovirus serotypes (rAd26 prime / rAd35 boost)	ďD	Yes, 1×10^{11} VP (Prime boost)	Not tested	Boost immunization required	99
DNA/rAd5 (Prime/Boost)	GP+NP	Yes DNA: 4 mg (3 doses)/rAd5: 1 \times 10 10 PFU, 1 \times 10 11 PU	Not tested	Boost immunization required, pre-existing immunity	57, 58, 190
Vesicular stomatitis virus (VSV)	dБ	$\rm Yes~1\times10^7~PFU$	$\rm Yes2\times10^7PFU$	Safety (replication competent)	74–76, 79–81
Human parainfluenza virus type 3 (HPIV-3)	GP, GP+NP	Yes $4 \times 10^6 ^{TCID50}$ (single dose) $2 \times 10^6 ^{TCID50}$ (Prime/boost)	Not tested	Boost immunization required, Safety (Replication competent)	86, 191
Venezuelan Equine Encephalitis virus (VEE) replicon	ďD	$\rm Yes~1\times 10^7~FFU$	Not tested	Boost immunization required	192
Virus-like particles (VLPs)	GP+NP+VP40	Yes 250 µg (3 doses)	Not tested	Boost immunization required	193

Abbreviations: VP: total number of virus particles (infectious and non-infectious); PFU: plaque forming units; PU: particle units; TCID50: median tissue culture infective dose; FFU: focus forming units.

Table 2

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Anti-Viral Compounds Currently Tested in Rodent Models of Ebola Infection

	Therapeutic targets	Prophylactic efficacy	Therapeutic efficacy	Concerns	References
FGI-103, 104 and 106	Unknown	Yes FGI-106: $2 \sim 5 \text{ mg/kg}$ BW * (single dose)	Yes FGI-103: 10 mg/kg BW *, (single dose) FGI-104: 10 mg/kg BW */ day (11 doses), FGI-106: 5 mg/kg BW * (3 doses)	Multiple doses required	131–133
NSC62914	Reactive oxygen species (ROS)	Partial protection 2 mg/kg BW *(3 times)	Partial protection $2 \sim 5 \text{ mg/}$ kg BW*(3 doses)	Multiple doses required	144
Small interfering RNAs (siRNA)	L polymerase + $VP24 + VP35$	Not tested	Yes $0.75 \sim 1 \text{ mg/kg BW}^*(7)$ doses)	Multiple doses required	122
Phosphorodiamid ate morphino oligo nucleotides (PMOs)	VP24+VP35; L polymerase + VP24 + VP35	Yes 5–50 µg (twice)	Yes $12.5 \sim 100 \text{ mg (11)}$ doses)	Multiple doses required	127, 128
Monoclonal neutralizing Antibodies (NABs)	Ebola Virion (KZ52)	Not tested	Yes 50 mg/kg BW * (single dose)	Multiple doses required	194
Triple monoclonal antibody cocktail	Ebola GP	Yes 100 µg (single dose)	Yes 100 µg (single dose)	Must be used early before/after exposure.	107
S-adenosyl-L-homocysteine hydrolase inhibitors	S-adenosyl-L-homocysteine hydrolase	Yes Ca-C ³ Ado: 80 mg/kg BW *, (single dose) C- NpcA: 1 mg/kg BW * (single dose)	Yes Ca-C ³ Ado: 80 mg/kg BW*, (3 doses) C-NpcA: 1 mg/kg BW* (3 doses)	Manipulation of host immune system	139, 140

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Table 3

Anti-Viral Compounds Currently Tested in NHP Models of Ebola Infection

Platform	Therapeutic targets	Prophylactic efficacy	Therapeutic efficacy	Concerns	References
Recombinant human activated protein C (rhAPC)	Abnormal coagulation	Not tested	Partial protection 2 mg/m²/h (i.v. infusion) until day 7 post-exposure	Low efficacy, manipulation of coagulant pathway, withdrawn from global market (2011)	147
Recombinant nematode anticoagulant protein C2 (rNAPC2)	Factor VIIa: tissue factor complex	Not tested	Partial protection 30 µg/kg BW */day until day 14 post-exposure	Low efficacy, manipulation of coagulant pathway	146
Small interfering RNAs (siRNA)	L polymerase + VP24 + VP35	Not tested	Yes 2 mg/kg BW*(7 doses)	Multiple doses required	123
Phosphorodiamidate morphino oligo nucleotides (PMOs) (LNP/ siRNA: TKM-Ebola)	VP24+VP35; L polymerase + VP24 + VP35	Not tested	Yes 12.5 ~ 200 mg (11 doses)	Multiple doses required	128
Monoclonal neutralizing Antibodies (NABs)	Ebola Virion (KZ52)	No	No	No efficacy	103, 194
Triple monoclonal antibody cocktail	Ebola GP	Not tested	Full protection 24H, Partial Protection 48H, 25 mg/kg BW * (3 doses)	Multiple doses required, must be used early after exposure.	106

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