Insights into Ebola Virus VP35 and VP24 Interferon inhibitory functions and their initial exploitation as drug targets

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Abstract: Upon viral infection, the interferon (IFN) system triggers potent antiviral mechanisms limiting viral growth and spread. Hence, to sustain their infection, viruses evolved efficient counteracting strategies to evade IFN control. Ebola virus (EBOV), member of the family Filoviridae, is one of the most virulent and deadly pathogen ever faced by humans. Etiological agent of the Ebola virus disease (EVD), EBOV can be undoubtedly considered the perfect example of a powerful inhibitor of the host organism immune response activation. Particularly, the efficacious suppression of the IFN cascade contributes to disease progression and severity. Among the EBOV-encoded proteins, the viral proteins 35 (VP35) and 24 (VP24) are responsible for the EBOV extreme virulence, representing the core of such inhibitory function through which EBOV determines its very effective shield to the cellular immune defenses. VP35 inhibits the activation of the cascade leading to IFN production, while VP24 inhibits the activation of the IFN-stimulated genes. A number of studies demonstrated that both VP35 and VP24 are validated target for drug development. Insights of the structural characteristics of VP35 and VP24 domains revealed crucial pockets exploitable for drug development. Considered the lack of therapy for EVD, restoring the immune activation is a promising approach for drug development. In the present review we summarize the importance of VP35 and VP24 proteins in counteracting the host IFN cellular response and discuss their potential as druggable viral targets as a promising approach toward attenuation of EBOV virulence.

Keywords: Ebola virus, Interferon, IFN production, IFN signaling, VP35, VP24, small molecules, FDA approved drugs

1. INTRODUCTION

Zaire Ebola virus (EBOV) is an enveloped virus with a linear, non-segmented, ssRNA- of approximately 19 kb in length, organized in seven linear genes which encode for the nucleoprotein (NP), viral protein (VP) 35, VP40, glycoprotein (GP), VP30, VP24 and the Large (L) RNA-dependent RNA polymerase [1,2]. This virus causes a highly contagious hemorrhagic fever in humans and non-human primates called Ebola virus disease (EVD). The most devastating EVD West African epidemic occurred among the years 2014-2016 standing as a landmark in EBOV history, resulting in more than 28000 cases and 10000 deaths.

EBOV is a zoonotic pathogen whose reservoir has not been definitively clarified, possibly due to the difficulty to isolate EBOV or viral RNA in animals that are infected at very low titer, while humans are only occasional hosts. After viral inoculation onto mucosal surfaces or skin's injuries, EBOV targets dendritic cells, monocytes and macrophages, then reaches the lymphatic system from which spread along multiple tissues and organs [1].

After an incubation period of generally 2–21 days, the first stages of the infection include an asymptomatic incubation followed by the onset of nonspecific symptoms consisting of fever, chills, fatigue, headache, myalgia and general malaise [3]. EVD subsequent manifestations are characterized by a multisystem involvement with systemic, gastrointestinal, respiratory, vascular and neurological disorders. During the peak of the illness the hemorrhagic complications (macropapular rash, petechiae, ecchymosis, mucosal hemorrhages) occur followed, in later stages, by shock, convulsions, severe metabolic disturbances, and death typically within about 10 days of symptom for hypovolaemic shock and multiorgan failure [1,4].

The EBOV ability to efficiently evade the interferon (IFN) response is a key for the pathogenesis of the disease. In particular, two proteins - VP35 and VP24 - are responsible for blocking the IFN production and signaling, respectively, provoking a rapid impairment of the early cellular defense machine [5–22]. In light of their contribution to the high virulence, they are attractive target for drug development [16,18,19,23–26]. In this review, we propose an overview of the molecular mechanisms adopted by EBOV to escape this response underling the impact that might has a VP35/VP24 target-based therapy.

2. THE IFN SYSTEM

In case of a viral infection, the defense mechanisms against infectious pathogens are regulated in concert through innate and adaptive immunities, the innate immune system being the first defense line defense. An efficient and successful innate response determines the consequential activation of

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the adaptive immune response that, contrarily to the innate, is specific and responsible for inducing and maintaining the immune memory. In most instances, thanks to this defense system virus-infected patients recover from viral infections and either eliminate the virus or incorporate it in a latent or persistent form without further problems [27,28].

The IFN system, as part of the innate immune response, serves as first-line protection against virus infections, inducing antiviral states in infected and neighboring cells and promoting the activation of the adaptive responses, including antigen presentation and natural killer cell functions.

Typically, viral infections are detected by sensor molecules within virus-infected cells that synthesize and secrete type I IFNs, IFN-α and IFN-β (IFN-α/β). Secreted IFNs circulate in the body where they exert antiviral activity limiting further viral growth and spread [27,29]. The activation of the IFN system begins with recognition of pathogen-associated molecular pattern (PAMP) by pattern recognition receptors (PRRs), that sense the presence of the invading pathogens. Activation of host PRRs by non-self nucleic acids such as those found in RNA viruses trigger a signaling cascade resulting in the production of IFN-α/β and the expression of hundreds of IFN-stimulated genes (ISGs), whose encoded proteins exert antiviral roles such as the inhibition of viral replication, transcription and translation as well as the degradation of viral nucleic acids and the alteration of the host cells lipidic metabolism [28–30].

Viral PAMPs mainly consist of nucleic acids that originate from different steps of the viral life cycle: uncoating process of newly-infecting virions, transcription of viral genes and replication of genomic intermediates. EBOV replication and transcription are primer-independent processes and the synthesis of viral RNA starts with a single nucleoside triphosphate, resulting in production of genomes, antigenomes and viral transcripts that bear 5'-ppp end [31]. These molecules could form secondary structures after self-hybridization or could anneal to complementary sequence resulting in dsRNA with blunt ends or exposed 5'-ppp, efficient triggers for PRRs activation [32].

