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Flaviviridae virus nonstructural proteins 5 and 5A mediate viral immune evasion and are promising targets in drug development

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Abstract

Infections with viruses in the *Flaviviridae* family have a vast global and economic impact because of the high morbidity and mortality. The pathogenesis of *Flaviviridae* infections is very complex and not fully understood because these viruses can inhibit multiple immune pathways including the complement system, NK cells, and IFN induction and signalling pathways. The non-structural (NS) 5 and 5A proteins of *Flaviviridae* viruses are highly conserved and play an important role in resisting host immunity through various evasion mechanisms. This review summarizes the strategies used by the NS5 and 5A proteins of *Flaviviridae* viruses for evading the innate immune response by inhibiting pattern recognition receptor (PRR) signalling pathways (TLR/MyD88, IRF7), suppressing interferon (IFN) signalling pathways (IFN-γRs, STAT1, STAT2), and impairing the function of IFN-stimulated genes (ISGs) (e.g. protein kinase R [PKR], oligoadenylate synthase [OAS]). All of these immune evasion mechanisms depend on the interaction of NS5 or NS5A with cellular proteins, such as MyD88 and IRF7, IFN-αRs, IFN-γRs, STAT1, STAT2, PKR and OAS. NS5 is the most attractive target for the discovery of broad spectrum Compounds against *Flaviviridae* virus infection. The methyltransferase (MTase) and RNA-dependent RNA polymerase (RdRp) activities of NS5 are the main therapeutic targets for antiviral drugs against *Flaviviridae* virus infection. Based on our site mapping, the sites involved in immune evasion provide some potential and promising targets for further novel antiviral therapeutics.

Keywords: *Flaviviridae* virus; nonstructural protein 5 and 5A; immune evasion; IFN induction; IFN-dependent signal pathways; antiviral therapeutics.

Abbreviations

AAF, IFN-α-activated factor; ATA, Aurintricarboxylic acid; BDV, Border disease virus; BPgV, GBV-D; BVDV-1, Bovine Viral Diarrhea Virus-1; C, capsid protein; CLRs, C-type lectin receptors; Core, core protein; CSFV, Classical swine fever virus; DENV, Dengue virus; dsRNA, double-stranded RNA; E, envelope protein; E1 and E2, envelope proteins 1 and 2; eIF-2α, eukaryotic initiation factor 2 alpha subunit; EMCV, encephalomyocarditis virus; GAF, IFN-γ-activated factor; GBV, GBV-B; HCC, hepatocellular carcinoma; HCV, Hepatitis C virus; HPgV, GBV-C; hSTAT2, human STAT2; ICTV, International Committee on Taxonomy of Viruses; IFN, interferon; IRAK4, IL-1 receptor-associated kinase 4; IRF5, IFN regulatory factor 5; ISDR, IFN sensitivity determining region; ISGs, IFN-stimulated genes; JAK, the Janus-activated kinase; JEV, Japanese Encephalitis virus; KUN, The Kunjin virus; LAPSDa/b, LAP-specific domains a/b; LGTV, Langat virus; LRRs, leucine-rich repeats; MAP, the mitogen-activated protein; mSTAT2, mouse STAT2; MTase, methyltransferase; Mx, myxovirus resistance protein; MyD88, the myeloid differentiation protein; NIs, the nucleoside analogue inhibitors; NLRs, nucleotide oligomerization domain (NOD)-like receptors; NNIs, the non-nucleoside analogue inhibitors; NS, nonstructural proteins; OAS, 2', 5'-oligoadenylate synthetase; PAMPs, pathogen-associated molecular patterns; PEPD, prolidase; PKR, double-stranded RNA-activated protein kinase; PRR, pattern recognition receptor; PTPs, cellular protein tyrosine phosphatases; RdRp, RNA-dependent RNA Polymerase; RLRs, retinoic acid-inducible gene I (RIG-I)-like receptors; RTP, ribavirin 5'-triphosphate; SIN, Sinefungin; SPgV, GBV-A; SPgVcpz, GBV-Ccpz; ssRNA, single-stranded RNA; STAT, signal transducers and activators of transcription; TAK1, TGF-β-activated kinase 1; TBEV, tick-borne encephalitis virus; TLRs, Toll-like receptors; TRAF, tumour necrosis factor receptor-associated factor 6; TRIF, TIR-domain containing adaptor inducing the IFN-β; Tyk2, tyrosine kinase 2;

WNV, West Nile virus; YFV, Yellow Fever virus; ZIKV, Zika virus.

1. Introduction

The latest report of the International Committee on Taxonomy of Viruses (ICTV) showed that the *Flavivirdae* family is divided into four genera—*Flavivirus, Hepacivirus, Pestivirus, and Pegivirus* (Davidson, 2009; Stapleton et al., 2011). *Flavivirdae* viruses are positive sense single-stranded RNA viruses. The *Flavivirus* genus is the largest genus of the *Flaviviridae* family and contains more than 70 members (Diamond, 2003; Ye et al., 2013; Nazmi et al., 2014), about 34 of which are mosquito-borne viruses and about 17 of which are tick-borne viruses (Diamond, 2003). Approximately 40 species of the *Flavivirus* genus contribute to human and animal disease (Diamond, 2003), including mosquito-borne viruses such as West Nile virus (WNV), Japanese encephalitis virus (JEV), dengue virus (DENV), yellow fever virus (YFV), and Zika virus (ZIKV), and tick-borne viruses including tick-borne encephalitis virus (TBEV) and Langat virus (LGTV). Hepatitis C virus (HCV) is a major member of the *Hepacivirus* genus and can cause serious human and animal disease (Macdonald & Harris, 2004). The *Hepacivirus* genus has recently been divided into 14 species (*Hepacivirus A-N*), with HCV renamed as *Hepacivirus C* (Smith et al., 2016). The *Pestivirus* genus includes bovine viral diarrhea virus-1 (BVDV-1), BVDV-2, border disease virus (BDV) and classical swine fever virus (CSFV) and some new species (Avalos-Ramirez et al., 2001; Mosena et al., 2016). *Pegivirus* genus is a new genus of *Flavivirdae* family proposed in 2011, which recently has been divided into 11 species (*Pegivirus A-K*). (Stapleton et al., 2011 , Smith et al., 2016) (See Table 1).

The *Flavivirdae* family viruses most harmful to human health are flaviviruses and HCV, which can cause fever, hemorrhagic fever, shock syndrome, viral encephalitis, arboviral encephalitis, cirrhosis or hepatocellular carcinoma (HCC) etc (Ye et al., 2013). As critical components of the innate immune pathway, IFNs serve as the first line of defence against invading viruses and are considered an effective treatment for flavivirus infection. However, flaviviruses have developed a variety of immune evasion strategies to establish infection and replication in the host, which lead to poor efficacy of IFN treatment. Almost all *Flaviviridae* non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) can suppress the IFN signal transduction pathway through a variety of evasion mechanisms.

In this review, the roles of the NS5 and 5A (NS5/5A) proteins of *Flaviviridae* viruses in interfering with IFN induction and IFN dependent signaling pathways, as well as the development of IFN-independent antiviral drugs that target MTase and RdRp activity of NS5, are summarized. We provide insight into how understanding of viral evasion of innate immunity will be beneficial for the development of the novel antiviral compounds according to their genome characters.

2. Genome structure of flaviviruses and Hepatitis C virus

Flaviviruses are enveloped viruses that have an approximately 11-kilobase (kb) positive sense single-stranded RNA genome and encode one unique open reading frame (ORF) that is flanked by a type 1 capped 5'-terminal non-coding region (NCR) and a 3'-terminal NCR (Chambers et al., 1990) (See Table 1). To gain access

to and replicate in target cells, flavivirus use a complex multistep process including entry, replication, assembly and maturation. Briefly, viral entry is initiated by attaching to the cell surface and entering the cell by receptor-mediated endocytosis. Low pH in the endosomal compartment triggers fusion of the viral and host cell membrane mediated by structural reorganization of E, which leads to the release of the nucleocapsid and viral RNA into the cytoplasm. The RNA genome is then directly translated at the rough endoplasmic reticulum (ER) into a single polyprotein precursor of about 3,000 amino acid residues that is eventually cleaved by cellular and viral proteases into ten mature products: the N-terminal end (capsid protein (C), transmembrane protein (prM) and envelope protein (E)) and seven NS proteins at the C-terminal end (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (Chambers et al., 1990; Davidson, 2009).

The HCV genome is about 9.6 kb, which encodes a polyprotein that differs from the *Flaviviruses* genus. The polyprotein of HCV includes three structural proteins (core protein (Core), envelope proteins 1 and 2 (E1 and E2) and seven NS proteins (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B)) (Rai & Deval, 2011; Wang et al., 2016). The protein corresponding to the HCV p7 in hepatitis GB virus B (GBV-B) is p13, which can be cleaved into p7 and p6 proteins; p7, but not p6, is important for virus viability (Stapleton et al., 2011).

The *Flaviviridae* family genome structure is shown in Fig. 1. The structural proteins of *Flavivirdae* viruses play roles in receptor binding, membrane fusion or viral assembly (Diamond, 2003), while the NS proteins participate in viral RNA

replication, and some special NS proteins play roles in viral assembly and interfere with host defence mechanisms (Lindenbach & Rice, 2003; Lin et al., 2006).

