INFECTIOUS DISEASES OF POVERTY

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Human Ebola virus infection in West Africa: a review of available therapeutic agents that target different steps of the life cycle of Ebola virus

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Abstract

The recent outbreak of the human Zaire ebolavirus (EBOV) epidemic is spiraling out of control in West Africa. Human EBOV hemorrhagic fever has a case fatality rate of up to 90%. The EBOV is classified as a biosafety level 4 pathogen and is considered a category A agent of bioterrorism by Centers for Disease Control and Prevention, with no approved therapies and vaccines available for its treatment apart from supportive care. Although several promising therapeutic agents and vaccines against EBOV are undergoing the Phase I human trial, the current epidemic might be outpacing the speed at which drugs and vaccines can be produced. Like all viruses, the EBOV largely relies on host cell factors and physiological processes for its entry, replication, and egress. We have reviewed currently available therapeutic agents that have been shown to be effective in suppressing the proliferation of the EBOV in cell cultures or animal studies. Most of the therapeutic agents in this review are directed against non-mutable targets of the host, which is independent of viral mutation. These medications are approved by the Food and Drug Administration (FDA) for the treatment of other diseases. They are available and stockpileable for immediate use. They may also have a complementary role to those therapeutic agents under development that are directed against the mutable targets of the EBOV.

Keywords: Ebola virus, Non-mutable host cell therapeutic targets for Ebola virus, Cocktail therapeutic intervention for RNA virus

Multilingual abstract

Please see Additional file 1 for translations of the abstract into the six official working languages of the United Nations.

Background

The recent outbreak of the human Zaire ebolavirus (EBOV) infection starting in West African countries has resulted in 15,351 infected patients, as of 18th of November 2014. A total of 5,459 deaths have been reported in six affected countries (Guinea, Liberia, Mali, Sierra Leone, Spain, and the United States of America) and two previously affected countries (Nigeria and Senegal) [1]. Apart from supportive care, neither a licensed vaccine nor a specific therapy is available for the treatment of the human EBOV infection [2]. The World Health Organization (WHO) has considered that it is ethically acceptable to offer unproven interventions that have shown promising results in laboratory and animal models, but have not yet been evaluated for safety and efficacy in humans as potential sources of treatment or prevention [3]. Several promising therapeutic agents have been identified for the treatment and immunization of the EBOV. These may include monoclonal antibody (mAbs)-based therapies (e.g. ZMapp), anti-sense phosphorodiamidate morpholino oligomers (PMO AVI-6002), lipid nanoparticle small interfering RNA (LNP-siRNA: TKM-Ebola), and an EBOV glycoprotein-based vaccine using live-attenuated recombinant vesicular stomatitis virus (rVSV-EBOGP) or a chimpanzee adenovirus (rChAd-EBOGP)-based vector. Human trial results of these agents would not be available until next year. Moreover, existing supplies of all these experimental medications and vaccines for compassionate use are either extremely limited or exhausted [4-6]. To

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combat such an unprecedented global public-health crisis before these experimental agents are available, alternative available interventions that can target different steps in the replication cycle of the EBOV should be explored in the management of the human EBOV infection as contingency preparation for the international dissemination of the EBOV outbreak in West Africa. We have reviewed currently available therapeutic agents that have shown to be effective in suppressing the proliferation of the EBOV in cell cultures or animal studies. We propose a therapeutic regimen to supplement the current supportive therapy aiming to reduce viral load, the most important factor in the determination of mortality. Through viral load suppression, we may be able to prolong a patient's survival in order to provide a better chance for the patient to develop natural immune defense against the EBOV.

Discussion

The genome of the Ebola virus

The EBOV is an enveloped filamentous RNA virus belonging to the family *Filoviridae*. The 19-kb linear, non-segmented, negative-sense, single-stranded RNA genome of the EBOV encodes seven structural proteins and two non-structural proteins in the following order within the genome: 3′ non-coding region (leader), nucleoprotein (NP), virion protein 35 (VP35), VP40, 3 glycoproteins (sGP/ssGP/GP1,2), VP30, VP24, RNA-dependent RNA-polymerase protein (L-polymerase), and 5′ non-coding region [7].

The glycoproteins of the Ebola virus

The EBOV genome encodes one transmembrane protein GP1,2 (GP1–GP2) and two secreted non-structural proteins: secretary glycoprotein (sGP) and small soluble glycoprotein (ssGP). A small soluble delta peptide (Δ-peptide) is secreted from EBOV-infected cells after the carboxyl-terminal cleavage of sGP [8]. GP1,2 is produced through transcriptional RNA editing as a precursor for 676 amino acid polyprotein (GP0), which is post-translationally cleaved by furin into two disulfide-linked subunits; a surface subunit, GP1; and a membrane-spanning subunit, GP2. GP1 contains the receptor-binding domain (RBD) for host cell attachment and a mucin-like domain to protect the RBD from humoral and cell-mediated immunity. The RBD responsible for receptor binding, viral entry, and cellular tropism is covered by a heavily glycosylated "glycan cap". The transmembrane GP2 contains a helical heptad-repeat region, transmembrane anchor, and a 4-residue cytoplasmic tail. The GP2 drives fusion of the viral membrane with the endosomal membrane of the target cell. This GP1–GP2 heterodimer then assembles as a trimer on the viral surface. This homotrimeric GP1,2 complex forms the spike on the envelope membrane of the mature viral particles. During processing, GP1,2 are unstable, and an abundant amount of a soluble non-virion form of GP1 and a scanty amount of GP1,2 are released into the circulation [9-12]. The virus-associated GP1,2 and not the other soluble glycoproteins released during the virus infection are responsible for primary target cell activation [13]. The highly glycosylated mucin-like region of GP1 is cytotoxic to the host cells [14]. The shedding of souble GP1,2-like protein due to cleavage of EBOV glycoprotein on the surface of EBOV-infected cells by tumor necrosis factor-alpha converting enzyme (TACE) can activate non-infected dendritic cells and macrophages to induce cytokine dysregulation and endothelial cell dysfunction [15]. The GP2 of the EBOV is able to counter the interferon (IFN)-inducible antiviral protein tetherin which restricts the VP40-dependent budding of the progeny viral particles from infected cells [16-18]. The sGP is produced from non-edited mRNA species through furin cleavage from a precursor pre-sGP. The sGP shares the N-terminal 295 amino acids with GP1, but differs in the carboxyl terminus by 69 amino acids. The sGP is released into the circulation in the form of homodimers in anti-parallel orientation [19] to evade an antibody-associated innate immune response [20,21]. The sGP has an anti-inflammatory function and impairs the transmigration and activation of neutrophils [22,23]. While the GP1,2 in its particle-associated form mediates endothelial cell activation and a decrease in endothelial cell barrier function, sGP protects the endothelial cell against cytokine-induced barrier dysfunction. The sGP constitutes at greater than 80% of the total GP synthesized during infection. Hence, the hypersecretion of the sGP may protect the EBOV against host humoral immune defense and the host endothelial cell against cytokine-induced cytotoxicity during the early phase of the EBOV infection [15,24,25]. Δ-peptide released in EBOV-infected cells joins cathepsins and integrins to inhibit further entry of the EBOV in a dose-dependent manner to prevent superinfection of EBOV-infected cells. Δ-peptide inhibits entry of both marburgviruses and the EBOV, indicating that they might interfere with a common pathway used by filoviruses to gain entry into target cells [26]. The ssGP of a yet undefined function is produced through transcriptional editing and secreted in the form of a disulfide-linked homodimer that is exclusively N-glycosylated. While ssGP appears to share similar structural properties with sGP, it does not appear to have the same anti-inflammatory function as sGP [22,23,27].