Two PRRs families are dedicated to specifically recognize dsRNA and differ for their cellular compartmentalization. The recognition of dsRNA in endosomes, lysosomes and extracellular surface is exerted by TLR-3, a member of the Toll-like receptor (TLR) family. In contrast, dsRNA recognition in the cytoplasm occurs through the retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) family, that includes RIG-I, the melanoma differentiation associated factor-5 (MDA5) and the laboratory of genetics and physiology-2 (LGP2) proteins [14,33].

On the one hand, the signal transduction at the base of the recruitment of dsRNA by TLR3 starts with the dimerization and phosphorylation of TLR3 [34] and the engagement of the adaptor Toll–interleukin (IL)-1-resistance (TIR) domain-containing adaptor inducing IFN-β (TRIF) which leads to the activation of IRF3 through its phosphorylation mediated by the kinases TBK-1 and IKKε [35–37]. On the other hand, the recognition of viral nucleic acids by cytosolic RLRs promotes the recruitment and activation of a mitochondrion-associated adaptor called CARD adaptor inducing IFN-β (Cardif)/virus-induced signalling adaptor (VISA)/ mitochondrial antiviral signalling protein (MAVS)/ IFN-β promoter stimulator protein 1 (IPS-1) [38–42]. The engagement of RIG-I to from the cytosol to mitochondrial membranes is mediated by the tripartite motif 25 alpha (TRIM25a) E3 ligase which is important for the interaction with MAVS [43,44]. The prosecution of the cascade is characterized by MAVS prion-like polymerization [45] and the recruitment of two adaptors, tumor necrosis factor (TNF) receptor-associated factor 3 (TRAF3) and the nuclear factor-kB (NF-kB) essential modifier (NEMO), which connect and regulate signaling between MAVS and the complex formed by TRAF family member-associated NF-kB activator (TANK)-binding kinase 1 (TBK-1) and inducible IkB kinase epsilon (IKK-ε) [35,46–49]. The formation of the complex TBK-1-IKK-ε-MAVS is essential for IRF phosphorylation and is strongly enhanced by the stimulator of interferon genes (STING), also called mediator of IRF-3 activation (MITA) and endoplasmic reticulum IFN stimulator (ERIS) [50–52]. Once phosphorylated IRF-3 and IRF-7 homo- or heterodimerize and associate with other transcription factors, driving the transcription of IFN-α/β promoter [53].

The signaling pathway activated in response to IFN-α/β has been deeply characterized [54–56]. IFN-α and IFN-β bind a heterodimeric receptor named IFN-α receptor (IFNAR), composed of two subunits, IFNAR1 and IFNAR2. IFNAR is shown to activate the receptor-associated protein tyrosine kinases Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2), which phosphorylate the cellular transcription factors signal transducer and activator of transcription 1 (STAT1) and STAT2 [57–59]. Phosphorylated STAT1 (P-STAT1) and STAT2 (P-STAT2) dimerize and assemble with IFN-regulatory factor 9 (IRF-9) to form a heterotrimeric complex, IFN-stimulated gene factor 3 (ISGF3) [57,58,60]. The translocation to the nucleus is dependent on the tyrosine phosphorylation of STAT1 which is at the base of the conformational change resulting in the recognition by a subset of the karyopherin-α (KPNα) family of nuclear transport factors [61,62]. The binding between P-STAT1 and a non-classical nuclear localization signal (ncNLS) motif of KPNα results in the nuclear transport of the complex [63]. In the nucleus, ISGF3 recognizes the IFN-stimulated response elements (ISRE), promoter of the ISGs [60,64,65].

3. EBOV EVASION OF IFN SYSTEM

The severity of EVD is mainly due to the massive viral replication and dissemination that are strictly dependent on the EBOV efficiency to evade the host innate immune response, particularly the IFN system [1,11]. The first evidences were observed in human umbilical vein endothelial cells where the induction of several immunomodulatory genes, including the major histocompatibility complex class I (MHC-I) family of genes, 2'-5' oligoadenylate synthetase, interleukin-6 (IL-6), PKR, IRF-1, and intercellular adhesion molecule-1 (ICAM-1) by dsRNA polyinosinic acid:polycytidylic acid (PIC) was dramatically suppressed by infection with EBOV and not by other negative-stranded RNA viruses [66]. In mock-infected cells, PIC induced the formation of protein–DNA complexes that specifically target the ISRE or GAS promoters, while infection with EBOV strongly blocked the formation of these PIC-induced complexes, suggesting a possible inhibition of STAT-1α function [66]. Moreover, the same EBOV infection did not induce these genes [66,67].

Addition of IFN-γ or IFN-α or their combination in EBOV infected cells led to a complete inhibition of IFN induction resulting in a level of MHC protein expression lower than that in the mock-infected not stimulated cells [67]. After these preliminary studies, much work has been done to deeply investigate the viral proteins responsible for the IFN inhibition and now it is well known that they are VP35 and VP24 [5–22,68].

3.1. VP35

The EBOV-encoded protein VP35, whose gene is positioned second on the 19 kb EBOV linear genome, is a protein of 340 amino acid residues long with a molecular mass of ~35 kDa. VP35 is a dsRNA binding protein that fulfills several important functions in the viral life cycle, acting i) as a component of the replication and transcription holoenzyme, ii) as an assembly factor of EBOV viral particles and iii) as a powerful antagonist of the host antiviral innate immune response [5,25,69–71].

VP35 allows the efficient impairment of the host defenses and the viral replication blocking the IFN-α/β production pathway at several points, both hiding and directly hitting the components of the pathway (Figure 1) [11,72]. The VP35 C-terminal domain is involved in suppression of the IFN response as demonstrated by the fact that this domain alone is sufficient to exert this property; therefore, it was named interferon inhibitory domain (IID) [10]. In addition, the N-terminal coiled-coil domain provides a critical VP35 oligomerization function enhancing IFN inhibition as its deletion or mutation abrogates IFN suppression [21,73,74].