3. NS5 of flaviviruses and NS5A of Hepatitis C virus

NS5 is the largest (about 900 amino acids) and most conserved protein of flaviviruses. The NS5 protein of all flaviviruses carries a methyltransferase (MTase) domain at the N-terminus and an RNA-dependent RNA polymerase (RdRp) domain at the C-terminus; between the two domains there is an interdomain linker (EI Sahili & Lescar, 2017). N-terminal MTases, including N7 MTase and 2'-O MTase, are required for capping of the viral RNA genome (GpppA-RNAm→7GpppAm-RNA), and C-terminal RdRp activity is required for the synthesis of viral -ssRNA (Davidson, 2009). The MTase domain can be subdivided into three subdomains: subdomain 1 (N-terminal extension), subdomain 2 (the core domain), and subdomain 3 (C-terminal extension). The RdRp domain also contains three subdomains: fingers subdomain, palm subdomain, and thumb domain. The MTase domain contains conserved motifs I-X, while the RdRp domain contains conserved motifs A-F (EI Sahili & Lescar, 2017). The protein corresponding to the *Flavivirus* genus NS5 in HCV is cleaved into two proteins NS5A and NS5B. NS5B possesses RdRp activity, while NS5A is an integral membrane protein and has no enzyme function. An N-terminal amphipathic alpha helix in NS5A is important for HCV RNA replication, and two phosphorylated forms of NS5A (p56 and p58) play different roles in virus replication cycle (Macdonald & Harris, 2004). Many studies have indicated that among all of the

Flaviviridae virus proteins, NS5 and NS5A play the most important role in immunosuppression (see Table2). Because NS5 and NS5A play vital roles in viral genome replication, as well down-regulating the host immune interferon response through interacting with host factors, they have been considered as promising antiviral targets for anti-flavivirus drug design.

4. IFN induction and IFN-dependent signal pathways

Host immune responses depend on the activation of signalling pathways triggered by pattern recognition receptors (PRRs), which detect viral pathogen-associated molecular patterns (PAMPs). The major PRRs include Toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), nucleotide oligomerization domain (NOD)-like receptors (NLRs), and C-type lectin receptors (CLRs) (Inohara et al., 2005; Rogers et al., 2005; Kawai & Akira, 2006, 2010; Takeuchi & Akira, 2010; Nazmi et al., 2014). Viral PAMPs include genomic DNA, single-stranded RNA (ssRNA), double-stranded RNA (dsRNA), RNA with 5'-triphosphate ends and viral proteins (Shizuo et al., 2006; Medzhitov, 2007; Nazmi et al., 2014). TLR-, RLR- and NLR-sensing pathways are important for host immunity against *Flaviviridae* infection (Gack & Diamond, 2016).

Once PAMPs bind to TLRs, two antiviral signalling pathways are activated: the myeloid differentiation protein (MyD88)-dependent pathway, which all TLRs except for TLR3 can utilize, and the TIR-domain containing adaptor inducing the IFN-α/β (TRIF)-dependent pathway utilized by TLR3 and TLR4 (Kawai & Akira, 2007). Different PAMPs can be recognized by different TLRs, which will further

trigger the signalling cascade in the MyD88-dependent and TRIF-dependent pathways (Kawai & Akira, 2007) (Fig. 2A). In the MyD88-dependent pathway, there are four signal transduction pathways that trigger and activate four types of transcription factors. The TGF-β-activated kinase (TAK) 1 complex activates the IKK complex, which further catalyses the phosphorylation of IκB proteins. The phosphorylated IκB proteins then degrade and activate the transcription factor NF-κB. The TAK1 complex also activates the mitogen-activated protein (MAP) kinases and mediates activation of transcription factor AP-1. Furthermore, the MyD88-IL-1 receptor-associated kinase (IRAK) 4-tumour necrosis factor receptor-associated factor (TRAF) 6 complex can phosphorylate and promote the activation of IFN regulatory factor (IRF) 5, and the MyD88-TRAF6-IRAK4-IRAK1 complex can mediate IRF7 phosphorylation and activation (Kaisho & Akira, 2006; Kawai & Akira, 2007; Yuk & Jo, 2011). Activated NF-κB, AP-1 and IRF5 translocate into the nucleus and induce inflammatory cytokine transcription, while activated IRF7 translocates into the nucleus and induces expression of type I IFNs and interferon-stimulated genes (ISGs) (Kawai & Akira, 2007).

Type I IFNs (IFN-α and IFN-β) and type II IFN (IFN-γ) recognize their cognate receptors, leading to activation of downstream signalling (Davidson, 2009) (Fig. 2B). Type I IFN receptors include IFN-αR1 and IFN-αR2 subunits, while type II IFN receptors include IFN-γR1 and IFNγR2 subunits. Different receptor subunits recruit distinct cellular tyrosine kinases of the Janus-activated kinase (JAK) family.

IFN-αR1 and IFN-αR2 associate with tyrosine kinase 2 (Tyk2) and JAK1, respectively. However, IFN-γR1 and IFN-γR2 associate with JAK1 and JAK2, respectively (Davidson, 2009). Phosphorylation of Tyk2/JAK1 and JAK1/JAK2 triggers the recruitment and phosphorylation of signal transducers and activators of transcription (STAT) proteins. (Bao & As., 2000; Bromberg & Jr, 2000; Shuai & Liu, 2003). Phosphorylated STAT1 and STAT2 heterodimers form the IFN-stimulated gene factor 3 (ISGF3) transcription complex and translocate into the nucleus to induce ISGs (Fu et al., 1990; Levy, 1997), while phosphorylated STAT1 and STAT1 homodimers form IFN-α-activated factor (AAF), also termed IFN-γ-activated factor (GAF) transcription complex and translocate into the nucleus to induce ISG expression (Takaoka & Yanai, 2006). ISGs such as double-stranded RNA-activated protein kinase (PKR), 2', 5'-oligoadenylate synthetase (OAS), and myxovirus resistance protein (Mx) play important antiviral roles against viral infection (Meurs et al., 1990; Clemens & Elia, 1997; Lin et al., 2009; Schoggins & Rice, 2011; Zhu et al., 2015).

5. Effect of NS5 proteins of different mosquito-borne flaviviruses on IFN-dependent signaling pathways

JEV

A 2004 study revealed that the phosphorylation of Tyk2 and STAT1 was inhibited in IFN-α–treated, JEV-infected BHK-21 cells (Lin et al., 2004). Further research in BHK-21 cells using an immunofluorescence assay demonstrated that JEV NS5 potently inhibited IFN-α/β signalling by blocking IFN-α-induced nuclear

translocation of STAT1 (Lin et al., 2006). In Vero cells stimulated with IFN-α, tyrosine phosphorylation of STAT1 and Tyk2 was reduced after infection with a JEV NS5-expressing recombinant Sindbis virus (Lin et al., 2006). Both STAT1 nuclear translocation in BHK-21 cells and STAT1 phosphorylation in Vero cells induced by IFN-α were almost completely and partially inhibited by the 762 or 667 N-terminal residues of JEV NS5. Deletion of the 143 C-terminal residues of NS5 did not affect the ability of NS5 to inhibit STAT1 tyrosine phosphorylation, suggesting that NS5 probably required an intact N terminus but not the 143 C-terminal residues to mediate its IFN-antagonistic activity (Lin et al., 2006). Another study suggested that the activity of certain unidentified cellular protein tyrosine phosphatases (PTPs) was probably involved in the blocking of IFN-α signalling (Lin et al., 2006), which was consistent with previous studies showing that STAT1 retention in the cytoplasm and loss of activity were mediated by PTPs (Shuai & Liu, 2003).

DENV 2

Ho et al. demonstrated that DENV2 could suppress IFNα/β, but not IFN-γ, signalling through down-regulation of STAT2 in human dendritic cells (Ho et al., 2005). Mazzon and others confirmed that DENV2 NS5 could bind STAT2, inhibit its phosphorylation, and induce degradation of STAT2 (Mazzon et al., 2009). Similarly, Ashour and others confirmed that DENV2 could bind to and reduce the expression level of STAT2 in infected Vero cells and U6A-STAT2-GFP cells (Ashour et al., 2009). Transient expression of the NS1-5 plasmid (containing all

nonstructural proteins) and NS1-4B plasmid (containing all nonstructural proteins except for NS5) in 293T cells further confirmed that DENV NS5 was necessary to reduce STAT2 expression. Subsequently, the results of an immunoprecipitation assay suggested that NS5 pulled down STAT2 but not STAT1 in 293T cells, confirming that NS5 specifically interacted with STAT2. Using western blotting, the results also suggested that the precursor form of NS5, cleaved by DENV viral protease, was required for STAT2 degradation. They also suggested that the first 10 amino acids at the extreme N terminus of NS5 were required for STAT2 degradation, but the first 202 amino acids were dispensable for STAT2 binding activity or blocking IFN signalling (Ashour et al., 2009). Subsequent studies showed that DENV2 NS5 could not degrade mouse STAT2 (mSTAT2) but could degrade human STAT2 (hSTAT2) in both U6A cells and BHK21 cells. This degradation did not require additional host factors such as IRF9 and STAT1, as proven in U2A cells (IRF9 deficient) and U3A cells (STAT1 deficient). In addition, NS5 could associate with hSTAT2 but not mSTAT and block hSTAT2-mediated IFN signalling (not mSTAT2-mediated IFN signalling) in 293T cells. Amino acid residues 181-200 of hSTAT2 were required for NS5-mediated association and degradation (Ashour et al., 2010). Later, Morrison and others identified the host protein UBR4 as important to promote DENV NS5-mediated STAT2 degradation through interacting with NS5. Meanwhile, Thr^{2} and Gly^{3} of DENV NS5 were required for NS5 binding to UBR4 (Morrison et al., 2013).