The life cycle of the Ebola virus

The EBOV, being a RNA virus with limited coding capacity, has utilized the host's unique metabolic pathway for its viral entry, replication, and egress. The entry of the EBOV into cells is initiated by interaction of the viral GP1 with host cell surface T-cell immunoglobulin and mucin domain 1 (TIM-1) receptors. Upon receptor binding, the EBOV is internalized into endosomes

primarily via macropinocytosis [28-30]. Within the acidified endosome compartment of the host cell, the heavily glycosylated GP1 is cleaved to a smaller 19-kDa fusogenic form by the low pH-dependent cellular proteases Cathepsin L (CatL) and B (CatB), exposing residues in the receptor binding site. This allows the binding of GP1 to cholesterol transporter Niemann-Pick C1 (NPC1), a step in the late endosome phase essential for virus-host membrane fusion and viral entry [31-34]. Cells where the NPC1 function has been biochemically disrupted or cells lacking NPC1 showed resistance to the EBOV infection. Cells from subjects with NPC1 disease were resistant to the EBOV because of defects in the NPC1 protein [35-38]. After complete fusion of the viral and host endosomal membranes via conformational change in GP2, viral RNA and its associated proteins are released into the host cell cytoplasm [39]. Once inside the cytoplasm of the host cell, the EBOV suppresses the innate immune response via VP35 and VP24 proteins [40], and hijacks transcription and translation for robust genome replication and the production of new virions. The ribonucleoprotein (RNP) complex that mediates transcription and replication of the EBOV genome comprises NP, VP35, VP30, and L protein [41-44]. VP30 is essential in the initiation of the EBOV transcription, but is not required for viral replication. However, dynamic phosphorylation of VP30 is an important mechanism to regulate the balance between the transcription and replication processes in the EBOV replication cycle [45-47]. This unique property of VP30 allows the development of a genetically stable VP30 deleted EBOV vaccine with protective efficacy in the mice and guinea pig models [48]. The matrix proteins VP40 and VP24 associated with the viral lipid coat are important for virus structure and stability. Both matrix proteins VP24 and VP40 contribute to the regulation of viral genome replication and transcription [49] and the budding of the virus [50-52], an important step prior to viral egress [53,54]. This distinct replication cycle of the EBOV serves as an attractive target for the development of therapeutic agents against the EBOV (see Figure 1 and Table 1).

Pathogenesis of the Ebola virus infection

Human EBOV hemorrhagic fever, characterized by uncontrolled viral replication together with immune and vascular dysregulation, has a case fatality rate of up to 90% [7]. Type I alpha/beta interferons (IFN-α/β), encoded by a single IFN-β and 13 homologous IFN-α genes in humans, represent an essential element of host defense against virus infections, including the EBOV [55]. The human EBOV infection is associated with robust IFN-α production—with plasma concentrations of IFN-α that greatly (60- to 100-fold) exceed those observed in other viral infections—but limited IFN-β production [56]. The

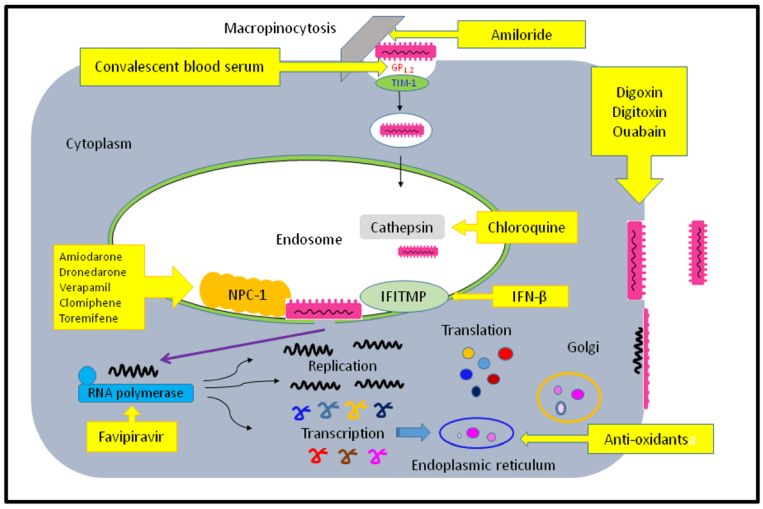

Figure 1 Schematic diagram showing the replication cycle of Ebola virus (EBOV): Upon receptor binding of EBOV GP_1 with host TIM-1 receptor, EBOV is internalized into endosome via macropinocytosis. Within the acidified endosome compartment of the host cell, under the action of the low pH-dependent cellular proteases cathepsins, the receptor binding site of GP_1 to cholesterol transporter Niemann-Pick C1 (NPC1) is exposed. This results in conformational change in GP_2 , leading to complete fusion of the viral and host endosomal membranes in the late endosome and the release of viral RNA and its associated proteins into the host cell cytoplasm. EBOV then hijacks transcription and translation for robust genome replication and viral protein production under the action of ribonucleoprotein polymerase complex (RNP polymerase). The accumulation of $GP_{1,2}$ in the endoplasmic reticulum leads to endoplasmic reticulum overload response (ER-overload) which, in turn, induces cytokine dysregulation via the activation of nuclear factor kappa B (NFκB) through the production of reactive oxygen species (ROS). New virions are released through ATP-dependent budding and egress from host cell membrane. Currently available therapeutic agents that target the different steps of the EBOV life cycle are described in Table 1.