VP35 was found to act at the cascade upper levels, hiding the presence of dsRNA replicative intermediates to the sensory action of RIG-I and MDA5. The central basic patch (CBP) of VP35 IID is involved in the interaction with dsRNAs, differently from the first basic patch (FBP) important for VP35 function in the replication complex. Crystallographic analyses solved the structures of EBOV IID in complex with 8 bp dsRNA [75] and Reston IID bound to an 18 bp dsRNA [76] and revealed that VP35 uses a bimodal strategy to bind the dsRNA. With a VP35 monomer binds the terminal nucleotides and the proximal phosphate backbone, while with a second monomer it binds the sugar-phosphate backbone of both dsRNA strands. These two binding modalities, termed as "end-capping" and "backbone-capping", respectively, lead the two VP35 monomers to assemble as an asymmetric dimer that binds with very high affinity to both blunt-ended and 5'-ppp dsRNA molecules and in a sequence-independent mode (Figure 2) [12,13,68,76,77]. The structural characteristics of the VP35 IID revealed the contribution of several amino acids for the dsRNA binding function. Residues that lie within the highly conserved CBP R305, K309, R312 and K339 directly interact with the dsRNA phosphate backbone. In addition, protein–protein interactions occurs between the R312, K319, R322 and K339 and the dimer interface (Figure 2). In the end-capping monomer I340 and F239 interact with the K339, Q274 and I278 residues, which in turn bind dsRNA terminal nucleotides (Figure 2) [75,76].

Mutations in residues R305, K309, R312, K319, R322, F239 and K339 correspond to a diminished or abolished suppression of IFN-β induction [13,16,20,68,75,78–80]. Notably, upon infection of guinea pigs with wild-type VP35

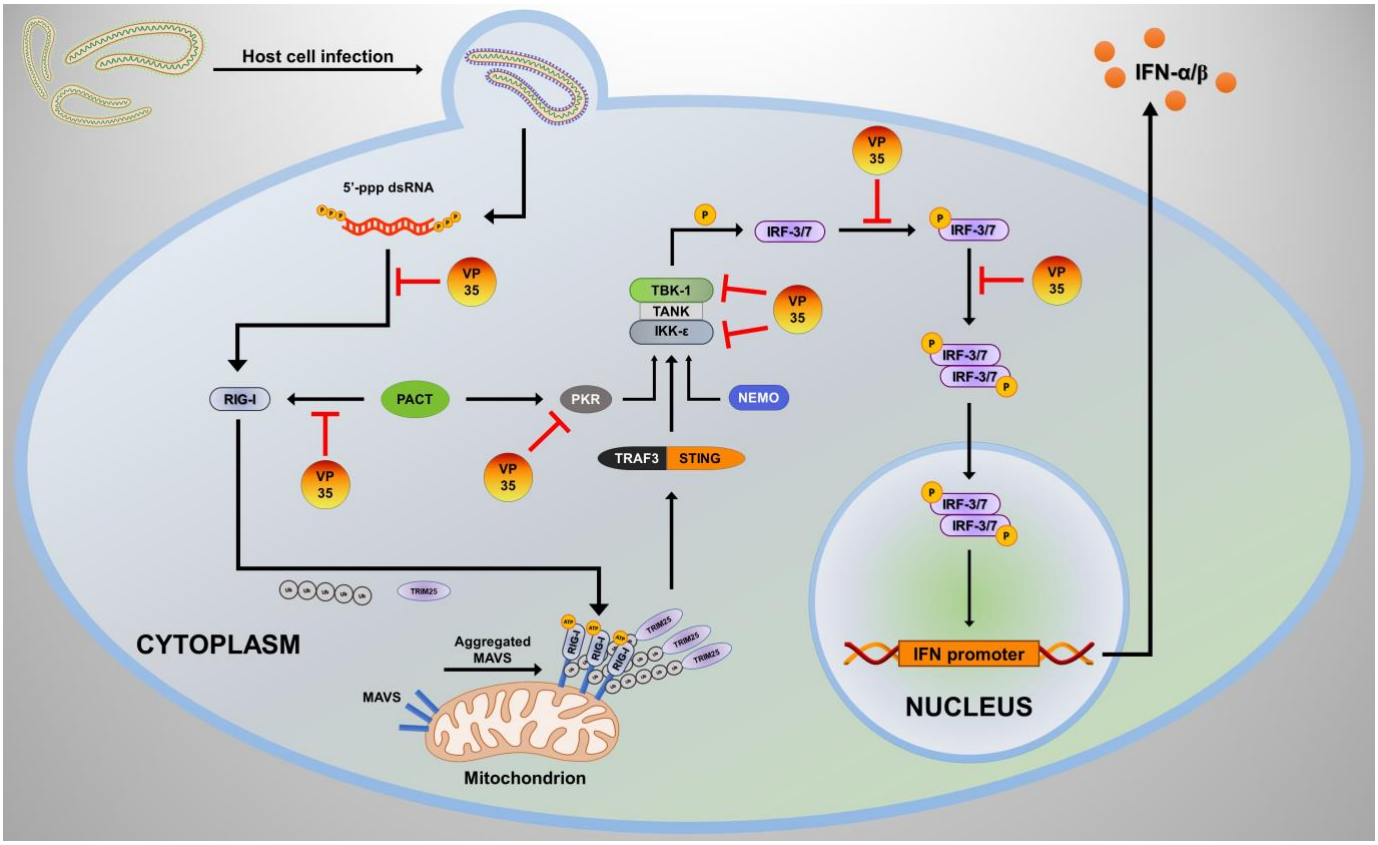

Figure 1. VP35 inhibition of IFN-α/β production.

a massive viral replication with death of all animals was observed within 5 to 7 days postinfection. In the contrary, the VP35 double point mutant R319A/ K322A (KRA) EBOV was completely avirulent. Viral RNA at 3, 5, or 17 days or later postinfection and any disease symptoms were detected upon infection with EBOV/VP35KRA, suggesting a very low virus replication in animals. When EBOV/VP35KRA infected guinea pigs were challenged with lethal EBOVwt, all the animals survived through 28 days postchallenge, confirming the EBOV/VP35KRA induction of an anti-EBOV state [16].