WNV

By early 2005, it had been well established that WNV could antagonize IFN-mediated signal transduction (Guo et al., 2005; Liu et al., 2005). Laurent-Rolle and others first demonstrated that the WNV NY99 strain NS5 protein could antagonize the host IFN-α/β response by inhibiting phosphorylation and nuclear translocation of STAT1 in Vero cells. Kunjin virus (KUN) is a naturally attenuated subtype of WNV, and the inhibition of STAT1 phosphorylation by KUN NS5 was significantly weaker than that by WNV-NY99 NS5 (Laurent-Rolle et al., 2010).

Based on the amino acids identified for LGTV NS5 (Park et al., 2007), site-specific mutations to alanine were made at the analogous amino acid residues in WNV-NY99 NS5 (Asn^{377}, Asn^{381}, Glu^{627}, Glu^{629}, Val^{631}, Ile^{632}, and Trp^{651}), as well as at the adjacent two residues (Glu^{376} and Trp^{382}). The inhibitory effects of mutated NS5 on phosphorylation of STAT1 demonstrated that residues Trp382, Val^{631}, Ile^{632}, and Trp^{651} of WNV NS5 were important for IFN antagonist function. However, the regions and amino acid residues of STAT1 responsible for the interaction with WNV NS5 have not yet been reported. Considering that 9 of 10 different amino acids were relatively conserved between WNV-NY99 NS5 and KUN NS5, the S653F mutation in KUN NS5 (KUN NS5:S653F), as well as the F653S mutation in WNV-NY99 NS5 (WNV-NY99 NS5: F653S), were constructed. The data showed that suppression of phosphorylated STAT1 by KUN NS5:S653F was greater than that by WNV-NY99 NS5: F653S in Vero cells, demonstrating that the residue at position 653 was a critical determinant for WNV NS5-antagonized IFN signalling (Laurent-Rolle et al., 2010).

Lubick and colleagues found that the NS5 of WNV-NY99 could bind to prolidase (PEPD), which is a cellular dipeptidase required for IFN-αR1 maturation and induction of ISG mRNA expression following IFN-β stimulation. The interaction of NS5 with PEPD was demonstrated by immunoprecipitation and confocal microscopy in HEK293 cells. This interaction could lead to inhibition of IFN-αR1 surface expression in HEK293 cells and decrease of phosphorylated STAT1 in IFN-β-stimulated HEK293 cells. In addition, the reduction of surface IFN-αR1 and phosphorylated STAT1 by KUN NS5:S653F were more evident than that induced by KUN (Lubick et al., 2015).

YFV

Laurent-Rolle and colleagues first showed that YFV NS5 protein could inhibit IFN-α/β signalling by reducing IFN-β-mediated ISRE-54-CAT promoter activation and ISG54 promoter activation in IFN-β-treated 293T cells (Laurent-Rolle et al., 2014). Immunoprecipitation experiments revealed that YFV NS5 could coprecipitate with STAT2 in IFN-β-stimulated and IFN-λ-stimulated 293T cells, but not in IFN-γ-stimulated 293T cells. Moreover, YFV NS5 colocalization with STAT2 could be observed by confocal immunofluorescence only in IFN-β treated Vero cells. Subsequent immunoprecipitation assays suggested that IFN-β promoted YFV NS5/STAT2 interaction by inducing tyrosine phosphorylation of STAT1, which in turn bonded with STAT2, resulting in a conformational change in STAT2 and exposure of the NS5 binding domain (Laurent-Rolle et al., 2014). The first ten amino acid residues in the N-terminus of YFV NS5 have been identified as

necessary for binding STAT2, using an NS5 mutant in IFN-β-treated 293T cells. Meanwhile, the Lys^{6} of YFV NS5 was found to be critical for the association of NS5 with ubiquitin that mediated the NS5-STAT2 interaction in IFN-β-stimulated 293T cells as well as the suppression of IFN-β -mediated antiviral activity in IFN-β-treated Vero cells (Laurent-Rolle et al., 2014). Further immunoprecipitation experiments indicated that the ubiquitination of YFV NS5 Lys^{6} by K63-linked polyubiquitination was necessary for the NS5-STAT2 interaction in 293T cells (Laurent-Rolle et al., 2014). The E3 ligase TRIM23 promotes this interaction by interacting with and polyubiquitinating YFV NS5 (Laurent-Rolle et al., 2014). However, no report has identified the YFV NS5-binding region of STAT2, although it was deemed likely to be similar to the DENV2 NS5-binding region of STAT2.

ZIKV

Grant and colleagues found that ZIKV could suppress type I IFN signalling through interaction with and degradation of STAT2 in human dendritic cells, similar to DENV NS5 (Grant et al., 2016). The proteasome pathway is necessary for the degradation of STAT2 during ZIKV infection, and ZIKV NS5 strongly interacts with hSTAT2 but not mSTAT2 in the cytoplasm. However, in contrast to DENV NS5, immune precipitation assays showed that ZIKV NS5 could not interact with UBR4, and ZIKV NS5-mediated STAT2 degradation did not depend on UBR4, as demonstrated in 293T cells with UBR4 deletion (Grant et al., 2016). The regions or sites required for ZIKV NS5 and STAT2 complex formation have not been investigated yet. The evasion mechanism of ZIKV was also different from

Spondweni virus (SPOV), which is the closest relative of ZIKV. SPOV NS5 did not inhibit STAT phosphorylation and translocation, and only weakly bound, but did not degrade, STAT2. However, expression of SPOV NS5 could inhibit ISRE-dependent gene (ISG54) reporter activation, similar to ZIKV NS5, possibly by binding IRF9 and hence interfering with ISGF3 interacting with ISRE promoter elements (Best, 2016; Grant et al., 2016).

6. Effects of NS5 proteins of different tick-borne flaviviruses on IFN induction and IFN-dependent signaling pathways

LGTV

LGTV NS5 was shown to inhibit phosphorylation of JAK and STAT in Vero cells after treatment with IFN-α/β or IFN-γ (Best et al., 2005). Subsequently, immunoprecipitation assays proved that, among various LGTV NS proteins, only NS5 could be coprecipitated with IFN-αR2, but not IFN-αR1, in Vero cells. Additionally, there was also an association between IFN-γR1 and NS5 and a weaker association between IFN-γR2 and NS5. Similar results were also confirmed in primary human monocyte-derived dendritic cells and murine bone marrow-derived dendritic cells pre-treated with IFN-α/β (Best et al., 2005).

Later, Park and others found that two non-contiguous regions of amino acid residues 374 to 380 and 624 to 647 of LGTV NS5 were important for suppression of JAK-STAT signalling by reducing IFN-β-mediated STAT1 phosphorylation in Vero cells. Amino acids Arg^{376}, Asp^{380}, Glu^{626}, Glu^{628} or Trp^{647} were determined to be critical for JAK-STAT inhibition (Campbell & Pletnev, 2000; Park et al., 2007).

The regions or sites required for LGTV NS5 complex formation with IFN-αR2 or IFN-γR1 have not yet been determined. Prolidase (PEPD) was required for IFN-αR1 maturation and accumulation, which is vital for the induction of IFN-β-stimulated genes; LGTV NS5 was found to bind PEPD and down regulate IFN-αR1 expression and inhibit phosphorylation of STAT1, similar to WNV NS5. The interaction of PEPD with NS5 could be abolished by mutating W647A in NS5 (W647A) (Lubick et al., 2015). Mapping of the NS5-PEPD interaction domain indicated that the binding domain resided in amino acid residues 216 to 233 and residues 256 to 441 (Lubick et al., 2015).

TBEV

hScrib is a protein widely expressed at the membrane of polarized mammalian cells that contains 16 leucine-rich repeats (LRRs), LAP-specific domains a/b (LAPSDa/b), and 4 non-identical PDZ domains (Bilder et al., 2000; Santoni et al., 2002). Werme and others demonstrated that the expression of NS5 protein in HEK293 and HeLa cells could inhibit IFN-α-stimulated STAT1 phosphorylation, and the knock-down of hScrib could restore STAT1 phosphorylation in IFN-α/γ stimulated HeLa cells, shown by immunofluorescence assay (Werme et al., 2008). The N-terminal domain of TBEV NS5 protein was the main region binding with the hScrib C-terminal region (amino acid residues 1100-1630, encodes PDZ 4 domain), which was shown by pull-down and yeast two-hybrid (Y2H) assays. A coimmunoprecipitation assay further proved that the intact Tyr^{222} and Ser^{223} residues of NS5 were indispensable for the interaction with hScrib and may play an

important role in the formation of the NS5-hScrib complex (Werme et al., 2008). It was suggested that the inhibition of STAT1 phosphorylation by TBEV NS5 was due to the interaction of NS5 with hScrib.

TBEV NS5 could also co-localize with PEPD, which was also reported for WNV and LGTV (Lubick et al., 2015). Mutation of TBEV NS5 (D380A) influences the co-localization of PEPD and weakens the inhibition of IFN-αR1 expression, phosphorylation of STAT1, and IFN-α/β-induced gene expression. These results demonstrate that Asp^{380} plays a key role in NS5-PEPD interaction. TBEV D380A mutants had greatly reduced virulence comparted to wild type TBEV, and replicated to low viral titers in serum and brain of infected mice (Lubick et al., 2015).