Table 1 Available therapeutic agents that target the different steps of the EBOV life cycle as shown in the diagram

Medication	Mechanism of action
Convalescent blood serum	Contain neutralizing antibodies to provide passive immunity.
Na^+/K^+ exchanger	Inhibit virus uptake by macropinocytosis.
- Amiloride	
Chloroquine[1]	Leads to alkalinization of endosomes and prevent the acid pH-dependent cleavage of Ebola virus $GP_{1,2}$ by endosomal proteases cathepsin B and L.
Cationic amphiphiles	Induce a Niemann-Pick C-like phenotype and block the entry of EBOV through late endosomes.
Amiodarone[1]	
Dronedarone[1]	
Verapamil[2]	
Clomiphene	
Toremifene[1]	
Interferon- beta (IFN-β)	Induce interferon-inducible transmembrane proteins (IFITMP) production to restrict entry of EBOV.
Favipiravir	Suppress viral RNA polymerase.
Na+/K+/ATPase pump inhibitors	Inhibit Na^+/K^+-ATPase that are important in the budding and egress of encapsulated EBOV.
Ouabain	
Digoxin	
Digitoxin	
Anti-oxidants	Suppress ROS-dependent NFκB activation and cytokine dysregulation induced by $GP_{1,2}$-induced ER-overload.
High dose N-acetylcysteine infusion	

[1]Chloroquine, Amiodarone, Dronedarone and Toremifene administration is associated with an increased risk of QT prolongation and *Torsades de pointes*.
[2]Verapamil should be avoided in patient with hypotension.

EBOV, protected from the host interferon response by its encoded VP35 and VP24 proteins [40,57-59], produced a heavy viral load [60-62], cytopathic damages [14,63,64], and cytokine dysregulation in humans [65-68]. The efficient productive replication of the EBOV inside monocyte and macrophages leads to a massive release of proinflammatory cytokines/chemokines and reactive oxygen species (ROS) [13,15,65,66,69-71], which in turn leads to diffuse endothelial cell dysfunction [72-76], disseminated intravascular coagulation [77-79], and vasomotor collapse [80-82]. The infection of the antigen presenting dendritic cells [83-86] and profound bystander apoptosis of lymphocytes [63,87-89] impairs the development of adaptive immunity [90,91] and EBOV-specific CD8+ T [92-94], as well as CD4+ T cells [95] that are important for the clearance of, and protection from, the EBOV infection. Infected monocyte-derived dendritic cells were impaired in the secretion of pro-inflammatory cytokines, the up-regulation of co-stimulatory molecules, and the stimulation of T cells [96]. Numbers of CD4+ and CD8+ T cells are substantially reduced in fatal human and nonhuman primate (NHP) infections before death [63,88,97].

Immune evasion by the glycoproteins of the Ebola virus: implications on passive immunization and vaccine development

The EBOV is able to counteract both humoral and cell-mediated immunity through its GP1,2 and sGP [11,98]. The overexpression of mature GP1,2 on the plasma membrane results in the masking of antigenic epitopes on GP1,2 itself and the shielding of MHC-I and integrin β, leading to evasion of antiviral immunity. Steric shielding of surface epitopes by the heavily glycosylated GP impairs the recognition and killing of EBOV-infected cells by the natural killer and cytotoxic CD8+ T cell during an acute viral infection. It may also contribute to the persistent infection in the natural reservoir host to perpetuate the spread of the EBOV [99-101]. The sGP can evade host antibody-mediated response through "antigenic subversion" by eliciting non-neutralizing antibodies that cross-react with GP1,2. Thus, the massive secretion of sGP by the EBOV may prevent effective neutralization of the virus during an EBOV infection and reduce the effectiveness of vaccines that rely upon neutralizing antibody responses against GP1,2 [20,21]. Some of the antibodies against GP1 may lead to enhancement of infectivity of the EBOV via interaction with complement component C1q, a phenomenon known as the antibody-dependent enhancement. The EBOV initiates infection by binding its GP1 to its specific human receptor sites on the surface of human cells. The interaction of C1q enhances binding between the virus-antibody complex and the C1q ligands on the cell surface, promoting interaction between the EBOV and its receptor. These infectivity-enhancing antibodies were virus species specific and were primarily correlated with immunoglobulin IgG2a and IgM levels, but not with IgG1 levels [102,103]. The presence of infectivity-enhancing antibodies against GP1,2 in the EBOV infection raises concerns about the effectiveness of GP-based EBOV vaccines, and the use of passive prophylaxis or treatment with GP-based antibodies [104,105].

Antibodies against GP1 of the EBOV can be neutralizing, enhancing, or non-neutralizing and non-enhancing. Neutralizing antibodies are produced in infection by the EBOV at a relatively low frequency [106]. Some anti-EBOV antibodies are known to be neutralizing in vitro but not protective in vivo, whereas other antibodies are known to be protective in animal models in vivo, but not neutralizing in vitro [107]. Investigations of anti-GP antibodies against the EBOV showed that non-neutralizing antibodies