Beyond the sequestration and hiding of RLR-activating RNAs, VP35 also employs several "hit" strategies to suppress the downstream RIG-I pathway and IFN-α/β induction [11,81]. EBOV VP35 was shown to block the Sendai virus-induced activation of ISG54 and ISG56 promoters which are directly activated by IRF-3, whose phosphorylation, dimerization and nuclear accumulation were also inhibited [15]. Further, it was demonstrated that VP35 blocked IRF-3 phosphorylation and nuclear translocation induced by TBK-1 and IKKε overexpression [82]. Notably, co-immunoprecipitation (co-IP) studies revealed VP35 ability to directly interact with TBK-1 and IKKε, via their more conserved kinase domains located within the N-terminal domain of the two proteins, decreasing their catalytic activity and, acting as substrate being phosphorylated by these kinases. The overexpression of VP35 resulted in a disruption of IKK-ε interactions with IRF-3, IRF-7 and MAVS [82]. In addition, the N-terminal half of VP35 physically interact with both IRF-3 and IRF-7 to promote their ubiquitin (Ub)-like modification by the two members of the small Ub-like modifier (SUMO) cascade PIAS1 (the SUMO E3 ligase protein, inhibitor of activated STAT) and Ubc9 (the SUMO E2 enzyme), thereby inhibiting the IRFs transcriptional function and subsequently suppressing the activation of IFN-β promoter [29,83].

VP35 also inhibits the RIG-I-induced TRIM6-mediated IFN-I production by counteracting the host ubiquitin system [84–86]. VP35 IID K309 ubiquitinated interacts with TRIM6, a member of the E3-ubiquitin ligase tripartite motif family that promotes the synthesis of unanchored poly-Ub chains required for activation of IKKε kinase, thus blocking its IFN induction function [87,88].

Furthermore, VP35 interacts with RNA-activated protein kinase (PKR) activator (PACT), a cellular dsRNA binding protein involved in stimulation of RIG-I ATPase activity thus facilitating its activation by dsRNA [8,25]. EBOV VP35 interacts with PACT disabling its interactation with RIG-I so that PACT activation of RIG-I and induction of IFN-α/β gene expression is impaired. Interestingly, mutations of residues that abolished VP35 dsRNA binding activity resulted in the disruption of VP35-PACT interaction and in the VP35 inability to block PACT–RIG-I interaction. This provides evidence that the same VP35 residues that mediate the binding with dsRNA are also involved in the direct interaction with PACT [8].

Finally, VP35 counteracts IFN-α/β induction inhibiting the activation of the dsRNA-activated kinase PKR [77,89]. PKR is activated by autophosphorylation upon binding to dsRNA or phosphorylation by PACT. In normal conditions, PKR is

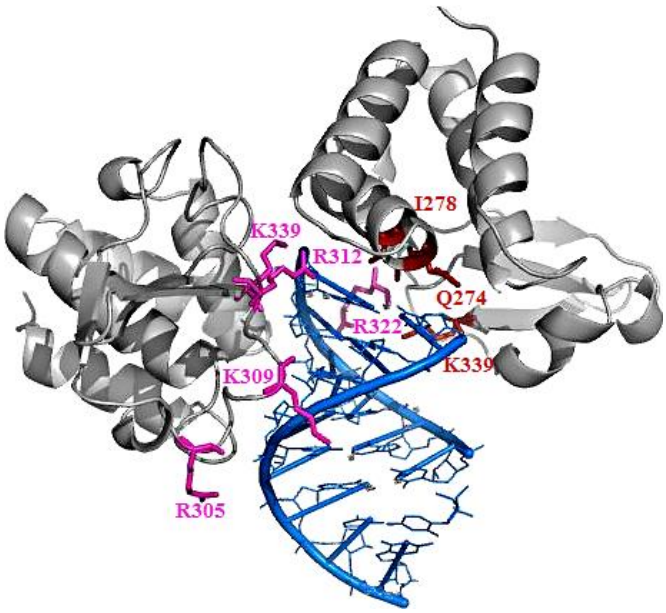

Figure 2. EBOV VP35 IID (gray) **in complex with 8 bp dsRNA** (blue). The two modalities of binding "end-capping" and "backbone-capping" are shown with residues labelled in red and pink,respectively.

expressed at basal level, but upon IFN induction its expression is upregulated and acts as an intracellular PRR, enhancing the IFN production [90–92]. After the interaction with dsRNA, the activated PKR phosphorylates the residue S51 of the α subunit of translation initiation factor eIF-2 (eIF-2α), which blocks protein synthesis [77]. The VP35 IID involved in dsRNA binding and IRF3 inhibition also mediates the inhibition of PKR enhancing the protein synthesis, but the mechanism seems to be dsRNA-binding independent [89]. Despite PACT is a PKR activator, no direct relation has been shown between the VP35 inhibition of PACT and PKR [11].

3.2. VP24

Ebola virus (EBOV) VP24 is also one of the seven proteins encoded by the viral genome. VP24 has been demonstrated to participate in different levels of the EBOV life cycle, including nucleocapsid formation, assembly and budding of the viral particles and viral replication [93–99]. Moreover, VP24 is known to contribute to the EBOV high virulence[24], in particular for its role in modulating the host response to infection [23]. Using reverse genetics, Ebihara et al. identified VP24 mutations correlated to the ability to evade type I IFN response that were responsible for the acquisition of high virulence of the adapted Mayinga strain in mice [23]. The main mechanism by which VP24 is able to hinder the cellular antiviral defense is the efficient suppression of the both IFN-α/β (Figure 3) and IFN-γ signaling [6]. This reflects the protein ability to interact with a step that is common to both cascades: the binding of karyopherin-α (KPNα) to phosphorylated STAT1 (P-STAT1) which is essential for the P-STAT1 nuclear translocation and hence activation of transcription of ISGs. In particular, VP24 binds specifically to the NP1-1 subfamily of KPNα (α1, α5 and α6) while does not interact with KPNα2, KPNα3 and KPNα4 [6,7], which are not involved in STAT1 nuclear transport (Figure 3).