7. Effects of HCV NS5A protein on IFN induction and signaling pathways

7.1 Effects of HCV NS5A protein on IFN induction

7.1.1 Inhibition of the TLR/MyD88 signalling pathway

Mouse macrophage RAW264.7 cell lines stably expressing HCV 1b structural and NS proteins were established by Abe and colleagues to determine the effect of HCV proteins on TLR function in immune cells (Abe et al., 2007). Their experiments showed that mRNA levels of TLR2, TLR3 and TLR4 were reduced, while TLR7 and TLR9 were enhanced, in NS5A-expressing cells (Abe et al., 2007). NS3, NS3/4A, NS4B and NS5A of HCV could inhibit the upregulation of IL-6, as detected by ELISA, in macrophage cell lines pre-treated with TLR agonists mCpG, R-837, LPS or PGN (the ligands for TLR9, TLR7, TLR4 and TLR2 respectively).

Finally, reduced expression of other proinflammatory cytokines such as IL-1α and chemokines such as CCL2 was also observed in NS5A-expressing cells (Abe et al., 2007). It was also suggested that MyD88 could be coimmunoprecipitated with HCV NS5 protein but not with other structural (Core, E1 and E2) and nonstructural proteins (NS3, NS3/4A, NS4B and NS5B) of HCV in 293T cells. In addition, HCV NS5 was shown to interact with MyD88 but not with other adaptor molecules such as TRAM, TIRAP or TRIF in 293T cells, confirming the specific interaction between NS5A and MyD88. Amino acid residues 240 to 280 of NS5A, which overlap with the IFN sensitivity determining region (ISDR) (amino acid residues 237-276) (Enomoto et al., 1995; Enomoto et al., 1996; Polyak et al., 1999) involved in inhibiting the MyD88-dependent signalling pathway, was identified as necessary for its interaction with MyD88. Subsequent studies in macrophages have suggested that the ISDR is required for inhibiting the MyD88-dependent signalling pathway. Moreover, researchers have found that MyD88 deletion mutants either lacking amino acid residues 1 to 50 or possessing amino acid residues 1 to 70 could both bind to NS5A in 293T cells. Thus, amino acid residues 50 to 70 in the death domain of MyD88 are necessary for the interaction with NS5A (Abe et al., 2007).

7.1.2 Impairment of IRF-7 activation

Phosphorylated IRF-7 can translocate into the nucleus, a step that is crucial for mediating activation of IFN-α4, IFN-α7, and IFN-α14 promoters (Lin et al., 2000). The NS5A protein of HCV 1b and 2a had the most potent inhibitory effect on IFR-7-mediated IFN-α14 promoter activity in immortalized human hepatocytes

(IHH) (Chowdhury et al., 2013). In the absence of IRF7, NS5A had no inhibitory effect against the IFN-α14 promoter. A previous study suggested that treating hepatocytes with poly (I-C) or IFN-α would translocate IRF-7 into the nucleus (Raychoudhuri et al., 2010). IRF-7 was retained in the cytoplasm of HCV-infected IHH treated with poly (I-C) or IFN-α, further demonstrating that HCV NS5A contributed to the retention of IRF-7 in the cytoplasm (Raychoudhuri et al., 2010). The association of HCV NS5A with IRF-7 was verified in Huh7 cells harbouring the HCV genotype 1b or HCV 2a subgenomic replicons and in HEK293 cells (Chowdhury et al., 2013). Two conserved Arg residues at 216 and 217 of NS5A were found by amino acid sequence alignment of different HCV genotypes. NS5A with mutations at Arg^{216} and Arg^{217} could not inhibit IRF-7-mediated IFN-α14 promoter activation, unlike wild-type NS5A. The HCV NS5A mutants failed to localize with IRF-7 in IHH and to physically interact with IRF-7 in 293 cells (Chowdhury et al., 2013). However, the specific interaction regions or amino acid residues in IRF-7 involved in the interaction with NS5A have not yet been mapped.

7.2 Effects of HCV NS5A protein on IFN-dependent signalling pathways

7.2.1 Inhibiting IFN-dependent signalling pathways

Kumthip and others demonstrated that HCV could strongly decrease IFN-α-induced ISRE signalling in an HCV JFH1-infected human hepatocellular carcinoma cell line (Huh7.5.1 cells) using an ISRE-luciferase reporter assay (Kumthip et al., 2012). The protein level of phosphorylated STAT1, not STAT2, in IFN-α-treated NS5A-expressing Huh7.5.1 cells was highly reduced (Kumthip et al.,

2012). Different immune inhibition levels were identified between the HCV genotypes (GTs) in Huh7.5.1 cells: GT1 (GT1a and GT1b) NS5A protein showed stronger inhibition of IFN-α-induced ISRE signalling, phosphorylation of STAT1 and mRNA expression of ISGs (PKR, OAS, and MxA) than GT3 (GT3a and GT3b) NS5A protein (Kumthip et al., 2012). Further, the results of immunoprecipitation assays demonstrated that GT1 NS5A had a higher STAT1 binding affinity than did GT3 NS5. The increased ability of GT1 NS5A over GT3 NS5A to suppress the IFN response was due to its stronger binding with STAT1 (Kumthip et al., 2012). Furthermore, immunoprecipitation experiments proved that the C terminus of NS5A (amino acid residues 237 to 447) was the main STAT1 interaction domain responsible for inhibiting type I IFN signalling and reducing the level of phosphorylated STAT1 and the downstream ISG response (Kumthip et al., 2012). The amino acid residues of STAT1 responsible for interaction with HCV NS5A have not yet been mapped.

7.2.2 Impairing ISG function

PKR

The PKR protein kinase is an important antiviral protein induced by IFN that inhibits viral replication by interfering with the host eukaryotic initiation factor 2 alpha subunit (*eIF-2α*). *eIF-2* is a protein kinase consisting of three subunits—α, β and γ (Kimball, 1999; Clemens, 2001; Erickson et al., 2001). PKR phosphorylates serine 51 on the alpha subunit of *eIF-2* and can perturb ternary complex formation,

thereby affecting the recycling process of *eIF-2* and blocking global protein synthesis, as well as inhibiting viral replication (Clemens, 2001; Joshi et al., 2013).

IFN-sensitive HCV genotype HCV-1a has evolved a mechanism to repress PKR through NS5A protein and avoid the antiviral effects of IFN (Gale et al., 1997). GST pull-down assays demonstrated that HCV-1a NS5A could specifically interact with PKR protein in vitro; amino acids 244-551 of PKR were necessary and sufficient for the interaction between NS5A and PKR (Clemens, 2001). Using the Y2H system, it was shown that HCV 1a/1b NS5A protein specifically interacted in vivo with the PKR protein catalytic domain mapped to amino acid residues 244 to 366 (Hanks et al., 1988; Bossemeyer, 1991; Taylor et al., 1993), and this interaction did not depend on dsRNA (Gale et al., 1997).

HCV 1a NS5A inhibits PKR activity and histone phosphorylation in vitro. In vivo, HCV 1a NS5A reversed PKR-mediated growth suppression in the yeast strain RY1-1, and the enormous increase in the level of monophosphorylated *eIF-2α* in RY1-1 cells further confirmed this conclusion. NS5A derived from the HCV-1a-deleted ISDR region (amino acid residues 237 to 276) failed to interact with PKR, and coexpression of NS5A-deleted ISDR with PKR failed to reverse PKR-mediated growth suppression in yeast strain RY1-1, demonstrating that the ISDR region of NS5A was necessary for the interaction with PKR and inhibition of PKR function in vivo (Gale et al., 1997). Later, in 1998, a minimal NS5A (HCV 1b)-binding region of PKR including amino acid residues 244 to 296 was identified, which was an important PKR dimerization domain (Gale et al., 1998; Tan et al.,

1998). Through phage genetic assay in *E. coli*, it was demonstrated that NS5A derived from HCV 1b inhibited PKR through disruption of the PKR dimerization process in vivo (Gale et al., 1998). Importantly, the PKR-binding region of NS5A (amino acid residues 237-302) derived from HCV 1b mapped not only to the ISDR but also to the adjacent C-terminal 26 amino acid residues, and this region was required for the interaction with PKR in vivo by the Y2H assay (Gale et al., 1998). This result demonstrated that the ISDR was indispensable but not sufficient for the NS5A-PKR interaction, complementing the authors' previous study in 1997.

Meanwhile, a previous study confirmed that mutations in the ISDR of the PKR-binding domain of HCV NS5A could influence IFN sensitivity of HCV-1b (Enomoto et al., 1995; N et al., 1996; Di, 1997; Polyak et al., 1999). Site-directed mutagenesis showed that a single ISDR point mutation of NS5A was not sufficient to abolish the NS5A-PKR interaction, while multiple ISDR mutations inhibited complex formation with PKR. The mutations of ISDR not only prevented NS5A from interacting with PKR but also inhibited the PKR-regulatory function of NS5A (Gale et al., 1998).