recognized GP epitopes in the sGP or non-essential mucin-like domain of GP1, while neutralizing antibodies were specific to RBD in GP1 or conformation-dependent epitopes at the base of the GP1,2 spike where GP1 meets GP2. Two neutralizing antibodies (KZ52 and JP3K11) against EBOV—that recognize conformation-dependent epitopes comprising residues in GP1 and GP2—were identified to have quite distinct mechanisms of neutralization. KZ52 is a human recombinant IgG1 neutralizing antibody derived from a human survivor of a natural EBOV infection during the 1995 outbreak in Kikwit, Democratic Republic of Congo. KZ52 has impaired recognition for the sGP and binding was dependent on the presence of GP2 residues which are not present in the sGP. KZ52 is able to inhibit cathepsin cleavage of GP1,2. JP3K11, a monkey derived neutralizing monoclonal antibody against EBOV, recognized the cleaved, fusion-active form of GP [108]. 16 F6 is a mice derived monoclonal IgG1 antibody that neutralizes Sudan EBOV by preventing the conformational changes in GP1,2 required for membrane fusion. Both 16 F6 and KZ52 recognize GP1–GP2-bridging epitopes at the base of the GP1,2 trimer, indicating that this overlapping epitope may be one of the key sites for neutralization of the EBOV, and is thus a target for immunotherapy and a key goal of vaccine design [109]. Antibody subclass may be another important factor in protection against the EBOV. IgG2 isotype may offer more effective protection against EBOV [110,111]. Although fully protecting guinea pigs from infection, KZ52 fails to slow viral replication and protect NHPs from the EBOV infection [112]. In contrast, rVSV-EBOGP [113-116] and rChAd-EBOGP [117-120]-based vaccination have demonstrated both prophylactic and post-exposure protection in NHPs [121]. This was previously attributed to the protective action of EBOV-specific CD4+ and CD8+ T-cell response induced by these vaccines in limiting infection and the inability of KZ52 to completely block all entries of the EBOV into cells and its subsequent explosive replication [112]. rChAd-EBOGP-based vaccination is able to generate potent humoral and cell-mediated responses. Significant antibody titers are detectable at 48 weeks post vaccination [122,123]. CD8+ cell-mediated immunity has been shown to play a critical role in protection against the EBOV infection in NHPs in rChAd-EBOGP-based vaccination [124]. On the other hand, humoral rather than the cell-mediated response contributes to protection against the EBOV infection in NHPs in rVSV-EBOGP-based vaccination [125,126].

Candidate vaccines expressing the EBOV GP or NP protect rodents and NHPs from the lethal EBOV infection [127-129]. Humoral and cell-mediated immune responses are working together to provide protection against the lethal EBOV infection. Either response alone may be able to limit virus replication but both arms of the immune response are required to clear the infection [97,130]. VP proteins (VP24, VP30, VP35, and VP40) are poor inducers of cell-mediated immunity and are inaccessible to the protective effect of VP-induced neutralizing antibodies because they are not found on the surface of virions or infected cells [131]. However, the genetic sites of these internal proteins are susceptible to siRNA and PMO interference. TKM-Ebola (a siRNA targeting L-polymerase, VP24, and VP35) can be administered intravenously or subcutaneously in a lyophilized lipid nanoparticle formulation. TKM-Ebola offers post-exposure protection against the EBOV infection in NHPs. The FDA has approved an "expanded access" program for the use of TKM-Ebola in patients with confirmed or suspected infections [132,133]. Anti-sense phosphorodiamidate morpholino oligomers AVI-6002 effectively reduce viral load, diminish virally-induced pathology, and improve survival of NHPs with the EBOV infection by targeting VP24 and VP35 mRNA. Through judicious placement of positive charges on the drug backbone, the drug is able to bind to a negative charge on the virus even if binding at one or more drug-virus base pairs are lost through mutation. This integration of dual targeting and charge complementation significantly lowers the likelihood of drug resistance through viral mutagenesis [134,135].

Available drugs that target the different steps of the Ebola virus life cycle

Currently available therapeutic agents that are effective in targeting the EBOV infection in cell or animal studies may include convalescent plasma, favipiravir, chloroquine, amiodarone, dronedarone, verapamil, clomiphene, toremifene, IFN-β, Na^+/K^+ exchangers, Na^+/K^+-ATPase pump inhibitors, and antioxidants. Except for convalescent plasma and favipiravir, most of the therapeutic agents under review are acting against the non-mutable targets of the host cells which participate in the replication cycle of the EBOV. They may also have a complementary role to conventional therapy in the management of the current EBOV outbreak in West African countries (see Table 1).

(1) Convalescent blood serum

The WHO issued a consensus statement that the use of whole blood therapies and convalescent blood serum needs to be considered as a matter of priority in the recent EBOV outbreak in West African countries [2]. The development of neutralizing antibodies and T-cell responses are important for recovery from the EBOV infection [97,136]. Patients who are able to mount an immune response to the EBOV will begin to recover in seven to ten days and start a period of prolonged convalescence [137]. In survivors, early and increasing levels of IgG, directed mainly against the NP and the VP40, were followed by the clearance of circulating viral antigen and activation of cytotoxic T cells. In contrast, fatal infection was characterized by

impaired humoral responses, with absent specific IgG and barely detectable IgM [63]. Convalescent blood has been shown to improve survival of EBOV-infected patients during the outbreak in Kikwit in 1995 [138]. Immunity against EBOV GP is sufficient to protect individuals against infection, and several vaccines based on EBOV GP are under development including recombinant adenovirus, parainfluenza virus, Venezuelan equine encephalitis virus, vesicular stomatitis virus, and virus-like particles [139]. Neutralizing human monoclonal antibodies is able to protect mouse and guinea pigs from lethal EBOV. However, the protection was achieved only by treatment shortly before or after viral infection [140-142]. The EBOV can rapidly mutate to produce antibody-escape mutants. Hence, antibody therapy may require hyperimmune polyclonal serum or a panel of monoclonal antibodies of different epitope specificities to be successful [143,144]. These studies have laid the foundation for subsequent clinical research on the development of monoclonal antibodies [145-148] and utilization of a monoclonal antibody cocktail such as MB-003 [149], ZMAb [150], and ZMapp [151] in the treatment of the EBOV infection in NHPs. It is interesting to note that all three monoclonal antibody cocktails include one antibody that binds to or close to the glycan cap and that two of the three monoclonal antibody cocktails include at least one antibody that binds the GP1/GP2 interface, indicating that these two regions may be especially important in protection against EBOV [148]. The treatment window of monoclonal antibody therapy can be extended by the co-administration of adenovirus-vectored interferon therapy. In a guinea pig model, monoclonal antibodies combined with adenovirus-vectored interferon given three days after infection resulted in 100% survival, a significant improvement over either treatment alone [152]. A subsequent study showed that such a combination therapy is capable of saving 100% of EBOV-infected NHPs when initiated after the presence of detectable viremia along with symptoms [153].