Mutation analysis with truncated KPNα1 demonstrated that

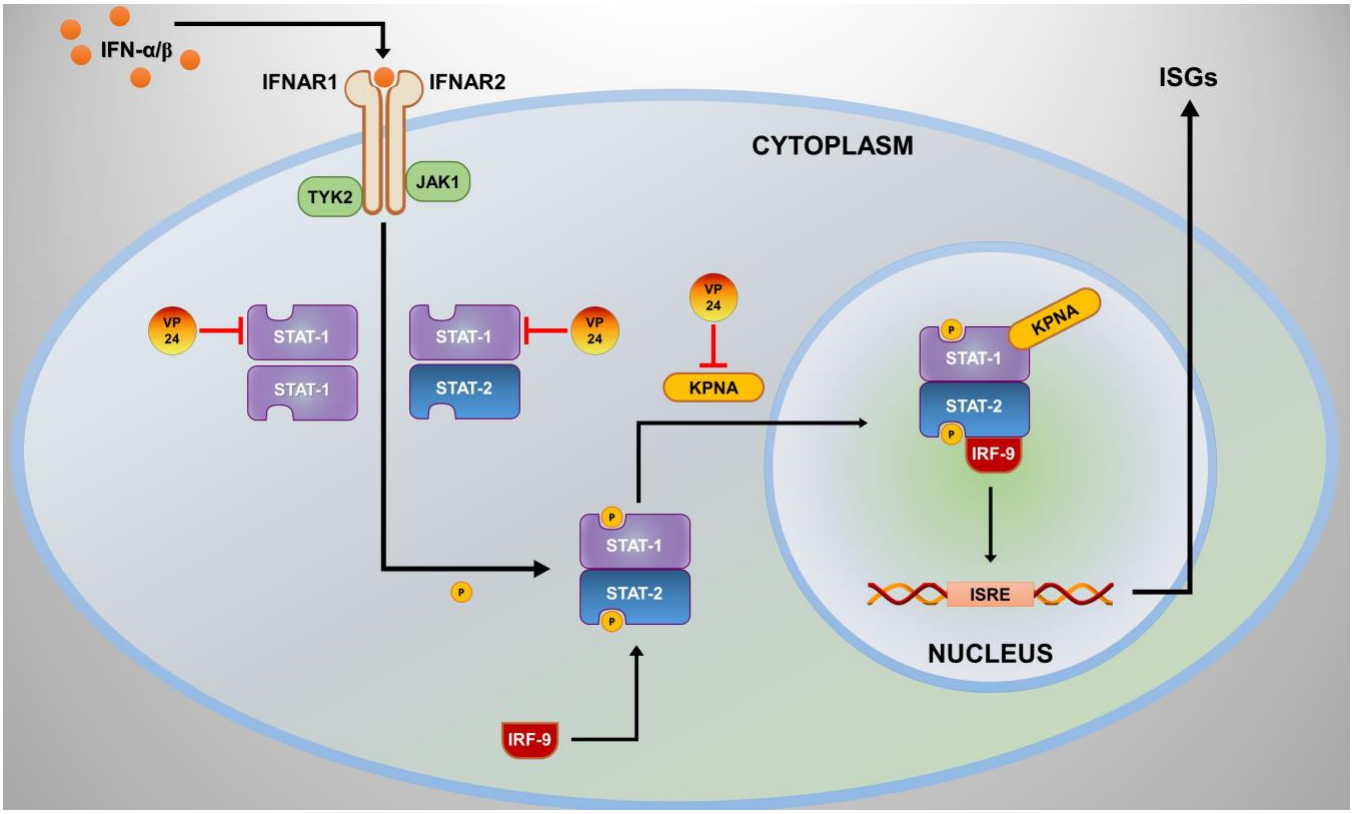

Figure 3. VP24 inhibition of IFN-α/β signaling.

residues 425 to 538 are implicated with the binding to P-STAT1, while residues 458 to 504 bind VP24, revealing that there is a competition between the two proteins for the binding to the same region of KPNα1 [22]. Subsequent studies identified two VP24 domains (26 to 50 and 142 to 146) responsible for blocking IFN-β gene expression and P-STAT1 nuclear translocation [7]. Combining a mutation in the first domain (W42A) and mutations at residues 142 to 146, ISGs promoter expression was increased more than 90% compared with wt VP24 and the interaction with KPNα1 decreased. Complete loss of binding was observed with the VP24 mutant K142A [7]. The crystal structure of KPNα5 interacting with VP24 has been determined [63]. Analysis of these residues demonstrated the hydrophobic property of the interface. KPNα5 residues 308-509 (KPNα5C) surrounding armadillo (ARM) repeats 7-10 (329-502) are sufficient for binding to VP24, however the minimal binding region was demonstrated to be within ARMs 8-10 (371-502) [63]. Three clusters of VP24 are mainly involved in the binding to KPNα5C and include residues N130, T131, N135, R137, T138 and R140 (Cluster 1), residues Q184, N185, H186 (Cluster 2) and L201, E203, P204, D205 and S207 (Cluster 3). Other residues required for the binding are L115, L121, D124, W125, T128 and T129 [63]. Mutations of residues 142 to 146 due to their proximity to cluster 1 probably contribute to conformational change of the binding site and consequent loss of IFN inhibitory function (Figure 4).

The ability of VP24 to inhibit the P-STAT1 nuclear transport is explained by the finding that VP24 specifically recognizes a non-classical nuclear localization signal (ncNLS) binding site on KPNα5 that partially overlaps with the ncNLS binding site for P-STAT1 [63]. Another suggested mechanism is the direct binding of VP24 with STAT1 [100].