OAS

OAS is another important antiviral molecule induced by IFN. It plays an important role in inhibiting viral replication through RNase L-dependent and RIG-I-dependent antiviral pathways (Zhu et al., 2015). HCV NS5A and OAS were first found to colocalize in the cytoplasmic perinuclear region of HeLa cells using a vaccinia virus-T7 hybrid expression system. GST pull-down assays showed that

NS5A could also physically interact with OAS in HeLa cells. Furthermore, coexpression of various NS5A deletion mutants showed that NS5A (1-148) (which contains neither the ISDR nor PKR-binding domains) could be pulled down by OAS and was involved in forming a complex with OAS in HeLa cells. GST-pull down assay showed that OAS (1-104), OAS (52-144) and OAS (184-275), but not OAS (1-60) or OAS (184-235), could pull down NS5A (1-148), demonstrating that two regions of OAS (amino acid residues 52-104 and 184-275) contributed to the physical interaction with NS5A (Taguchi et al., 2004). Furthermore, the F37L mutation of NS5A but not F37N, F37S and F37Y mutation significantly increased complex formation with OAS (Taguchi et al., 2004). The IFN antiviral activity assay demonstrated that NS5A (1-148) with F37N mutation, which interacted only weakly with OAS, was less effective in reducing the antiviral activity of IFN against encephalomyocarditis virus (EMCV) than wild-type NS5A (1-148) (Taguchi et al., 2004). There is a need for further functional analysis of the NS5A-OAS interaction. Taken together, the identification of virus-encoded antagonists will be beneficial for the development of vaccines and antiviral compounds.

8. Pharmacotherapy targets NS5 or NS5A against *Flaviviridae* virus infection

Flaviviridae viruses have diverse immune evasion strategies, which leads to poor efficacy of IFN treatment. For this reason, various IFN-independent antiviral drugs have been extensively studied in vitro or in vivo and were shown to block critical steps in the life cycle of *Flaviviridae* virus. However, at present there is no effective universal drug available against *Flaviviridae* virus infection.

To date, many inhibitor compounds targeting host metabolic and post-translational modification pathways such as the lipid and nucleotide metabolic pathways and the carbohydrate modification pathway (Krishnan & Garcia-Blanco, 2014), as well as the key host proteins involved in viral replication such as IRF3, DDX3, and AXL (Pattabhi et al., 2015; Brai et al., 2016; Rausch et al., 2017), have been evaluated. There has been considerable recent interest in identification of direct-acting antivirals (DAAs) that specifically inhibit critical steps (proteins essential for viral replication) in the viral life cycle; however, development of DAAs relies on detailed biochemical and structural characterization of target proteins.

Viral proteins including E protein, NS2B/NS3 protease, NS3 helicase, NS4B protein and NS5 can serve as therapeutic targets for antiviral drugs against *Flaviviridae* virus infection (Xie et al., 2011; Kok, 2016; Eyer et al., 2017; Rausch et al., 2017; Vidotto et al., 2017). As the most conserved protein in flaviviruses, NS5 plays an important role in viral RNA synthesis, with N-terminal MTase enzymatic activity and C-terminal RdRp activity. NS5 is the most attractive target for the discovery of broad spectrum compounds for treatment of *Flaviviridae* virus infection. Targeting the MTase or RdRp activity of NS5 is the most promising approach for drug design (Davidson, 2009a; Krishnan & Garcia-Blanco, 2014; Kok, 2016; EI Sahili and Lescar, 2017).

Two types of compound have been used to target NS5: nucleoside analogue inhibitors (NIs) and non-nucleoside analogue inhibitors (NNIs) (see Table 3). NIs

mainly mimic the natural substrates of the enzyme and inhibit enzyme activity after phosphorylation by cellular kinases. NIs usually inhibit all serotypes of the virus through binding to active enzyme sites. However, NIs can interfere with some cellular processes through interactions with host proteins, which lead to serious cell toxicity (EI Sahili and Lescar, 2017). The NNIs mainly affect conformational changes of the enzyme through binding to specific allosteric pockets. Unlike treatment with NIs, virus resistant variants may emerge during NNI treatment because the allosteric pockets are more susceptible to mutation than the active enzyme site (EI Sahili and Lescar, 2017).

HCV

HCV infection is one of the main causes of liver disease worldwide. Recent advances in molecular biology have led to the development of novel small molecules that target specific HCV proteins. These drugs, which include ribavirin, protease inhibitor (telaprevir or boceprevir), NS5B polymerase and NS5A inhibitors, are at various stages of clinical development. The inhibitors of HCV NS5A exhibit broad genotype activity and have excellent antiviral potency. NS5A inhibitors have all been labelled with the "asvir" suffix (Blight et al., 2000). Elbasvir is an important HCV inhibitor that inhibits replication of HCV genotypes 1 and 4 in vitro by targeting the HCV NS5A protein (Coburn et al., 2013; Al-Salama & Deeks, 2017). Its antiviral mechanism is still unknown. Balapiravir is a triisobutyrate ester prodrug of 40-azidocytidine (R1479) developed for HCV patients but re-purposed for DENV. The emerging NS5A inhibitors like velpatasvir and pibrentasvir have

greater activity against HCV than the earlier NS5A inhibitors such as ledipasvir and ombitasvir (Blight et al., 2000). The enzyme of HCV NS5B can be inhibited by NIs and NNIs. Both NIs and NNIs of NS5B polymerase inhibitors are designated by the "buvir" suffix. The first NI inhibitor of the NS5B polymerase was Sofosbuvir (SOF), which reduced the replication of HCV viral genomic RNA by mimicking the polymerase substrate, leading to false termination of the newly synthetized HCV RNA chain (see Table 3). Dasabuvir (DCV) is an NNI of NS5A that has high antiviral activity against genotypes 1 to 4 of HCV both in vivo and in vitro and is also active against genotypes 5 and 6. As reported, NNIs exhibited weaker antiviral activity and were effective against a more limited spectrum of genotypes than NIs, and have only a low barrier to antiviral resistance (Powdrill et al., 2010a; Powdrill et al., 2010b; Sarrazin et al., 2016). However, multiple drug regimens can be considered in future to protect against selection of resistant HCV variants. For example, dasabuvir, the only approved NNI, could be used in combination with ritonavir-boosted paritaprevir and ombitasvir against HCV genotype 1 isolates.

DENV

NIs provide the greatest promise for potential dengue therapeutics, which gave the evidence that the NS5 inhibitor has cross-species activity, most likely due to the similar crystal structure of NS5 among *Flaviviridae*. Benarroch and colleagues found that the NI ribavirin 5'-triphosphate (RTP) could bind the GTP-binding pocket of DENV NS5 MTase and inhibit RNA cap 2'-O-methylation of DENV in vitro (Benarroch et al., 2004) (Table 3). They found that it could also inhibit N7 and

2'-O MTase activities, as well as replication of DENV2 and YFV by interacting with the SAM-binding site of MTase in vitro (Dong et al., 2010) (see Table 3). Compound 10 is an NNI of MTase that inhibits N7 and 2'-O MTase activities of DENV3 and WNV by interacting with residues in the SAM-binding pocket and inducing conformational changes of amino acids residues lining the pocket (Lim et al., 2011). The interaction site (2-3aa) of DENV2 NS5 with UBR4 and STAT2 was close to the inhibitor sites of Compound 10 (Fig. 3). Therefore, it is possible that Compound 10 may affect DENV NS5 interaction site leading to inability of the virus to inhibit UBR4 and STAT2 function. Ribavirin is a synthetic guanosine analogue that inhibits dengue methyltransferase and HCV replication (Tomlinson et al., 2011; Chang et al., 2011). Other inhibitors, like NSC12155, NSC125910, NSC306711 and NSC610930, were reported to target the SAM-binding pocket of MTase and inhibit the replication of DENV2, DENV3 and YFV in vitro (Brecher et al., 2015; Thiel et al., 2015; Duan et al., 2017) (see Table 3). However, because the core domain of NS5 MTase is conserved not only in *flaviviruses* but also in human MTase, designing inhibitors to specifically target viral MTase presents a challenge. NITD107 was the first identified RdRp inhibitor that targeted the RNA tunnel (Noble et al., 2013) (see Table 3, Fig. 3). It induced conformational changes in DENV3 RdRp by interacting with the RNA binding sites of the RNA tunnel, which inhibited RdRp activity (Noble et al., 2013). Metal ion chelation is another potential approach for drug design against RdRp activity by targeting the GDD motif of the RdRp domain. DMB220, a novel inhibitor of RdRp, acts as a chelating agent for

divalent metal ions in the catalytic site of DENV RdRp and inhibits replication of DENV serotypes 1-4 in vitro (Xu et al., 2015) (see Table 3). Notably, a novel drug-target, named the "N pocket", was identified in the DENV RdRp domain through fragment-based screening via X-ray crystallography (Lim et al., 2016; Noble et al., 2016). JF-31-MG46 was identified as an RdRp inhibitor by targeting "N pocket" and inhibits the RdRp activity of DENV3 and DENV4 in vitro (Noble et al., 2016). Meanwhile, more inhibitors targeting the "N pocket" were reported, such as Compound 27 and Compound 29, and they could inhibit the replication of DENV serotypes 1 to 4 in vitro (Yokokawa et al., 2016) (see Table 3). There are still some drugs that can inhibit the RdRp activity of *Flaviviridae* viruses, but the targets in RdRp are still unknown. For example, NITD203 and NITD008 are both NIs of RdRp that can protect mice from DENV2 infection in vivo and in vitro through inhibiting DENV2 RdRp activity (Chen et al., 2010; Chen et al., 2015) (see Table 3).