(2) Favipiravir (T-705; 6-fluoro-3-hydroxy-2-pyrazinecarboxamide)

Favipiravir is a broad-spectrum inhibitor of viral RNA polymerase that is able to inhibit the replication of many RNA viruses. It is registered in Japan for the treatment of influenza virus infection [154,155]. Favipiravir is able to suppress the replication of the EBOV in cell culture. Favipiravir, initiated at day 6 after EBOV infection, induced rapid virus clearance, reduced the biochemical parameters of disease severity, and prevented a lethal outcome in 100% of mice lacking the Type I interferon receptor [156]. Oral favipiravir taken twice daily for 14 days is able to give 100% protection against an aerosol EBOV infection in an immune-deficient mice model [157,158]. The survival benefit was suboptimal in NHPs. Only one of the six animals tested survived. Studies using dosages that are two to five times higher and have duration longer than shown in influenza studies are being conducted for the human EBOV infection [5]. BCX4430, a synthetic adenosine analogue with a viral RNA polymerase inhibitor function, is active against the EBOV and Marburg virus in rodent and cell culture. BCX4430 completely protects NHPs from the Marburg virus infection when administered as late as 48 hours after infection [159,160].

(3) Chloroquine

The antimalarial drug chloroquine is able to increase endosomal pH. An acidic endosomal environment is important for the pH-dependent activation of cysteine proteases CatB and CatL, the proteases responsible for the cleavage of EBOV GP1,2 essential for endosomal virus-host membrane fusion [35,39,161-163]. However, proteolytic processing of the EBOV glycoprotein has been demonstrated to be not critical for EBOV replication in cell culture [164] or NHPs [165]. A recent study using a CatB and CatL deficient mouse model for the study of the EBOV infection demonstrates that CatB and CatL activity is not absolutely required for EBOV replication. The EBOV glycoprotein cleavage seems to be mediated through a broader spectrum of proteases making therapeutic approaches targeting limited proteases unlikely to be beneficial to combat EBOV infections [166]. A broad-spectrum small molecule that targets the CatL cleavage of the EBOV and inhibits the entry of a wide variety of viruses has recently been identified. It has been examined for the potential to develop into a potent broad-spectrum antiviral medication [167].

(4) Cationic amphiphiles

Multiple cationic amphiphiles including amiodarone, dronedarone, verapamil, clomiphene, and toremifene have been identified as potent inhibitors of the entry of the EBOV in an NPC1-dependent fashion [38,168]. Amiodarone used for the treatment of atrial fibrillation and ventricular cardiac arrhythmia can induce lipidosis with features similar to Niemann-Pick C disease [169]. Amiodarone and dronedarone, having basic pKa and high water solubility at acidic pH, accumulates within late endosomal compartments, blocking fluid-phase endocytosis, proteolysis and lipid trafficking, and inducing a Niemann-Pick C-like phenotype. In contrast to the Niemann-Pick type-C disease, they are not alleviated by cholesterol removal [170,171].

Amiodarone, at concentrations that are routinely reached in human serum during anti-arrhythmic therapy (1.5–2.5 μg/ml), is a potent inhibitor of filovirus cell entry through late endosomes (IC50 0.25 μg/ml for EBOV), when induced as a Niemann-Pick C-like phenotype. Significant inhibition is observed in most endothelial and epithelial cells (e.g. macrophage, monocyte, vascular

endothelial cell), except for primary hepatocyte and fibroblast. The inhibitory effect of amiodarone on the entry of the EBOV was dose-dependent and reversible upon removal of the drug. Prolonged exposure to amiodarone will not lead to a compensatory change in the host cell. A similar inhibitory property is observed with the amiodarone-related agent dronedarone and the L-type calcium channel blocker verapamil [38,168,172,173].

Both clomiphene and toremifene have anti-EBOV activity in both the Vero E6 (interferon-deficient African green monkey kidney epithelial cells) and HepG2 (human hepatocellular carcinoma) cell lines. The anti-EBOV activity of clomiphene and toremifene is dependent not on its estrogen receptor antagonistic action but upon the ability of both drugs to induce a Niemann-Pick C-like phenotype to inhibit viral entry at late endosome. Clomiphene and toremifene do not disrupt the interaction between primed GP1 and NPC1, but mediate the entry block indirectly through NPC1 by targeting other endosomal/lysosomal proteins involved in the cholesterol uptake pathway whose functions may be regulated by NPC1. Clomiphene and toremifene at 60 mg/kg every other day have been shown to result in a 90% and 50% survival rate, respectively, in EBOV-infected mice compared with 100% mortality in the control group in an in vivo murine EBOV infection model. They are effective in both male and female mice [38,174]. However, the therapeutic dose against EBOV cannot be achieved with the oral clomiphene dose used for inducing ovulation in humans [175-177]. The therapeutic dose against EBOV with tolerable side effects can be achieved with toremifene at an oral dose used in the human trial for the treatment of advanced carcinoma of the breast [178-181]. Toremifene is well absorbed and >99.5% bound to plasma protein. Toremifene undergoes extensive liver metabolism and enterohepatic recirculation. The majority of the toremifene dose is excreted as metabolites in feces. The long terminal half-life of oral toremifene may be due to both plasma protein binding and enterohepatic recirculation [182,183].

(5) Interferon-beta

Interferon-induced transmembrane proteins (IFITMs) are expressed basally in the absence of IFN induction in both primary tissues and cell lines [184]. An IFITM is able to inhibit the entry of viruses to the host cell cytoplasm; permit endocytosis, but prevent subsequent viral fusion; and release viral contents into the cytosol. The human IFITM locus is located on chromosome 11 and composed of four functional genes: IFITM1, IFITM2, IFITM3, and IFITM5. IFITM4p is a pseudogene. Viruses that are restricted by IFITM proteins tend to fuse with host cell membranes in a late endosome or lysosome that precedes the induction of Type I IFN in infected cells. Viral escape from restriction by IFITM proteins could be more challenging than for antagonizing inhibitory factors that function at later stages of the virus life cycle because the opportunity for de novo synthesis of viral inhibitors is not available. All four human IFITM proteins are induced robustly by both Type I and Type II IFNs. IFITM1 is active against multiple viruses, including the EBOV and hepatitis C viruses [185-187]. IFN-β is able to induce interferon-inducible transmembrane protein production to restrict entry of the EBOV [188]. Early post-exposure treatment with IFN-β significantly increased survival time of rhesus macaques infected with a lethal dose of the EBOV, although IFN-β alone failed to alter the mortality rate. IFN-β treatment was associated with a trend towards lower plasma and tissue viral burden and pro-inflammatory cytokines production [56].