Using an *in vitro* binding assay, Zhang et al. reported that a purified EBOV VP24 bound directly a truncated form of STAT1 lacking the phosphorylation site at Y701, although it was not determined for full length non-phosphorylated STAT1 or P-STAT1 [100]. The finding, however, was not confirmed by cell-based experiments that did not detect

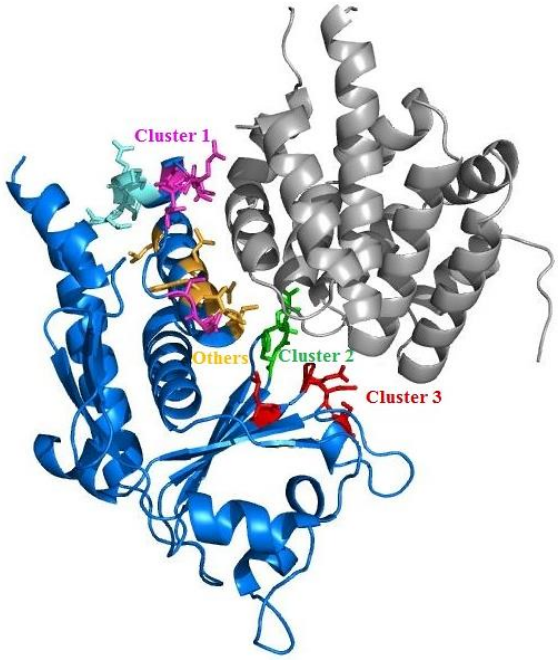

Figure 4. EBOV VP24 (blue) **in complex with KPNα5C** (gray)**.** Residues implicated in the binding are grouped in Cluster 1 (pink), Cluster 2 (green) and Cluster 3 (red), while the other residues contributing to IFN signaling inhibition are shown in yellow.

interaction between VP24 and non-phosphorylated STAT1 or P-STAT1 [63].

In addition to its role within the JAK/STAT cascade, it has been shown that VP24 blocks the cellular response to type I IFN also counteracting the p38 MAP kinase pathway inhibiting the IFN-β–induced phosphorylation of p38-α [101]. The p38 MAP kinase cascade is activated after hepatitis C virus infection inducing the cellular antiviral state. The finding that also VP24 blocks this response suggests that at least two mechanisms are possible for the IFN inhibition by VP24. There are, in fact, mutations critical for virulence in mouse-adapted EBOV not affecting the JAK/STAT cascade that might be related to this other IFN inhibitory function [22].

4. ANTIVIRAL APPROACHES TO TARGET VP35 AND VP24 IFN INHIBITORY FUNCTION

Mutational studies on both VP35 and VP24 confirmed the correlation between virulence and the ability to suppress the IFN antiviral response, validating them as excellent targets for drug development [16,23]. As already mentioned, it has been extensively demonstrated that mutations of residues principally involved in the function of IFN antagonism, as residues R319, K322 of VP35 and T50I of VP24 are critical for the acquisition of the high virulence in animal models and thus can contribute to the progression of the infection even in humans[16,23]. Finding a therapy targeting the IFN inhibitory functions of VP35 and VP24 leading to the restoration of the IFN response has an obvious impact in the treatment of EVD. Although the increasing and constant effort in the identification of VP35 and VP24 inhibitors and despite recent progresses have been made in developing novel techniques to test their functions, nowadays no approved drugs are available against these promising pharmacological targets. These studies involved *in vitro*, *in vivo* and *in silico* approaches aimed to identify potential inhibitors among small molecules and FDA-approved drugs, plant extracts or compounds, oligomers, peptides and immune therapies [16,18,19,63,102–113]

4.3. Small molecules and FDA approved drugs

An ensemble-flexible strategy was used to dock a 80,000 compound library on the crystallographic structure of VP35 (PDB ID: 3FKE). The search space for docking specifically included the CBP of VP35 IID and the residues known to be involved in the dsRNA binding: R305, K309, R312, K319, R322 and K339 of which residues R305, K339, K309 and R322 were designated as flexible. Among compounds with the highest predicted affinity, ZINC05328460 (Figure 5) showed the greatest inhibition of VP35-dsRNA binding (IC_{50}: 4 μM) [105].

Seven new VP35 potential inhibitors (cpd 1 – 7) were identified using a pharmacophore model (HypoA of 4-acetyl-3-hydroxy-1-phenyl-1H-pyrrol-2(5H)-one derivates) with 3D QSAR prediction and molecular docking method [104].

Using a pharmacophore based virtual screening and docking study, the FDA-approved deslanoside and digoxin and the deslanoside analogue ZINC77291634 potentially showed high binding affinity for VP35 (PDB ID: 3FKE) with predicted binding energies of -12.9 kcal/mol, -12.2 kcal/mol, and -10.0 kcal/mol, respectively, and for VP24 (PDB ID: 4M0Q) (-12.0 kcal/mol, -11.6 kcal/mol and -9.8 kcal/mol respectively). In contrast, ZINC85911633 (-9.8 kcal/mol) showed a predicted high energy only in complex with VP24 [108].

Furthermore, a screening of FDA approved drugs demonstrated that amodiaquine (Figure 5) and cloroquine, for which anti-EBOV activity was previously demonstrated [114], had higher LibDock scores when docked on the VP35 structure (PDB ID: 4IBI) [115]. Treatment of EVD patients with a combination of amodiaquine-artesunate resulted in an increased survival [116]. In contrast, treatment of animals or EVD patients with cloroquine did not resulted in an increase of the survival benefit [117–119].