WNV

In past years, a number of inhibitors were developed to protect the host from WNV infection. Because of their poor drug-like properties, no promising preclinical small molecule inhibitor candidates have been developed; there are currently no marketed drugs or clinical candidates for treatment or prevention of WNV infection in humans. However, a number of inhibitors developed for other *Flaviviridae* such as DENV and HCV exhibited cross-inhibition of WNV, suggesting the possibility of re-purposing these antivirals for WNV treatment (Lim et al., 2013a). The NI

Sinefungin (SIN) was identified as a broad-spectrum inhibitor of MTase activity (Dong et al., 2008). It inhibits N7 and 2'-O MTase activities and WNV replication in vitro by targeting the SAM-binding pocket (Dong et al., 2008; Chen et al., 2013). Aurintricarboxylic acid (ATA) was identified as an NI targeting MTase by Milani and colleagues. It inhibits N7 and 2'-O MTase activities of WNV and DENV2 by targeting the RNA-binding sites of the MTase domain in vitro (Milani et al., 2009). NITD-008 and NITD203 were found to inhibit the replication of multiple flaviviruses such as WNV, YFV, and HCV in vitro (Chen et al., 2010) (see Table 3, Fig. 3). BG-323, an effective inhibitor of DENV with low toxicity, also inhibits WNV (Kunjin) replication in cells. The broad spectrum NI 5-aza-7-deazaguanosine was also reported to inhibit replication of WNV and DENV (Ono et al., 2003). In addition to small molecule-based inhibitors, increasing attention is being paid to therapeutic antibodies for WNV treatment (Lim et al., 2013a). This approach has not only produced candidates in clinical trials for treatment of WNV infection, but also helped to understand that antibodies that are required for an effective flavivirus vaccine.

Zika Virus

Research on the crystal structure of ZIKV NS5 revealed conserved features of ZIKV NS5 MTase and RdRp with other Flavivirus, suggesting the use of flavivirus antiviral inhibitors for treating ZIKV infections (Stephen et al., 2016; Duan et al., 2017). For example, compounds known to inhibit DENV MTase and RdRp show similar inhibition potency for ZIKV MTase and RdRp. Because the binding mode

of RTP was similar to that of RNA analogues in ZIKV MTase and the binding sites in the DENV GTP-binding pocket were almost conserved in ZIKV MTase, RTP may serve as an antiviral drug against ZIKV infection by targeting ZIKV MTase (Duan et al., 2017). Moreover, Compound 10 might inhibit ZIKV MTase activity because the residues involved in the interaction with ZIKA MTase are conserved in DENV MTase (Duan et al., 2017). In addition, 7-deaza-2'-C methyladenosine (7DMA) was shown to reduce replication of Zika virus and delay disease progression in virus-infected mice (Joanna et al., 2016). Comparing the binding sites of NITD107 in DENV RdRp with that of ZIKV RdRp, Duan and colleagues found that almost all residues associated with interaction were conserved, suggesting that NITD107 may serve as an inhibitor of ZIKV RdRp (Duan et al., 2017). It was proposed that Compound 27 and Compound 29 have antiviral activity against ZIKV by targeting ZIKV RdRp, because the corresponding binding sites associated with interaction between JG-31-MG546/Compound 29 and DENV RdRp were conserved in ZIKV (Yokokawa et al., 2016; Duan et al., 2017). Meanwhile, the NNI DMB213 was found to inhibit ZIKV RdRp activity and viral replication through chelating divalent metal ions in the catalytic site of ZIKV RdRp in vitro (Xu et al., 2017). NITD008 was found to have antiviral activity against ZIKV both in vivo and in vitro (Xie et al., 2016; Deng et al., 2016; Shan et al., 2016).

To date, much inhibitor screening and design against flavivirus has focused on virus entry (E), viral protease (NS3), viral enzymatic activities (MTase, and RdRp), and other approaches such as therapeutic antibodies. Although inhibition of NS5

MTase and RdRp remains one of the most promising antiviral approaches, there are several key challenges in developing NS5 inhibitors for therapeutics. NIs have proven to be the most used antiviral regimens clinically and have several advantages over NNIs, such as a higher barrier to development of antiviral resistance (Delang et al., 2011). However, the toxicity of NIs is unpredictable and is often missed in vitro. An additional potential drawback to the use of NNIs in antiviral therapy is that the biding pockets must be well conserved, and mutations in or near the NNI-specific pocket could lead to diminution of inhibitory action (Lim et al., 2013b). Therefore, there is an urgent need to develop a new antiviral therapy with broad spectrum and no cell toxicity. In this regard, the immune evasion strategies used by flavivirus (JEV, WNV, TBEV) and HCV NS5 to antagonize the host IFN-α/β response by inhibiting the phosphorylation and nuclear translocation of STAT1 are of interest (Fig. 3). Both DENV-2 and YFV NS5 inhibit IFN-α/β signalling by binding STAT2 (1-10aa residues) and inhibiting its phosphorylation, resulting in the degradation of STAT2. Moreover, WNV, TBEV and LGTV NS5 were found to bind PEPD, down regulate IFN-αR1 expression, and inhibit the phosphorylation of STAT1 (see Table 2, Fig. 3). Figure 3 shows sites involved in immune evasion and drug that target these sites as reported previously; some of these are in close proximity or overlap with each other. We feel this discovery has great potential in the development of novel antiviral drugs that better activate host antiviral immune responses to control viral infection.

9 Conclusion

The balance between virus proliferation and host immune defence decides the outcome of infection. *Flaviviridae* viruses can cause serious infections in humans and animals, aided by their possession of multiple immune evasion mechanisms mediated by different viral proteins. There is no doubt that NS5 or NS5A protein is the most important protein in immune evasion mechanisms, especially against IFN induction and signalling pathways. Understanding how viral immune evasion mechanisms interact with cellular proteins and specific binding sites, and how these evasion mechanisms result in disease, need further investigation at both cellular and organismal levels. Such an understanding will contribute to development of improved immunocompetent animal models for evaluation of therapeutic methods or drugs for controlling flaviviruses and HCV infection. The conserved MTase and RdRp activities in *Flaviviridae* viruses serve as attractive targets for pharmacological discovery, with the goal being to develop novel drugs with low or no toxicity, high efficiency and broad spectrum against *Flaviviridae* viruses. This review provides new insights into the development of novel therapeutics, biological analogues or antiviral peptides that target immune evasion mechanisms employed by viruses, thereby improving host innate immunity. This approach has great potential to lead to development of drugs with little or no toxicity and the capacity to effectively control flavivirus infection.

Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Figure Legends

Fig. 1. Genome organization of four genera of *Flaviviridae* family. (A) The RNA genome of *Flavivirus* genus encodes three structural proteins: capsid protein (C), transmembrane protein (prM) and envelope protein (E), and seven nonstructural proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5. (B) The polyprotein of the *Hepacivirus* genus is different from that of the *Flavivirus genus*, which contains three structural proteins: core protein (Core), envelope proteins 1 and 2 (E1 and E2), and seven NS proteins: p7/p13, NS2, NS3, NS4A, NS4B, NS5A, and NS5B. The protein corresponding to the HCV p7 in GBV-B is p13. (C) The polyprotein encoded by *Pestivirus* genus contains a N terminal autoprotease (N^{pro}), and four structural proteins: capsid protein (C), envelope protein with ribonuclease activity (E^{rns}), envelope proteins 1 and 2 (E1 and E2), and seven NS proteins,: p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B. (D) The RNA genome of *Pegivirus* genus encodes two structural proteins: envelope proteins 1 and 2 (E1 and E2), and seven NS proteins: p7/x, NS2, NS3, NS4A, NS4B, NS5A and NS5B. The p7 protein in GBV-D (BPgV) is not clear compared with GBV-A (SPgV), GBV-C (HPgV), and GBV-Ccpz (SPgVcpz).

Fig. 2. Immune evasion strategies of *Flaviviridae* virus nonstructural proteins 5 and 5A involved in TLR/MyD88-dependent and Type I or II interferon signalling pathways. (A) In TLR/MyD888-dependent signalling pathway, there are four main signal transduction pathways that trigger and activate four transcription factors: NF-κB, AP-1, IRF5, and IRF7. The activated transcription factors translocate into the

nucleus and induce the expression of inflammatory cytokines and IFNs. The HCV NS5A protein is involved in inhibiting the TLR/MyD88 signalling pathway through binding to MyD88, and HCV NS5A also impairs IRF-7 activation through the interaction with each other. (B) Type I IFN (IFN-α and IFN-β) or type II IFNs (IFN-γ) recognize their cognate receptors IFN-αR1/2 or IFN-γR1/2, causing the phosphorylation and activation of the associated tyrosine kinases of Janus-activated kinase (JAK) family and signal transducers and activators of transcription (STAT) proteins; phosphorylated STAT proteins form transcription complexes and translocate into the nucleus to induce IFN-stimulated gene (ISG) expression. The NS5A of HCV and NS5 of Langat virus (LGTV), tick-borne encephalitis virus (TBEV), Japanese encephalitis virus (JEV), dengue virus (DENV), West Nile virus (WNV), yellow fever virus (YFV), Zika virus (ZIKV), and Spondweni virus (SPOV) can disturb the IFN signalling pathway directly or indirectly by interacting with different host factors.

Fig. 3. Schematic diagram of the amino acid sites of Flaviviridae virus nonstructural proteins 5 and 5A which involved in immune evasion or targeted by reported inhibitors.