(6) Na^+/K^+ exchangers (amiloride and its derivatives)

Amiloride and its derivatives are used as potassium-sparing diuretics to treat hypertension and congestive heart failure. Apart from inhibiting epithelial Na^+ channel and cellular Na^+/K^+ exchangers, these drugs could also affect the function of other less well-defined ion-exchangers (Na^+/Ca^{2+} and Na^+/Mg^{2+}), and disturb the equilibrium of other ions, such as Mn^{2+} [189-192]. The entry of the EBOV into host cells is the first step of infection and a crucial determinant of pathogenicity. Upon receptor binding between GP1 and host TIM-1 receptors, the EBOV is internalized into endosomes primarily via the macropinocytic pathway. Amiloride is able to inhibit the uptake of many viruses that utilize the macropinocytic pathway for host cell entry [193-196]. Amiloride at non-cytotoxic dosages leads to potent dose-dependent inhibition of the entry and infection of the EBOV [197,198]. Amiloride can lead to dose-dependent inhibition of RNA synthesis. This may be due to a direct blockage of a nucleotide entry tunnel or catalytic site, or due to its effect on the equilibrium of Mg^{2+} and Mn^{2+} that are essential co-factors for polymerase activity and nucleotide insertion [199,200]. These novel antiviral mechanisms of amiloride may uncover new targets for drug discovery against the EBOV.

(7) Na^+/K^+-ATPase pump inhibitors (ouabain, digoxin, and digitoxin)

Adenosine triphosphate (ATP) is essential in multiple steps in the replication cycle of many viruses. Na^+/K^+-ATPase pump is located in the plasma membrane of all animal cells to maintain the cell membrane potential. Budding of enveloped viruses is a complex phenomenon that requires concerted actions of many viral and host components. ATP may affect multiple steps in the budding process [201]. ATP is required for the assembly and maturation of a number of enveloped viruses such as the influenza virus, vaccinia virus, retrovirus, and herpes simplex virus. The

Na^+/K^+-ATPase pump inhibitors, ouabain, Lanatoside C, strophanthidin, and digoxin are able to inhibit the replication of the influenza virus, Newcastle disease virus, and vesicular stomatitis virus through an interferon-independent mechanism [202]. Digoxin and Lanatoside C have been shown to inhibit vaccinia virus replication at non-cytotoxic doses [203]. Ouabain has shown antiviral activity against the influenza virus [204], herpes simplex virus [205], Sendai virus [206], murine leukemic virus [207], cytomegalovirus porcine reproductive and respiratory syndrome virus [208], and human cytomegalovirus virus [209]. One common feature shared by these viruses is that they all possess a lipid envelope. The EBOV is an enveloped filamentous RNA virus. The secondary matrix protein VP24—apart from its role in the evasion of host immune response, nucleocapsid formation, and regulation of replication—has an important role in viral budding and egress. Na^+/K^+-ATPase ATP1A1 is detected to have a close interaction with VP24 of EBOV during replication. Ouabain, at a non-cytotoxic concentration of 20nM, is able to suppress the replication of the EBOV in human MRC-5 cells [210,211]. Among the three cardiac glycosides that may include digoxin, digitoxin, and ouabain, only digoxin is commonly used in clinical practice. Ouabain, because of its poor oral availability, is used primarily as a research tool. Further research should be conducted to investigate whether digoxin and other Na^+/K^+-ATPase inhibitors might play a role in the management of the EBOV or other enveloped virus infections.

(8) Antioxidants

The virus-associated glycoprotein GP1,2 is responsible for the activation of human macrophages [13]. The highly glycosylated mucin-like region of the GP1 subunit of GP1,2 is cytotoxic to the host cells [14]. The mucin-like region in GP1 leads to an accumulation of GP1,2 at the endoplasmic reticulum, induces endoplasmic reticulum stress [212], and activates nuclear factor kappa B (NF-κB) [213]. Mutations of the EBOV that lead to an enhanced accumulation of GP1,2 in the endoplasmic reticulum were significantly more cytotoxic than wild-type virus [214]. In human cells, the accumulation of protein in the endoplasmic reticulum will lead to endoplasmic reticulum overload response (ER-overload) which activates NF-κB through the production of ROS [215]. As a major transcription factor for antiviral and immune stimulatory activities, NF-κB is thought to play an important role in the induction of pro-inflammatory molecules, such as interleukin-1β (IL-1β), and tumor necrosis factor α (TNF-α), upon cellular responses against a virus infection [216]. The cytokine dysregulation of the EBOV involves massive ROS, NF-κB, TNF-α, and IL-1β activation [65,66]. The effectiveness of antioxidant therapy for the EBOV infection indicates the importance of ROS in the pathogenesis of the EBOV [217]. The activation of NF-κB by ER-overload is ROS-dependent [218]. NF-κB-induced cytokine dysregulation of novel H1N1 pneumonia has been shown to be suppressible by high-dose N-acetylcysteine (NAC) antioxidant therapy at 100 mg/kg continuous infusion daily [219]. Given the poor oral availability of NAC in the range of 6% to 10% in humans [220], a therapeutic dose of NAC equivalent to the intravenous route can hardly be delivered by oral preparation. NAC is a category B drug for pregnancy and is affordable, with a wide therapeutic window. NAC has an established safety profile even in high doses and prolonged use in humans [221-223].