Another FDA approved drug, indinavir, known to inhibit the HIV protease, was identified to potentially interact with

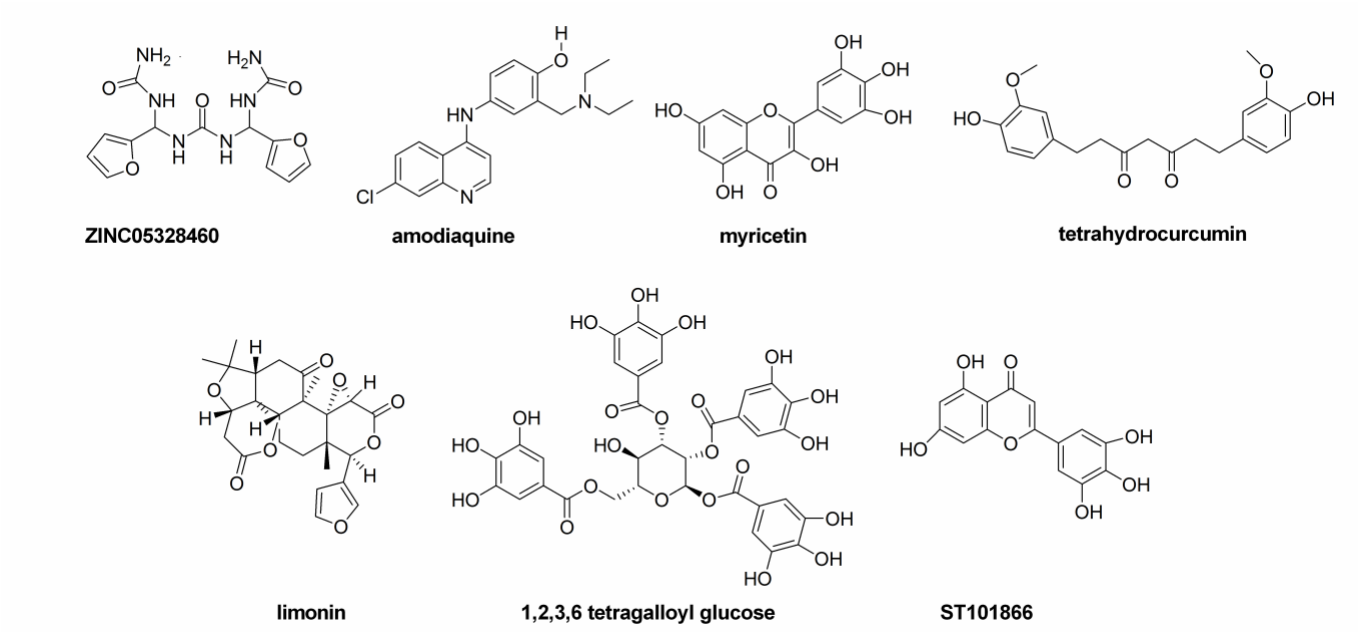

Figure 5. VP35 and VP24 inhibitors.

VP24 (PDB ID: 4M0Q) using the same atoms to form hydrogen bonds with both HIV protease and VP24 [120].

Moreover, a fragment-based drug approach led to the identification of a fragment compound, L595, with high predicted affinity for VP24 (PDB ID: 4U2X) (-54.2 kcal/mol). L595 pharmacological and toxicity prediction also confirm its potential as VP24 inhibitor [106].

4.4. Plant extracts and natural compounds

A luciferase reporter gene assay was developed to quantify the inhibition on IFN production by EBOV VP35 (Cannas et al. 2015) and was used to test the extract from the leaves of *Asphodelus microcarpus* Salzm.et Vivi against the viral protein. The extract was able to counteract the IFN VP35 inhibitory effect, partially restoring IFN production [18,121].

Furthermore, *Limonium morisianum* Arrigoni extract was reported to inhibit the VP35-dsRNA binding in a biochemical fluorescence-based assay (IC_{50} value of 19.2 μg/mL). Among the extract components, the (-)-epigallocatechin 3-O gallate (EGCG) and the flavonol myricetin (Figure 5) were able to suppress the VP35-dsRNA binding with IC_{50} values of 43.5 μM and 2.7 μM, respectively [122].

Among natural products, curcumin, curcuminoids and the metabolite tetrahydrocurcumin (Figure 5) were shown by molecular dynamics simulations to potentially bind EBOV proteins. The best predicted binding energy against EBOV VP35 (PDB ID: 4IBB) was displayed by tetrahydrocurcumin [123].

Another docking study performed by Setlur et al. suggested that herbal leads limonin (Figure 5) and gummosin reported high predicted binding energy of -9.7 kcal/mol and -9.1 kcal/mol, respectively, for both VP35 (PDB ID: 4IJE) and VP24 (PDB ID: 4M0Q) [124]. In addition, samarcandin were reported to have high docked energy (-9.4 kcal/mol) in complex with VP24, while polyanthin (-9.0 kcal/mol) with VP35 (Setlur, Naik, and Skariyachan 2016).

Docking studies revealed a potential high VP24-KPNα5 complex (PDB ID: 4U2X) binding affinity to plant polyphenols such as epigallocatechin gallate, 1,2,3,6-tetragalloyl glucose (1,2,3,6-TGG) (Figure 5) and theaflavin-3,3'-digallate [112]. In addition, flavonoids gossypetin and taxifolin, for which antiviral properties are already known, were found to potentially interact with VP35 (PDB ID: 3FKE) and VP24 (PDB ID: 4M0Q), while the flavonoid ST101866 (Figure 5) was predicted to bind specifically VP24 [113,125–128].

4.5. Oligomers and peptides

Iversen et al. reported the designing of phosphorodiamidate morpholino oligomers (PMOs) as anti-EBOV therapy [102]. PMOs physically bind to mRNA sequence acting as steric inhibitors of the translation process. Treatment of mice and guinea pigs with PMOs and PMOs modified, named PPMO and PMOplus, against VP35 and VP24 led to an increase in animal survival, differently from PMOs developed against GP and L targets [102]. Combinations of PMOplus resulted to enhance the efficacy on animal survival. A survival of 61% in non-human primates (NHPs) was observed after administration of the combination of PMOplus against VP35 (AVI-7539) and PMOplus against VP24 (AVI-7537), termed AVI-6002, while the maximal survival (100%) was observed after treatment with AVI-6003, a combination of AVI-7287 (VP24) and AVI-7288 (NP). Subsequently, to evaluate which PMOs alone was sufficient to protect against EBOV in NHPs, AVI-7537 and AVI-7539 alone were compared to AVI-6002. The study revealed that the AVI-7537 alone was sufficient to confer protection against EBOV while not the same was observed for AVI-7539 [103].