The viral strains include DENV1-4 (AAB70694.1; AAC59275.1; AAT75224.1; AAA42964.2), WNV(AAM81752.1), YFV(CAA27332.1), ZIKV(Q32ZE1), JEV(AAA81554.1), LGTV(AAF75260.1), TBEV(AAA86870.1), and HCV(Q99IB8). The experimental confirmed amino acid sites interacted with host immune molecules are highlighted with pink background. The domains or regions involved in

immune evasion are underlined with color bars (Yellow bars for OAS, Light Blue bars for STAT1 and STAT2, Green bars for PKR, Blue bars for MyD88). The targeting amino acid sites for Compound 10 were shown in Yellow characters. The targeting amino acid sites for Ribavirin 5'-triphosphate (RTP) were shown in Light Green characters. The targeting amino acid sites for Aurintricarboxylic acid (ATA) were shown in Green characters. The targeting amino acid sites for NSC12155 were shown in Orange characters. The targeting amino acid sites for Sinefungin were shown in Pink characters. The targeting amino acid sites for NITD107 were shown in Purple characters. The targeting amino acid sites for Compound 27 were shown in Light Blue characters. The targeting amino acid sites for JF-31-MG46 were shown in Blue characters. The targeting amino acid sites for DMB220 were shown in Brown characters. The targeting amino acid sites for DMB213 were shown in Red characters. The target sites for Ribavirin 5'-triphosphate (RTP), Aurintricarboxylic acid (ATA), Compound 10, NSC12155 and Sinefungin were included in MTase domain, while the sites for NITD107, Compound 27, JF-31-MG46, DMB220 and DMB213 located in RdRp domain. The linker region between MTase domain and RdRp domain is indicted with Gray background.

Table 1. Species of *Flaviviridae* Family

Flaviviridae Family	Characteristic	Species	Reference
Flavivirus genus	Mosquito-borne flaviviruses, the genome is approximately 11 kb in size.	Dengue virus (DENV), Yellow fever virus (YFV), West Nile virus (WNV), Japanese encephalitis virus (JEV), Tick-borne encephalitis virus (TBEV), Zika virus (ZIKV) *et al*.	(Diamond *et al*, 2003)
	Tick-borne encephalitis complex of viruses, the genome is approximately 11 kb in size.	Tick-borne encephalitis (TBEV); Langat virus (LGTV); Powassan virus; Kyasanur Forest disease virus; Omsk hemorrhagic fever virus; Louping ill virus *et al*.	(Diamond *et al*, 2003)
Hepacivirus genus	The genome is approximately 9.6 kb in size.	*Hepacivirus A-N*.	(Macdonald *et al*, 2004; Stapleton *et al*, 2011; Smith *et al*, 2016)
Pestivirus genus	The genome is approximately 12.3 kb in size.	Bovine Viral Diarrhea Virus-1 (BVDV-1); BVDV-2; Border disease virus (BDV); Classical swine fever virus (CSFV); Giraffe-1; Reindeer-1; Pronghorn antelope; Bungowannah virus and "HoBi"-like virus.	(Avalos-Ramirez *et al*., 2001; Mosena *et al*., 2016)
Pegivirus genus	The genome is approximately 9.6 kb in size.	*Pegivirus A-K*.	(Stapleton *et al*, 2011; Smith *et al*, 2016)

Table 2. Immunosuppressive effects of flaviviruses NS5 and HCV NS5A and its targeted host factors

Virus genera	Immune escape mechanism	Virus NS5/NS5A	Function regions/sites of NS5/NS5A	Targeted host molecule	Binding regions of host molecule	Evading mechanism by interaction	Reference
Flavivirus genus	Suppressing IFN-dependent signaling pathways	JEV NS5	aa1-762	PTP (unidentified)	Unknown	Inhibiting STAT1, Tyk2 phosphorylation and STAT1 nuclear translocation	(Lin et al. 2006)
		DENV2 NS5	aa1-10, Thr^{2} and Gly^{3}	hSTAT2	aa181-200	Degrading hSTAT2	(Mazzon et al. 2009; Ashour et al. 2009, 2010; Morrison et al. 2013)
		WNV NY99 NS5	Trp^{382} or Val^{631}, Ile^{632} or Trp^{651} or Phe^{653}	Unknown	Unknown	Inhibiting STAT1 phosphorylation and nuclear translocation	(Laurent-Rolle et al. 2010)
			Phe^{653}	PEPD	Unknown	Down-regulating IFN-αR1 expression	(Lubick et al. 2015)
		YFV NS5	aa1-10	STAT2 TRIM23	Unknown	Inhibiting ISRE signaling and ISGs transcription	(Laurent-Rolle et al. 2014)
		ZIKV NS5	Unknown	hSTAT2	Unknown	Degrading hSTAT2	(Grant et al. 2016)
		SPOV	Unknown	Unknown	Unknown	Inhibiting ISRE signaling and ISGs transcription	(Grant et al. 2016)

		LGTV TP21 NS5	aa374-380 and aa624-647	IFN-αR2, IFN-γR1, IFN-γR2	Unknown	Inhibiting JAK and STAT phosphorylation	(Best et al. 2005; Park et al. 2007)
			Trp^{647}	PEPD	aa216-233 and aa256-441	Down-regulating IFN-αR1 expression	(Lubick et al. 2015)
		TBEV NS5	Tyr^{222} and Ser^{223}	hScrib	aa1100-1630	Inhibiting STAT1 phosphorylation	(Werme et al. 2008)
			Asp^{380}	PEPD	Unknown	Down-regulating IFN-αR1 expression	(Lubick et al. 2015)
Hepacivirus genus	Inhibiting IFN induction	HCV 1b NS5A	aa240-280	MyD88	aa50-70	Inhibiting proinflammatory cytokines and chemokines	(Abe et al. 2007) (Chowdhury et al. 2013)
		HCV 1b/2a NS5A	Arg^{216} and Arg^{217}	IRF-7	Unknown	Retain IRF-7 in the cytoplasm	
	Suppressing IFN-dependent signaling pathways	HCV 1/3 NS5A	aa237-447	STAT1	Unknown	Inhibiting STAT1 phosphorylation and ISRE Signaling and ISGs transcription	(Kumthip et al. 2012)
		HCV 1a/1b NS5A	aa237-276	PKR	aa244-366	Inhibiting PKR and histone phosphorylation	(Gale et al. 1997)
		HCV 1b NS5A	aa237-302	PKR	aa244-296	Disrupting the PKR dimerization process	(Gale et al. 1998)
		HCV NS5A	aa1-148	OAS	aa52-104 and aa184-275	Inhibiting antiviral activity of IFN	(Taguchi et al. 2004)

Table3. Nucleoside analogue inhibitors (NIs) and non-nucleoside analogue inhibitors (NNIs) of MTase and RdRp activities

Activity	Target	Inhibitor (NI/NNI)	Virus (In vivo/In vitro)	Reference
MTase	GTP-binding pocket	Ribavirin 5'-triphosphate (NI)	DENV (In vitro)	(Benarroch *et al*, 2004)
	RNA-binding site	Aurintricarboxylic acid (NI)	DENV2 and WNV (In vitro)	(Milani *et al*, 2009)
	SAM-binding pocket	Sinefungin (NI)	WNV, DENV2 and YFV (In vitro)	(Dong *et al*, 2008; Chen *et al*, 2013)
		Compound 10 (NNI)	DENV3, WNV and ZIKV (In vitro)	(Lin *et al*, 2011)
		NSC12155/ NSC125910/ NSC306711 / NSC610930 (NI)	DENV2, DENV3 and YFV (In vitro)	(Brecher *et al*, 2015; Thiel *et al*, 2015)
RdRp	The RNA tunnel	NITD107 (NNI)	DENV3 (In vitro)	(Noble *et al*, 2013)
	GDD motif	DMB220 (NNI)	DENV1-4 (In vitro)	(Xu *et al*, 2015)
		DMB213 (NNI)	ZIKV (In vitro)	(Xu *et al*, 2017)
	N-pocket	JF-31-MG46 (NNI)	DENV3 and DENV4	(Noble *et al*, 2016)
		Compound27/Compound29 (NNI)	DENV1-4 (In vitro)	(Yokokawa *et al*, 2016)
	Others	NITD203 (NI)	DENV2 (In vitro and in vivo), WNV, YFV and HCV (In vitro)	(Chen *et al*, 2010)
		NITD008 (NI)	DENV2 and ZIKV (In vitro and in	(Chen *et al*, 2010;

	vivo)	Deng *et al*, 2016)
Sofosbuvir (NI)	HCV, DENV and ZIKV (In vitro)	(Potisopon *et al*, 2017)

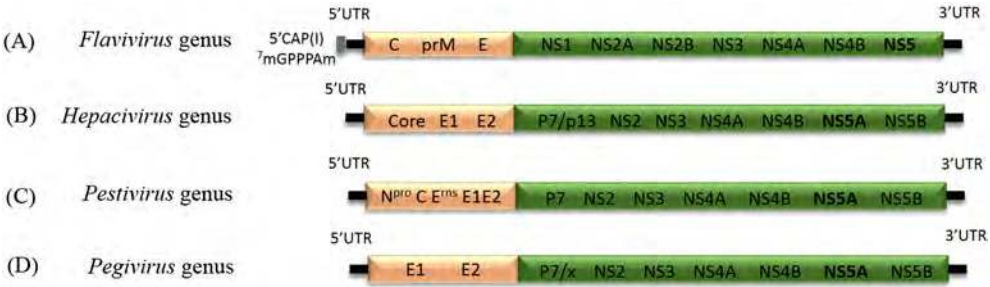
(A) Flavivirus genus
5'UTR
5'CAP(I)
7mGPPPAm
C prM E
NS1 NS2A NS2B NS3 NS4A NS4B NS5
3'UTR
(B) Hepacivirus genus
5'UTR
Core E1 E2
P7/p13 NS2 NS3 NS4A NS4B NS5A NS5B
3'UTR
(C) Pestivirus genus
5'UTR
Npro C Erns E1E2
P7 NS2 NS3 NS4A NS4B NS5A NS5B
3'UTR
(D) Pegivirus genus
5'UTR
E1 E2
P7/x NS2 NS3 NS4A NS4B NS5A NS5B
3'UTR