Cytokine dysregulation is a common feature in the EBOV infection and is associated with an enhanced mortality [65-68]. Antiviral medications directed against the mutable viral determinants of the EBOV cannot directly prevent cytokine dysregulation. The early endothelial vascular damage characteristic of the EBOV infection is not a direct effect of virus-induced cytolysis of endothelial cells, but is due to cytokine dysregulation resulting from massive release of proinflammatory cytokines/chemokines and ROS by infected macrophage and monocytes [70-72]. Lymphocytes are resistant to the EBOV infection. Cytokine dysregulation may also contribute to the diffuse bystander apoptosis of lymphocytes [63,87-89]. With the safety profile of NAC, if the therapeutic efficacy of a high-dose NAC antioxidant therapy to manage EBOV-induced cytokine dysregulation is confirmed, it may revamp the future management of the EBOV infection.

Proposed prophylactic and therapeutic regimen against the Ebola virus infection

There is a desperate need for a viable treatment regimen in Africa to engender hope and encourage people with symptoms and their close contacts to seek medical treatment, so as to limit the spread of the disease. This also helps to recruit and maintain adequate medical staff who are at high risk of contracting the disease. A proposed regimen against the human EBOV infection based on available medications and information from in vivo animal testing and in vitro cell culture is attached (see Tables 2 and 3). This regimen contains a cocktail of currently available medications that can target the different steps in the replication cycle of the EBOV aiming to suppress viral proliferation. It has been shown that viral load is major contribution to survival in both human and animal studies [60-62,136]. Through viral load suppression, we may be able to prolong a patient's survival in order to allow the development of natural body immune defense against the EBOV.

The EBOV has undergone a rapid mutation during its spread through humans [224-226]. The EBOV is an RNA virus the replication of which is highly error prone with nearly one viral mutation occurring during each cycle of replication. This extremely high mutation rate leads to

Table 2 Proposed therapeutic regimen for the prophylaxes and treatment of human EBOV infection based on available therapeutic medications and information from in vivo animal testing and in vitro cell culture

Therapeutic regimen based on available medications for ebola virus prophylaxes and treatment	
Ebola virus	**Available medications**
Prophylaxis[1]	Amiodarone (macrophage, monocyte & endothelial cell)
Post Needle Stick Injury Prophylaxis	IFN-β + amiodarone (macrophage, monocyte & endothelial cell) + toremifene (liver)[2,3] + favipiravir[4] ± convalescent blood serum
Treatment	Amiodarone (macrophage, monocyte & endothelial cell) + toremifene (liver)[2,3] + favipiravir[4] + high dose N-acetylcysteine infusion[5] + convalescent blood serum + supportive care

[1]1 ml of blood may contain 10^9 to 10^{10} virions in terminally ill patient. Prophylactic amiodarone therapy may protect macrophage, monocyte and endothelial cells immediately from EBOV during needle stick injury and accidental exposure and allow time for the consideration of IFN-β, toremifene, favipiravir and convalescent blood serum therapy.
[2]Amiodarone is unable to protect hepatocyte from EBOV infection.
[3]Both amiodarone and toremifene can increase the risk of QT prolongation and *Torsades de pointes*.
[4]The recommended dosage for treatment of human EBOV infection may be 2 to 5 times higher than influenza studies. Please confirm the recommended dose with the drug company.
[5]N-acetylcysteine intravenous infusion at 100 mg/kg/day to control cytokine dysregulation (e.g. add 5 g of intravenous preparation of N-acetylcysteine into each liter of intravenous replacement fluid).

significant genetic and antigenic diversity that allows the EBOV population to evolve resistance to antiviral medications and vaccines [227,228]. A combination therapy has been used in the treatment of RNA virus infections, such as the human immunodeficiency virus (HIV) [229,230] and hepatitis C [231,232] to minimize the development of drug resistance. Given the broad cell tropism and high replication rate of the EBOV due to the potent suppression of both innate and adaptive immune responses of the host, patients with the EBOV infection have an extremely high viral load. The selective pressure in the presence of the high mutation rate and viral load during the human EBOV infection make the evolution of the EBOV viral strains resistant to a single drug inevitable. The currently available medications in the proposed regimen—which is a treatment regimen containing a cocktail of antiviral medications targeting the different steps of the EBOV replication in order to achieve maximal suppression of viral replication and to prevent the rapid development of resistance to favipiravir, the only drug in the regimen that is directed against a mutable target of the EBOV—has been shown to reduce the replication of the EBOV. [233-235].

The current EBOV vaccine (rVSV-EBOGP and rChAd-EBOGP) and therapeutic agents (ZMapp, TKM-Ebola, PMO AVI-6002, and favipiravir) under development are directed against the mutable targets of the EBOV, and their effectiveness is limited by viral mutation. The EBOV, being a RNA virus with limited coding capacity, has utilized the host's unique metabolic pathway for its viral entry, replication, and egress. Most of the therapeutic agents in this current review are directed against non-mutable targets of the host which is independent of viral mutation. These medications are FDA-approved for the treatment of other diseases. They are available and stockpileable for immediate use. They may also have a complementary role to those therapeutic agents under development that are directed against the mutable targets of the EBOV.

The primary target of the EBOV is the mononuclear phagocytic system. The spectrum of target cells increases to include endothelial cells, fibroblasts, hepatocytes, and many other cells during the advanced stage of the disease [6,236,237]. The EBOV may produce a viral load of up to 10^{10} virions per ml serum in terminally ill patients [80]. Oral amiodarone prophylaxis, by inducing a Niemann-Pick C-like phenotype in the cells of the mononuclear phagocytic system, may prevent viral entry into these cells during needle stick injury. Through protection of the mononuclear system by our prophylaxis and cocktail therapy, we hope to offer a better chance of survival to these patients by allowing them to develop a natural body immune defense against the EBOV infection. The liver, containing the largest number of fixed tissue macrophages (Kupffer cells), as part of the reticuloendothelial immune defense system of the body, is a major target for the EBOV infection [238,239]. The EBOV replicates to high titer in the liver [240]. Hepatic apoptosis may play a role in the pathogenesis of the EBOV infection [88]. Toremifene is added to the treatment regimen for hepatic protection because amiodarone does not exert inhibitory action against the EBOV in hepatocyte. However, both amiodarone and toremifene can increase QTc and the risk of Torsades de pointes. Therefore electrocardiogram should be carefully monitored if both drugs are to be used. Amiodarone, favipiravir, and toremifene are available and stockpileable in oral preparations. These properties are advantageous in outbreak situations and contingency planning of a potential EBOV epidemic or pandemic. The avoidance of intravenous administration will prevent needle stick injury in healthcare workers caring for the infected patients.