In vitro inhibition of VP35 and VP24 translation was also mediated by locked nucleic acid (LNA)-modified phosphorothioate (PS) antisense oligonucleotides (ASOs) which exhibit increased binding affinity and specificity compared to other ASOs such as morpholinos. LNA ASOs dose-dependently reduced VP35 and VP24 expression in a concentration range of 1–25 nM [129].

Another strategy to identify novel viral proteins inhibitors is the use of the RaPID system that allows the selection of high-affinity binders from an mRNA library of non-standard peptides [111]. By this approach, recently, a macrocyclic peptid binder for VP24, eVpeD2 (Figure 6) has been identified. It represents the first molecule inhibiting the VP24–KPNα5 protein–protein interaction (PPI) *in vitro*. The peptide was able to block the PPI VP24-KPNα5 with an EC50 of 9 μM [111].

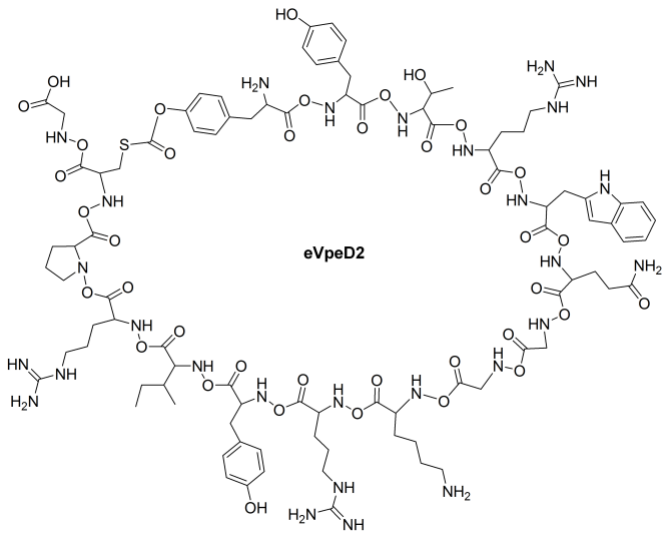

Figure 6. Macrocyclic peptide binding VP24.

Furthermore, Tanaka et al. used the capillary electrophoresis-systematic evolution of ligands by exponential enrichment (CE-SELEX) to generate natural and artificial nucleic acid aptamers with high-affinity for VP24. Since the aptamers shared a common binding site with KPNα1 for the interaction with VP24, they showed to antagonize VP24 binding to KPNα1 at sub-nanomolar range (Kd ≈ 10−10 M) [130].

4.6. Transbodies

Conventional antibody therapies used for EVD treatment are directed against GP1/2 inhibiting the processes of viral entry and fusion and secreted GP [131–135]. The inability of targeting viral proteins localized within the cytoplasm is a limit of antibodies, whose dimensions and hydrophilicity do not allow to penetrate the cellular membrane[136]. Cell-penetrating peptides (CPPs) were developed to the aim of

facilitating the entry of active compounds into cells, thanks to the electrostatic interactions between their positive charge and the negatively charge of membrane components [137,138]. Seesuay et al. used as CPP the nona-arginine (R9) to mediate the transport of human single chain variable antibodies fragments (HuscFvs) or tranbodies directed against VP35 that specifically recognize the IID function [107]. HepG2 cells transduced with a lentivector carrying VP35 were treated with R9-HuscFv3 and R9- HuscFv8 and gene expression of IFNβ1 promoter and EIF2AK2, encoding for PKR, were evaluated. Cell treated with both transbodies showed an overexpression of both genes compared to the expression of IFNB1 and EIF2AK2 in not treated VP35-transducted cells [107]. Homology modeling and docking studies revealed that R9-HuscFv3 interacts with IID residues of the CBP (R305, K309, R312, R322, and K339), the end-cap motif (F235, F239, and I340), and other residues known to interact with the dsRNA (P233, T237, Q274, C275, I278, A306, and S310), also with D271 [107]. R9-HuscFv8 was shown to interact with the CBP residues R322 and K339, the end-cap residue I340 and with S272, C275, I278, and Q279, important for the VP35-dsRNA binding, and other residues (E269, Q270, and D271) [107]. The obstruction of these residues by both transbodies lead to the suppression of IFN inhibitory function of VP35 and it is responsible for the host innate immunity rescue. In addition, the lack of cytotoxicity renders transbodies-based therapy a promising approach compare to the current antibodies treatments [107,135].

CONCLUSION

The IFN system is of crucial importance preventing viral recognition and thus the progression of infection. A number of RNA viruses, including EBOV, encode proteins that display IFN-antagonism properties circumventing the host innate immune system. Among those proteins, EBOV VP35 and VP24 are involved in targeting the RLRs pathway and the JAK/STAT cascade, respectively [5–22,68].

The impact of this counteraction is particularly evident for EVD since the fatal outcome has been related to the ability to subvert the type I IFN response that causes the systemic spread of the virus and excessive cytokine production and mediators that lead to the impairment of the vascular system and failure of adaptive immunity [4]. Currently, there is no FDA approved drug or therapy for the treatment of EVD [26]. Several agents have been tested in animal models and some of them have advanced into clinical trials [26]. The majority of them are directed against viral entry and replication [26]. However, an effective alternative strategy for EBOV drug development is the restoration of the host innate response by targeting VP35 and VP24, principal keys of IFN evasion and determinants of virulence. A number of *in silico* studies, including molecular docking, virtual screening and pharmacophore mapping, have shown great potential in predicting potential interactions between small molecules and VP35 and VP24 residues involved in the IFN inhibitory function. Some have been tested also in biochemical and cellular assays showing to be active at low micromolar range. Other agents have proven to be effective both in cell culture assays and in patients, leading to an increase of survival rate. In this review, we summarized the impact that the inhibition of IFN response has in EBOV pathogenesis, bringing to light the role of VP35 and VP24 and suggesting that novel strategies aimed to the development of an IFN-restoring based therapy are new interesting modes to identify novel EVD treatments.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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