Figure 1

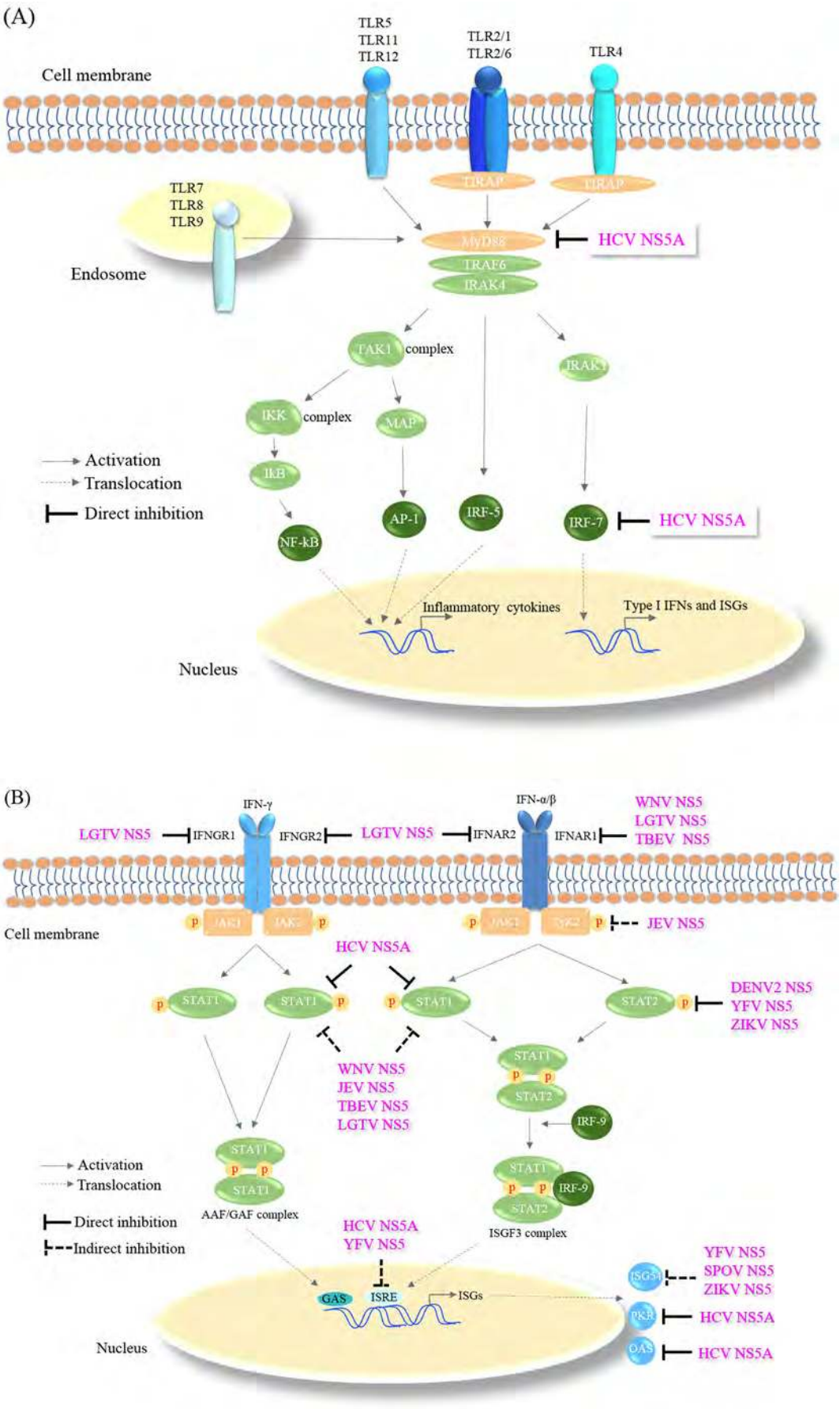
(A)
Cell membrane
TLR5
TLR11
TLR12
TLR2/1
TLR2/6
TLR4
TIRAP
TIRAP
TLR7
TLR8
TLR9
Endosome
MyD88
HCV NS5A
TRAF6
IRAK4
TAK1 complex
IRAK1
IKK complex
MAP
IkB
NF-kB
AP-1
IRF-5
IRF-7
HCV NS5A
Activation
Translocation
Direct inhibition
Inflammatory cytokines
Type I IFNs and ISGs
Nucleus
(B)
IFN-γ
IFN-α/β
LGTV NS5
IFNGR1
IFNGR2
LGTV NS5
IFNAR2
IFNAR1
WNV NS5
LGTV NS5
TBEV NS5
Cell membrane
JAK1
JAK2
TYK2
JEV NS5
HCV NS5A
STAT1
STAT1
STAT1
STAT2
DENV2 NS5
YFV NS5
ZIKV NS5
WNV NS5
JEV NS5
TBEV NS5
LGTV NS5
STAT1
STAT2
IRF-9
STAT1
STAT1
AAF/GAF complex
STAT1
STAT2
IRF-9
ISGF3 complex
Activation
Translocation
Direct inhibition
Indirect inhibition
HCV NS5A
YFV NS5
GAS
ISRE
ISGs
YFV NS5
SPOV NS5
ZIKV NS5
ISG54
PKR
HCV NS5A
OAS
HCV NS5A
Nucleus

Figure 2

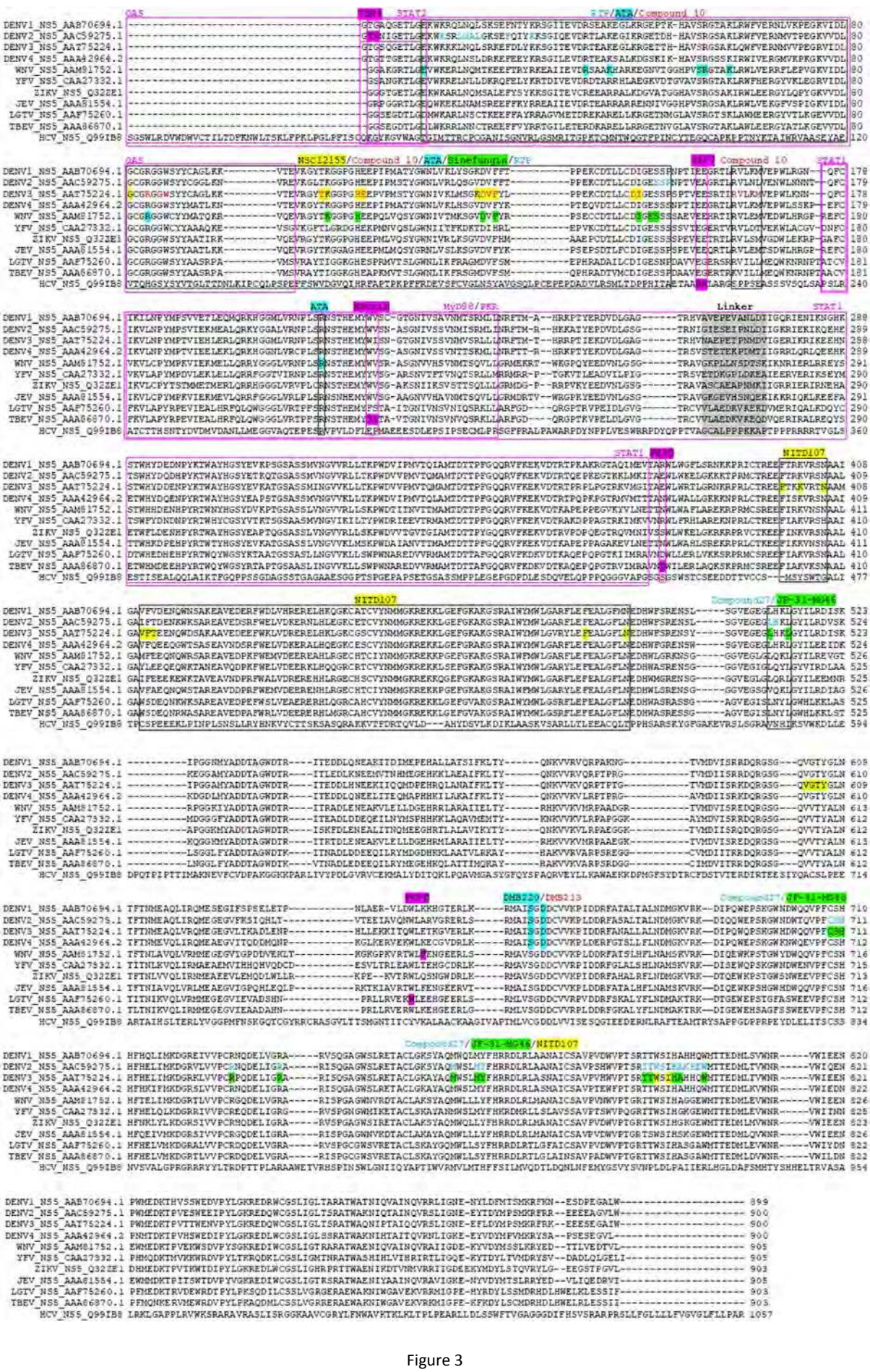

Figure 3