IFN-β may have potential as an adjunctive post-exposure therapy for high-risk exposure, such as needle stick injury, by inducing IFITM1 to limit entry of the EBOV. Post-exposure IFN-β treatment was associated with a trend towards lower plasma and tissue viral burden and pro-inflammatory cytokines production [56]. The reduction in viral load and cytokine dysregulation coupled with optimal supportive therapy may improve the chance of survival of the host to allow the development of natural immunity to control the underlying EBOV infection. IFITM1 is active against multiple viruses, including the EBOV [185,188] and hepatitis C

Table 3 Prophylaxis regimen for healthcare worker after needle stick injury

Regimen	Oral[1]	Intravenous[4]
Central venous line	**Not required**	**Required**
Interferon-beta	6 million international units (MIU) prefilled pen via intramuscular injection (IMI) weekly for 3 weeks.[2]	6 MIU intravenous infusion over 2 hour daily for up to 3 weeks[3] or 6 MIU prefilled pen IMI weekly for 3 weeks.
Amiodarone	600 mg p.o. twice daily for 8 days (loading) then maintenance 600 mg p.o. daily for further 3 weeks.	150 mg into 100 ml D5 over 10 minutes followed by 360 mg infusion over 6 hours then 540 mg infusion over 18 hours D1.[4] Amiodarone 720 mg infusion daily or 600 mg p.o. twice daily for further 7 days followed by 600 mg p.o. maintenance daily for further 3 weeks.
Toremifene	800 mg p.o. on Day 1 (loading) then 400 mg p.o. daily.[5]	800 mg p.o. on Day 1 (loading) then 400 mg p.o. daily.[5]
Favipiravir	1800 mg p.o. twice daily on Day 1 (loading doses) then 800 mg p.o. twice daily.[6]	1800 mg p.o. twice daily on Day 1 (loading doses) then 800 mg p.o. twice daily.

[1]Oral regimen are for those workers who are already on amiodarone prophylaxis with a loading dose of amiodarone 600 mg p.o. twice daily for 8 days followed by maintenance amiodarone 600 mg p.o. daily. Electrocardiogram and thyroid function should be monitored.
[2]Monitor for side effect of thrombocytopenia and proteinuria.
[3]Intravenous dosage of IFN-β that are used for human hepatitis C virus infection to induce IFITM1 to limit viral entry.
[4]Intravenous regimen is for those workers who have not been on amiodarone prophylaxis and agreed for the insertion of a central venous line for drug administration. Intravenous amiodarone should be administered via central venous line to avoid phlebitis. The dosage for treatment of frequently recurring ventricular fibrillation and hemodynamically unstable ventricular tachycardia is recommended because it can achieve therapeutic drug level immediately after the first dose of amiodarone.
[5]http://www.pulmcrit.org/2014/08/could-estrogen-receptor-antagonists.html.
[6]Dosage for the treatment of human influenza virus infection in human Phase 3 trial of Favipiravir (FAVOR Study). http://www.clinicaltrials.gov/show/NCT02008344. The recommended dosage for treatment of human EBOV infection may be 2 to 5 times higher than influenza studies. Please confirm the recommended dose with the drug company.

[186,187,241,242]. Interferon induced IFITM1 plays an important role in the treatment of human HCV infection by inhibiting entry of HCV into the host cell [243]. Six million international units (MIU) of IFN-β intravenous administration is as effective as a three MIU twice-daily regimen for treatment of the HCV infection [244], but has lesser side effects that require discontinuation of the medication [245,246]. As the aim of IFN-β therapy in our regimen for post needle stick prophylaxis against the EBOV infection is to induce IFITM1 to limit viral entry, the dose of IFN-β for the post needle stick prophylaxis [247,248] or induction therapy [249,250] for HCV infection in humans is chosen. Once infection is fully established, IFN-β are replaced by convalescent blood serum and high-dose NAC infusion for providing passive humoral immunity and for the control of ROS-dependent NF-κB-induced cytokine dysregulation respectively.

Summary

The EBOV is classified as biosafety level 4 pathogen and is classified by Centers for Disease Control and Prevention as a category A agent of bioterrorism with no approved therapies and vaccines for its treatment but carrying a high potential for large-scale dissemination. Recent political, economic, military, and religious turbulence around the world raises concerns that the EBOV might be used as an agent of bioterrorism [251-253]. The recent EBOV epidemic is spiraling out of control in West Africa. The containment measures that worked in the past, such as isolating those who are infected and tracing their contacts, have failed due to an exponential rise in infected patients. Although the short-term (three- and six-week) probability of international spread outside the African region is small, the risk of the extension of the outbreak to other African countries followed by international dissemination on a longer time scale is not negligible, indicating that this public health emergency has the potential to grow to extraordinarily destructive dimensions [254,255]. Although several promising therapeutic agents and vaccines against the EBOV are undergoing the Phase I human trial, the current epidemic might be outpacing the speed at which drugs and vaccines can be produced [5]. To combat such an unprecedented global public-health crisis before these experimental agents are available, alternative available interventions capable of managing the enhanced viral replication and cytokine dysregulation of the human EBOV infection should be explored and stockpiled as contingency preparation for the worst-case scenario of an impending human EBOV pandemic [256].

Like all viruses, the EBOV largely relies on host cell factors and physiological processes for its entry, replication, and egress which, in turn, lead to cytopathic damage, cytokine dysregulation, and death of the host. These non-mutable key steps inside the host may be novel targets for future therapeutic strategies against these rapidly mutating viruses. If the efficacy of amiloride, digoxin, amiodarone, and high-dose NAC antioxidant therapy against the human EBOV infection is confirmed, the availability and affordability of these stockpileable

agents make them ideal medications in pandemic situation and in countries with limited resources. They may have a complementary role to other antiviral medications to prevent the emergence of resistant strains. This may also signify a major breakthrough in future management of the EBOV infection.

Additional file

Additional file 1: Multilingual abstracts in the six official working languages of the United Nations.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
KYL and GWYN contributed to the conception, drafting, and writing of the paper. KYL, GWYN and FC revised the draft paper. All authors read and approved the revised paper.

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